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Signaling in induced resistance by arbuscular mycorrhizal fungi against insect pests in tomato

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Departamento Microbiología del Suelo y la Planta

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Memoria presentada por Laura Dejana para optar al título de Doctora por la Universidad de Granada dentro del Programa de Doctorado en Biología Fundamental y de Sistemas

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Esta Tesis Doctoral ha sido realizada en el Departamento de Microbiología del Suelo y la Planta de la Estación Experimental del Zaidín (EEZ) del Consejo Superior de Investigaciones Científicas (CSIC) de Granada, dentro del grupo de investigación de Micorrizas.

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Los resultados presentados en esta Tesis Doctoral han sido publicados en las siguientes revistas internacionales o están en vías de publicación:

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The results presented in this PhD Thesis have been published in the following international journals or are in the process of being published:

Autores/Authors:	**Laura Dejana**, Beatriz Ramírez-Serrano, Javier Rivero, Jordi Gamir, Juan A. López Ráez, María J. Pozo
Título/*Title*:	**Phosphorus Availability Drives Mycorrhiza Induced Resistance in Tomato**
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Título/*Title*:	**Tomato Cell Wall-Associated Kinase SlWAK1 impacts primary and secondary metabolism and plant resistance against herbivore attack**
Fecha/*Date*:	En preparación/*In process*

Autores/Authors:	**Laura Dejana**, Alvaro López-Garcia, Juan M. García, Estefania Berrio, Thomas Roitch, Jordi Gamir, María J. Pozo
Título/*Title*:	**Mycorrhizal colonization finetunes primary metabolism and primes plant defenses upon herbivory in wild type and WAK1 deficient tomato**
Fecha/*Date*:	En preparación/*In process*

Asimismo, parte de los resultados obtenidos durante esta Tesis Doctoral han sido presentados en los siguientes congresos y reuniones científicas:

/

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Conferences and Presentations

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Working groups meetings

19-24/08/2018. MIRA Kick-off Meeting (oral presentation), Proitzer Mühle, Germany.

04-03-09/03/2019. MIRA Meeting (oral presentation), Granada, Spain.

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El doctorando / The *doctoral candidate* **Laura Dejana** y los directores de la tesis / *and the thesis supervisor/s*: **María José Pozo Jiménez y Jordi Gamir Felip**

Garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

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Guarantee, by signing this doctoral thesis, that the work has been done by the doctoral candidate under the direction of the thesis supervisor/s and, as far as our knowledge reaches, in the performance of the work, the rights of other authors to be cited (when their results or publications have been used) have been respected.

Lugar y fecha / *Place and date*:

Granada, 18 de abril de 2023

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Resumen/Summary

RESUMEN

La agricultura intensiva se ha basado en gran medida en el uso de fertilizantes químicos y pesticidas para proteger los cultivos de plagas y enfermedades y aumentar la productividad, lo que ha llevado a la pérdida de biodiversidad y degradación del ecosistema. Por lo tanto, este uso excesivo ya no es sostenible y es crucial encontrar alternativas, sostenibles con el medio ambiente, a las técnicas tradicionales de manejo de cultivos.

Una de las estrategias más prometedoras para reducir la aplicación de productos químicos es el uso de microorganismos beneficiosos, como los hongos formadores de micorrizas arbusculares (HMA). Estos tienen propiedades biofertilizantes y bioprotectoras, mejorando la salud y producción de las plantas sin comprometer el medio ambiente. Por lo tanto, representan una excelente alternativa a los fertilizantes químicos y pesticidas en la agricultura sostenible (Mącik et al., 2020; Pozo et al., 2021).

Las micorrizas arbusculares (MA) son asociaciones mutualistas formadas entre las raíces del 71% de las plantas vasculares terrestres y hongos del phylum *Glomeromycota* (Brundrett y Tedersoo, 2018). Los HMA son biótrofos obligados que desarrollan un micelio externo en el suelo y otro interno a través del cual colonizan las raíces de las plantas hospedadoras. Esto representa una estrategia de adaptación para aumentar el suministro de agua y nutrientes minerales a la planta, mejorando especialmente la absorción de fósforo. A cambio, las plantas transfieren carbono fijo en forma de azúcares y lípidos a los HMA (Bago et al., 2000; Salmerón-Santiago et al., 2021).

El fósforo (P) es uno de los nutrientes más importantes para el crecimiento y desarrollo de las plantas (Malhotra et al., 2018), y su disponibilidad también regula el establecimiento de la simbiosis MA. Para algunas plantas, incluyendo el tomate, altos niveles de P en el suelo inhiben el establecimiento de la micorriza (Ferrol et al., 2018). Por lo tanto, uno de los principales desafíos para la aplicación de HMA como bioestimulantes en la agricultura es encontrar niveles de P compatibles con el establecimiento y funcionamiento de la micorriza sin comprometer la productividad de las plantas. Comprender cómo el P influye en las simbiosis de micorriza y los posibles beneficios para la planta hospedera es esencial para optimizar la aplicación de HMA en la agricultura.

Además de aumentar la absorción de nutrientes, la simbiosis de MA mejora la tolerancia y resistencia de las plantas a múltiples estreses causados por factores abióticos y bióticos (Mitra et al., 2021). Esto se logra generalmente mediante la mejora de la plasticidad fenotípica y metabólica de la planta hospedadora (Rivero et al., 2018). Esta es una ventaja importante en

entornos heterogéneos, donde la asignación precisa de recursos limitados entre el crecimiento y las respuestas de defensa es crucial para la supervivencia de las plantas. El precondicionamiento o "*priming"* de las defensas, lleva a una activación más rápida y más fuerte de los mecanismos de defensa de la planta cuando ésta se enfrenta a un estrés. Es una estrategia muy eficiente para hacer frente al estrés, puesto que supone un menor coste energético que la activación constitutiva de los mecanismos de defensa (Conrath et al., 2006; Martinez-Medina, 2016; Mauch-Mani et al., 2017). Se ha demostrado que la simbiosis micorrícica puede desencadenar el priming de las defensas en la planta hospedadora, y se considera como el mecanismo principal responsable de la mayor resistencia de las plantas micorrizadas frente a patógenos y plagas, conocida como Resistencia Inducida por Micorrizas (MIR), y también opera en la resistencia inducida por otros microorganismos beneficiosos (Pozo y Azcón-Aguilar, 2007; Pieterse et al., 2014). Es importante destacar que la resistencia inducida por microorganismos depende del genotipo de la planta y del microorganismo, así como de las condiciones ambientales, por lo que la eficacia de la protección es altamente dependiente del contexto (Lee-Diaz et al., 2021). Esta variabilidad es una limitación importante cuando se intenta predecir la efectividad de los bioinoculantes en el campo en términos de protección de cultivos. Por tanto, resulta clave comprender la base molecular de la resistencia inducida y el priming de las defensas, e identificar los factores clave que regulan estos mecanismos. Comprender cómo diversos factores, como la disponibilidad de nutrientes, afectan a la MIR es esencial para optimizar el manejo de los HMA como bioinoculantes en la agricultura sostenible.

El *priming* de las defensas de las plantas por microorganismos beneficiosos generalmente suele ser dependiente de la ruta de señalización coordinada por del ácido jasmónico (JA) (Pieterse et al., 2014), y este es el caso también de la MIR (Mora-Romero et al., 2014). El JA es el principal regulador de las respuestas de defensa de las plantas contra patógenos necrotrófos y herbívoros, coordinando la activación de respuestas de defensa que incluyen proteínas anti-nutricionales y metabolitos tóxicos (Chen et al., 2019), y es un modulador central en las interacciones a tres vías entre plantas, microorganismos y artrópodos (Gruden et al., 2020). La activación de cascadas de señalización de fitohormonas que coordinan las respuestas de defensa de las plantas dependen del reconocimiento de posibles agresores (Pieterse et al., 2012). Esto se logra mediante la detección de moléculas asociadas al agresor (PAMPs, HAMPs, etc.) o moléculas propias que indican daño celular, conocidas como patrones moleculares asociados a daño (DAMPs) (Tanaka y Heil, 2022). Uno de los DAMPs mejor caracterizados en plantas son los oligogalacturonidos (OGs); estas moléculas se liberan de las paredes celulares de las plantas tras una herida o el ataque de patógenos a través de la actividad

de enzimas poligalacturonasas, lo que desencadena respuestas de defensa y promueve la inmunidad en diferentes especies de plantas, incluyendo el tomate (Bergey et al., 1999; Orozco-Cardenas et al., 1999; Ferrari et al., 2013; Benedetti et al., 2015). De hecho, los OGs inducen rápidamente mecanismos de defensa en tomate, dando lugar a una mayor resistencia sistémica contra patógenos (Gamir et al., 2020), y las plantas micorrizadas responden de manera más eficiente a este elicitor (Gamir et al., en preparación). Sin embargo, el papel de la percepción de OGs durante la herbivoría y en MIR debe ser estudiado con más detalle.

La quinasa asociada a pared 1 (WAK1, del inglés *wall-associated receptor kinase 1*) es el principal receptor de OG en Arabidopsis y su ortólogo en tomate, SlWAK1 también parece tener una alta afinidad por los oligómeros derivados de pectina según ensayos in silico de acoplamiento molecular (Kohorn, 2016; Kurt et al., 2020). WAK1 tiene un papel en la percepción de daños y en la activación de defensas en diferentes especies de plantas, incluyendo el tomate (Stephens et al., 2022). Por ejemplo, la expresión de *SlWAK1* se indujo en hojas de tomate por elicitores derivados de bacterias, y las plantas de tomate con silenciamiento de *SlWAK1* muestran síntomas de enfermedad más intensos contra la infección bacteriana (Rosli et al., 2013; Zhang N. et al., 2020). Sin embargo, hasta donde sabemos, no se han realizado estudios sobre el papel de SlWAK1 en las respuestas de defensa de las plantas contra los herbívoros.

Por lo tanto, la presente Tesis Doctoral se centra en **investigar los principales mecanismos de defensa y señalización que regulan el impacto de los hongos micorrícicos arbusculares en la resistencia de las plantas frente a los insectos herbívoros, y cómo el contexto abiótico -en particular la disponibilidad de nutrientes- puede influir en los efectos de esta interacción**. Para lograr este objectivo general, **se explora la reprogramación del metabolismo primario y secundario asociado a la resistencia inducida por micorrizas (MIR) bajo diferentes niveles de fertilización con fósforo y en un genotipo de tomate alterado en la percepción de daño (*Slwak1*).** Para lograr este objetivo general, la Tesis de Doctorado ha abordado 3 objetivos específicos. El **Objetivo específico I** explora cómo la disponibilidad de fósforo modula la MIR contra el herbívoro generalista *Spodoptera exigua* y el patógeno foliar *Botrytis cinerea* en plantas colonizadas por el hongo HMA *Funeliformis mosseae.* El **Objetivo específico II** se centró en analizar el papel de la quinasa asociada a la pared SlWAK1 en la regulación del metabolismo primario y secundario de la planta en respuesta al herbívoro *S. exigua*, y su contribución a la resistencia de la planta. Por último, en el **Objetivo específico III** se evalúó el papel potencial de SlWAK1 y la regulación del metabolismo de carbohidratos en la resistencia frente a *S. exigua* en plantas colonizadas por diferentes hongos micorrícicos (*F. mosseae* y *Clareidoglomus etunicatum*).

En primer lugar, se exploró cómo el fósforo, siendo un elemento clave en el funcionamiento de la simbiosis micorrícica, la nutrición y la inmunidad de la planta, puede contribuir a la dependencia del contexto de la MIR. Este estudio se presenta en el **Capítulo 1** (Dejana et al., 2022), donde se evaluó el impacto de diferentes niveles de fertilización con fósforo en la MIR en plantas de tomate colonizadas por *F. mosseae* e infestadas por *S. exigua* o infectadas por *B. cinerea,* en comparación con plantas no micorrizadas.

La fertilización con fósforo afectó significativamente el valor nutricional de las plantas y sus defensas, el desarrollo de enfermedades y la supervivencia de las larvas, aunque estos efectos fueron modulados por el estado micorrízico de la planta. De hecho, el aumento de la resistencia contra *B. cinerea* y *S. exigua* en plantas colonizadas por *F. mosseae* dependió de la disponibilidad de fósforo, ya que no se observó protección bajo la condición de menor contenido de fósforo en comparación con las plantas no micorrizadas. Por el contrario, en condiciones moderadas de deficiencia de fósforo, las plantas micorrizadas mostraron una mayor resistencia, con peor desempeño de las orugas y una reducción de la infección por patógenos en comparación con las plantas no micorrizadas. Por lo tanto, los resultados confirman que el fósforo es un factor importante para MIR y que contribuye a la dependencia del contexto de ésta.

Para explorar si las diferencias observadas estaban relacionadas con cambios en el valor nutricional de la planta o en la regulación de sus defensas, se analizaron las respuestas tempranas de defensa mediante la aplicación de OGs. Se encontró que el *priming* de las defensas en las plantas micorrizadas – determinado por la mayor inducción de genes marcadores de defensa dependiente de JA- solo ocurrió a niveles adecuados de fósforo, pero no en las condiciones de más deficiencia. La diferencia en las respuestas de las plantas micorrizadas y no micorrizadas bajo diferentes niveles de fósforo sugieren que la micorriza ajusta finamente el balance entre crecimiento y defensa priorizando una u otra dependiendo de la disponibilidad de fósforo. La regulación diferencial de los genes *JAZ* en plantas micorrizadas, que codifican reguladores clave de la señalización del JA, apunta a un papel importante de esta ruta en la modulación del equilibrio entre crecimiento y defensa en las plantas micorrizadas.

Aunque no se han descrito previamente los OGs en respuesta a la herbivoría, siempre se había asumido que estas moléculas se liberan durante el ataque de herbívoros como consecuencia de los daños derivados de la masticación (Aljbory et al., 2018; Erb y Reymond, 2019). Los resultados del **Capítulo 1** muestran la mayor expresión en plantas micorrizadas de genes de defensa frente a herbívoros regulados por JA al ser estimulados por OGs. Estos resultados, junto con la idea de que los herbívoros masticadores, por su manera de alimentarse,

puedan liberar OGs desencadenando respuestas a las heridas e induciendo PG endógenas, apoyan esta suposición. Para explorar más profundamente si la percepción de daños (OGs) influye en las respuestas de las plantas a herbivoría, se analizó la regulación y función de SlWAK1 durante herbivoría por S. exigua (**Capítulo 2**).

Primero, se determinó que *SlWAK1* está regulado positivamente a nivel transcripcional por la aplicación de OGs y en respuesta a la herbivoría. Después se realizó un análisis funcional del gen mediante el uso de la línea mutante de tomate *Slwak1*, alterada en la región promotora de *SlWAK1* (Meco et al., 2020). Los resultados confirmaron que la planta mutante tenía menores niveles de expresión de *SlWAK1* que el fenotipo silvestre (WT, del inglés *wild type*) tanto en ausencia como en presencia de herbivoría.

Para profundizar en el posible papel de SlWAK1 en las respuestas a herbivoría, se monitorizó el desarrollo del herbívoro masticador generalista *S. exigua* en plantas WT y *Slwak1*. Las larvas alimentadas con la planta mutante *Slwak1* mostraron un mejor rendimiento que aquellas alimentadas con la planta WT. El análisis transcripcional mostró que *Slwak1* no estaba afectada en la ruta del JA, pero mostraba una activación deficiente de las rutas de biosíntesis de auxinas, fenilpropanoides y alcaloides. El análisis posterior de los perfiles metabólicos confirmó una reducción en *Slwak1* en la acumulación de compuestos defensivos de las rutas de fenilpropanoides, como flavonoides, lignanos, alcaloides y terpenos, tanto antes como después de la herbivoría, en comparación con el WT. Además, se detectaron cambios profundos en el metabolismo primario en la planta mutante *Slwak1*. Los niveles basales de azúcares, intermediarios del ciclo del ácido tricarboxílico y actividades enzimáticas relacionadas con el metabolismo de carbohidratos mostraron niveles alterados en la mutante. El mutante también mostró una regulación diferente del metabolismo y distribución de carbohidratos en respuesta a *S. exigua.* Por lo tanto, los resultados revelan una importante desregulación del metabolismo de carbohidratos en el mutante, que sugiere un estado reducido de energía que podría explicar los defectos en la activación de la inmunidad en estas plantas. Los resultados señalan la importancia del receptor SlWAK1, probablemente a través de la percepción de OG, para activar adecuadamente las respuestas de defensa frente a herbivoría en la planta y para una apropiada coordinación del metabolismo primario y secundario.

En base a los resultados descritos en el **Capítulo 1** y los resultados previos en nuestro grupo que apuntan a un "priming" de la respuesta a OGs en plantas micorrizadas, se planteó la hipótesis de que el gen *SlWAK1*, a través de su función en la regulación de la inmunidad de las plantas, pueda tener un papel en la MIR. Por lo tanto, en el **Capítulo 3** se analizaron las respuestas de defensa del tomate contra *S. exigua*, utilizando plantas WT y el mutante *Slwak1*,

inoculadas con dos hongos micorrícicos diferentes: *F. mosseae* o *Clareidoglomus etunicatum*. Se observó protección de la planta por la micorriza en ambos genotipos, lo que demuestra que la simbiosis MA tiene un impacto significativo en el rendimiento del herbívoro, independientemente del receptor SlWAK1. El análisis de la expresión génica mostró que, efectivamente, la MIR se asoció en ambos genotipos con una mayor inducción, en respuesta a herbivoría, de la expresión de genes de defensa regulados por JA en plantas micorrizadas. El análisis del perfil metabólico reveló una mayor acumulación de metabolitos secundarios defensivos específicos, pertenecientes a las rutas de lignanos, alcaloides y fenilpropanoides, especialmente en las plantas colonizadas por *C. etunicatum* en ambos genotipos. Es importante destacar que la simbiosis micorrícica tuvo un impacto importante en el metabolismo primario de la planta. Además, la alteración del metabolismo primario en las plantas mutantes *Slwak1*, mostrada en el Capítulo 2, se revirtió parcialmente por la colonización micorrícica. En el **Capítulo 3**, se muestra, por tanto, que la simbiosis mitiga los efectos de la deficiencia de *Slwak1* y compensa la regulación ineficiente en el mutante del metabolismo de carbohidratos y su distribución en planta.

Bajo herbivoría, las respuestas de las plantas mutantes no micorrizadas siguieron direcciones opuestas a las del WT (Capítulo 2), mientras que las plantas micorrizadas mostraron un metabolismo de carbohidratos más eficiente en ambos genotipos (Capítulo 3). En el WT, la simbiosis MA mejoró la fuerza del tejido sumidero en las hojas dañadas y la partición de C en comparación con las plantas no micorrizadas. En el mutante, la simbiosis revirtió la dirección del flujo de azúcares en comparación con las plantas mutantes no micorrizadas, mostrando patrones similares a las plantas WT. Esto sugiere que la colonización micorrícica puede mitigar los defectos en la regulación del metabolismo primario del mutante, restaurando la partición adecuada de C en respuesta a la herbivoría.

En general, la investigación realizada en esta Tesis Doctoral muestra que las simbiosis micorrícicas ayudan a las plantas a enfrentar condiciones subóptimas al mitigar los efectos negativos de factores ambientales externos y desequilibrios internos. La simbiosis MA puede mejorar el equilibrio entre crecimiento y defensa según las condiciones de estrés a las que la planta tenga que enfrentarse. La colonización micorrícica prioriza el crecimiento cuando los recursos son limitados, pero redirige eficientemente los recursos hacia la defensa durante un ataque, lo que resulta en una respuesta más rápida y más fuerte en comparación con las plantas no micorrícicas. Además, la simbiosis MA puede compensar las deficiencias en las respuestas de la planta, como se ha mostrado en plantas mutantes con expresión reducida de *SlWAK1* bajo ataque de herbívoros. En estos casos, la colonización micorrícica mejora las defensas de la planta

mediante la activación de rutas hormonales y biosíntesis de metabolitos secundarios relacionados con la defensa, y regula eficientemente la partición de carbono, revirtiendo el fenotipo del mutante a niveles más cercanos a los de las plantas silvestres.

En conclusión, los hallazgos de esta investigación sugieren que la simbiosis micorrícica puede desempeñar un papel crucial a la hora de amortiguar posibles efectos negativos y en la modulación de las respuestas de las plantas a través de la reprogramación transcripcional y metabólica para hacer frente al estrés predominante. Por lo tanto, la simbiosis micorrícica representa una solución prometedora y efectiva para mitigar el impacto de condiciones ambientales extremas y/o variables, y mejorar la resistencia de las plantas contra posibles agentes de estrés. Estos beneficios potenciales hacen que el de los HMA una herramienta valiosa para enfrentar de manera sostenible los desafíos planteados por el cambio climático y mejorar la resiliencia de las plantas en diferentes ecosistemas

SUMMARY

Common methods in intensive agriculture to protect crops from diseases and increase productivity largely rely on the use of chemical fertilizers and pesticides which led to biodiversity loss and ecosystem degradation. Thus, their excessive use is no longer sustainable for crop production and finding environmental-friendly alternatives to traditional crop management techniques is crucial.

One of the most promising strategies to reduce chemical application is the use of beneficial microorganisms, such as soil-borne Arbuscular Mycorrhiza-forming Fungi (AMF). They have biofertilizer and bioprotective properties, improving plant fitness and production without compromising the environment. Thus, they represent an excellent alternative to chemical fertilizers and pesticides in sustainable agriculture (Mącik et al., 2020; Pozo et al., 2021).

Arbuscular mycorrhizas (AM) are mutualistic associations formed between the roots of 71% of terrestrial vascular plants and fungi from the phylum *Glomeromycota* (Brundrett and Tedersoo, 2018). AMF are obligate biotrophs that colonize the root cortex of host plants and develop an external mycelium that overgrows the soil surrounding plant roots. It represents an adaptation strategy to increase the supply of water and mineral nutrients to the plant, especially improving plant phosphorous uptake. In exchange, plants transfer fixed carbon in the form of sugars and lipids to the AMF (Bago et al., 2000; Salmerón-Santiago et al., 2021).

Phosphorous (P) is one of the most important nutrients for plant growth and development (Malhotra et al., 2018), and its availability also regulates AM symbiosis establishment (Smith and Smith, 2011). For some plants, including tomato, high P levels in the soil inhibit mycorrhizal establishment (Ferrol et al., 2019). Thus, one of the major challenges for the application of AMF as biostimulants in agriculture is to find P levels compatible with mycorrhiza establishment and functioning without compromising plant productivity. Thus, unraveling how P influences mycorrhizal symbioses and the potential benefits for the host plant is essential to optimize the application of AMF in agriculture.

Besides increasing nutrient uptake, the AM symbiosis enhances plant tolerance and resistance to multiple stresses caused by both abiotic and biotic factors (Mitra et al., 2021). This is usually achieved by improving the phenotypic and metabolic plasticity of the host plant (Rivero et al., 2018). This is an important advantage in heterogeneous environments, where precise allocation of limited resources between growth and defense responses is critical for plant survival. “Defense priming” -leading to more rapid and stronger activation of plant defense

mechanisms upon challenge- is a cost-efficient strategy to cope with stress, in contrast to constitutive activation of plant defense mechanisms (Conrath et al., 2006; Martínez-Medina 2016; Mauch-Mani et al., 2017). Mycorrhizal symbiosis has been shown to trigger defense priming in different plant-stress systems, and this is considered as the main mechanism operating in the induced resistance by mycorrhizas (Mycorrhiza Induced Resistance (MIR) and other beneficial microbes) (Pozo and Azcón-Aguilar 2007; Pieterse et al., 2014). Noteworthy, microbe-induced resistance is dependent on the plant and microbe genotypes and the environmental conditions, so the protection is highly "context-dependent" (Lee-Díaz et al., 2021). This is a handicap when trying to predict the performance of bioinoculants in the field regarding crop protection. Thus, a major challenge in plant research is understanding the molecular basis of induced resistance and defense priming, and identifying key factors modulating these mechanisms. Understanding how factors, such as nutrient availability, affect MIR is essential for the optimized management of bioinoculants in sustainable agriculture.

The priming of plant defenses by beneficial microorganisms is usually mediated by the jasmonic acid (JA) signaling pathway (Pieterse et al., 2014), and this is the case also with MIR (Mora-Romero et al., 2014). JA is the main regulator of plant defense responses against necrotrophic pathogens, wounding and herbivores, coordinating de activation of defense responses including antifeedant proteins and toxic metabolites (Chen et al., 2019), and is a central modulator in three-way interactions between plant-microbes and arthropods (Gruden et al., 2020). The activation of phytohormone signaling cascades to coordinate plant defense responses depends on the recognition of potential aggressors (Pieterse et al., 2012). This is achieved through the detection of molecules associated to the challenging organism (PAMPs, HAMPs, etc) or self-molecules that indicate cellular damage, known as damage-associated molecular patterns (DAMPs) (Tanaka and Heil, 2021). One of the best-characterized plant DAMPs are the oligogalacturonides (OGs); these molecules are released from plant cell walls by wounding or pathogen attack through the cleavage activity of polygalacturonases enzymes, triggering plant defense responses and promoting immunity in different plant species -including tomato- (Bergey et al., 1999; Orozco-Cardenas et al., 1999; Ferrari et al., 2013; Benedetti et al., 2015). Indeed, OGs rapidly induced defense mechanisms in tomato plants leading to systemic resistance against pathogens in leaves (Gamir et al., 2020), and a primed response to this elicitor has been observed in mycorrhizal plants (Gamir et al., in preparation). However, the role of OG perception during herbivory and in MIR remains to be explored.

The wall-associated receptor kinase 1 (WAK1) is the main OG receptor in Arabidopsis and docking analyses point to a high affinity of the SlWAK1 for pectin oligomers in tomato plants

(Kohorn, 2016; Kurt et al., 2020). WAK1 has a role in damage perception and activation of plant defenses in different plant species, including tomato (Stephens et al., 2022). For example, *SlWAK1* expression was induced in tomato leaves by bacteria-derived elicitors, and *SlWAK1* tomato-silenced plants display enhanced disease symptoms against bacterial infection (Rosli et al., 2013; Zhang N. et al., 2020). However, to the best of our knowledge, no studies on SlWAK1 role in plant defense responses against herbivores have been performed.

Thus, the present Doctoral Thesis focuses on **investigating the main defense mechanisms and signaling components mediating the impact of the beneficial arbuscular mycorrhizal fungi on plant resistance to insect pests, and how the abiotic context -in particular nutrient availability- may shape the effects of the interaction.** To achieve this general objective, **I explore the reprogramming of primary and secondary metabolism associated with mycorrhiza-induced resistance (MIR) under different P fertilization levels and in a tomato genotype altered in damage perception.** To achieve this main objective, **the Ph.D. Thesis has been divided into 3 objectives. Objective I** explores how phosphorous availability modulates MIR against the generalist herbivore *Spodoptera exigua* and the foliar pathogen *Botrytis cinerea* in plants colonized by the AMF *Funneliformis mosseae*. **Objective II** unravels the role of the wall-associated kinase SlWAK1 in the regulation of the plant's primary and secondary metabolism in response to herbivory by *S. exigua* and its contribution to plant resistance. Finally, **objective III** evaluates the potential role of SlWAK1 and the regulation of carbohydrate metabolism on plant resistance against *S. exigua* in plants colonized by different mycorrhizal fungi (*F. mosseae* and *Clareidoglomus etunicatum*).

First, I explore how P, as a key element in mycorrhizal symbiosis functioning, plant nutrition and immunity, may contribute to the context dependency of MIR. This study is presented in **Chapter 1** (Dejana et al., 2022), where it was evaluated the impact of different P fertilization levels on MIR in tomato plants colonized by *F. mosseae* and infested by *S.exigua* or infected by *B. cinerea* compared with non-mycorrhizal plants.

P fertilization significantly affected plant nutritional value, plant defenses, disease development and caterpillar survival, but these effects were modulated by the mycorrhizal status of the plant. Indeed, enhanced resistance against *B. cinerea* and *S. exigua* of *F. mosseae*-colonized plants depended on P availability, as no protection was observed under the lowest P condition compared with non-mycorrhizal plants. On the contrary, at moderate P deficiency, AM plants showed higher resistance -worst caterpillar performance and reduced pathogen infection- than non-mycorrhizal ones. Then, the results confirm that P is indeed a crucial factor influencing the context-dependent nature of MIR.

In order to explore whether the observed differences were related to changes in the nutritional value of the plant or in the regulation of plant defenses, we analyzed early plant defense responses by applying the damage-associated molecules OGs. We found that priming of plant defenses in mycorrhizal plants -higher induction of JA-related defense marker genes in AM plants in response to OG- occurred only under sufficient P levels, but not under the lowest P condition. The differential responses of AM and non-mycorrhizal plants under different P fertilization levels suggest that mycorrhiza fine-tunes the plant growth vs defense prioritization depending on P availability. The differential regulation of *JAZ* genes, coding for key regulators of JA signaling, points to a role of the JA pathway in the modulation of the growth-defense trade-off in mycorrhizal plants.

Although OGs have not been described previously in response to chewing herbivory, it has always been assumed that these molecules are released under herbivore attack (Aljbory et al., 2018; Erb and Reymond, 2019). The results in **Chapter 1** –higher expression of JA-related genes coding for antifeedant proteins that impair herbivore fitness upon OGs elicitation– and the fact that chewing herbivores, for their feeding guild, may release OGs, triggering wound responses and inducing endogenous PG, points to confirm this assumption. Hence, to explore deeply how damage perception influences plant responses to herbivory, likely through the deficient perception of OGs, the regulation and function of SlWAK1 were analyzed (**Chapter 2**).

First, I analyzed whether *SlWAK1* is transcriptionally regulated by OG application and in response to herbivory. We found that both -OG application and local herbivory- upregulated the expression levels of *SlWAK1.* Then, to explore the function of the gene, the mutant tomato plant *Slwak1,* altered in the promoter region of *SlWAK1*, was used (Meco et al., 2020). We found that the mutant had reduced expression levels of *SlWAK1* compared with the wild-type (WT) in the absence as well as upon herbivory.

To gain a deeper understanding of the SlWAK1 role in plant responses to herbivory, I monitored the performance of the generalist chewing herbivore *S. exigua* in both WT and *Slwak1* plants. The larvae fed on the mutant *Slwak1* displayed a better performance than those feeding in WT. Transcriptional analysis displayed that *Slwak1* was not impaired in the jasmonic acid pathway, but showed deficient activation of auxin, phenylpropanoid and alkaloid biosynthetic pathways. Subsequent analysis of the metabolic profiles confirmed a reduced accumulation of defensive compounds belonging to phenylpropanoid, as flavonoids, lignan, alkaloid and terpene pathways in *Slwak1* -before and after herbivory- compared to WT. Noteworthy, we found profound changes in primary metabolism in the *Slwak1* mutant. Basal levels of sugars, tricarboxylic acid cycle intermediates and enzymatic activities related to

carbohydrate metabolism were altered in the mutant. The mutant also showed a differential response of the carbohydrate metabolism and allocation in response to *S. exigua* attack. Thus, the results reveal important deregulation of the carbohydrate metabolism in the mutant, pointing to a reduced energy status that may explain the defects in plant immunity activation. The results highlight the importance of SlWAK1 receptor, likely through OG perception, for properly activating plant defense responses against herbivory and its relevance in coordinating primary and secondary metabolism.

In light of the results described in **Chapter 1** and previous results in our group pointing to a primed response to OG in mycorrhizal plants, we hypothesized that the *SlWAK1* gene, through its function in regulating plant immunity, may have a role in MIR. Thus, in **Chapter 3** I evaluated tomato defense responses against *S. exigua,* using tomato WT and the mutant *Slwak1,* inoculated by two different mycorrhizal fungi: *F. mosseae* or *Clareidoglomus etunicatum*. We observed host protection by mycorrhiza in both genotypes, demonstrating that AM symbiosis significantly impacts herbivore performance, regardless of the SlWAK1 receptor. Transcriptional analysis showed that MIR was associated to increased expression of JA-regulated defense genes in AM plants under herbivory in both genotypes. The metabolic profiling performed revealed an accumulation upon herbivory of specific defensive secondary metabolites -belonging lignan, alkaloid and phenylpropanoid pathways- especially in *C. etunicatum* colonized plants of both genotypes. Noteworthy, mycorrhizal symbiosis has an important impact on the plant's primary metabolism. Moreover, the impairment of *Slwak1* mutant plants in primary metabolism, shown in Chapter 2, was partially reverted by mycorrhizal colonization. In **Chapter 3,** we showed that the symbiosis buffers the effects of *Slwak1* deficiency and compensates for the inefficient regulation of carbohydrate metabolism and partitioning of the mutant.

Upon herbivory, non-mycorrhizal mutant plant responses followed opposite directions than the WT (Chapter 2), while mycorrhizal plants displayed a more efficient carbohydrate metabolism in both genotypes (Chapter 3). In the WT, the AM symbiosis improves the sink strength in damaged leaves and C partitioning compared with non-mycorrhizal plants. In the mutant, the symbiosis reverted the direction of sugar flux as compared to the non-mycorrhizal mutant plants displaying similar patterns to WT plants. This suggests that mycorrhizal colonization can mitigate the defects of the mutant's primary metabolism regulation, restoring proper C partitioning in response to herbivory.

Overall, the investigation carried out in the present Doctoral Thesis shows that mycorrhizal symbioses help plants to cope with suboptimal conditions by mitigating the negative effects of external environmental factors and internal imbalances. AM symbiosis can improve

the growth-defense trade-off according to the stress condition the plant has to face. Mycorrhizal colonization prioritizes growth when resources are limited, but efficiently redirects the resources towards defense during an attack, resulting in a faster and stronger response compared to non-mycorrhizal plants. Furthermore, the symbiosis may compensate for deficiencies in plant responses, as shown in mutant plants with reduced expression of *SlWAK1* under herbivore attack. In such cases, mycorrhizal colonization enhances plant defenses by priming defense-related hormone pathways and secondary metabolite biosynthesis and, efficiently regulating carbon partitioning, reverting the phenotype to levels closer to wild-type.

In conclusion, the research findings suggest that mycorrhizal symbiosis can play a crucial role in buffering potential negative factors and shaping plant responses through transcriptional and metabolic reprogramming toward the major stress. Thus, mycorrhizal symbiosis represents a promising and effective solution for mitigating the impact of extreme and/or variable environmental conditions and enhancing plant resistance against potential attackers. These potential benefits make the use of AMF a valuable tool in addressing the challenges posed by climate change and improving plant resilience in different ecosystems.

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Introduction

INTRODUCTION

1. THE IMPORTANCE OF BIOINOCULANTS IN CROP PRODUCTION

Traditional methods used to protect crops from diseases and increase productivity have been largely based on the use of pesticides and fertilizers. However, intensive applications of these chemicals have led to environmental pollution in several agroecosystems, such as eutrophication and contamination of aquifers, and leaving harmful residues in the food chain negatively affecting consumers' health (Sharma N. et al., 2017; Evans et al., 2019). Moreover, the current climate change scenario challenges the future of natural resources and agricultural production, likely reducing yields in the long run (Smith et al., 2016). In addition, the anthropogenic misuse of land as monocultures has caused biodiversity loss and increased invasive species, forcing plants to adapt to new challenges (Liu et al., 2018). Thus, there is an urgent need to improve crop productivity whilst preserving agroecosystems and mitigating the effect of climate change. Indeed, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) establish that a priority in the 2030 Agenda is improving and developing practices that match human and environmental health with sustainable crop production (World Health Organization, 2020). Toward this aim, the application of environmentally friendly beneficial microorganisms as **bioinoculants** might solve fundamental and applied issues related to crop production by increasing plant efficiency in obtaining nutrients and water and, at the same time, increasing plant tolerance to adverse conditions (Parnell et al., 2016; Pozo et al., 2021).

1.1 Beneficial microorganisms as biofertilizers and bioprotectors to reduce the use of chemicals

Beneficial microorganisms are common in soils, and they can interact with plants to increase their growth (acting as biofertilizers) and resistance (acting as bioprotectors) against biotic or abiotic stresses (Mącik et al., 2020; Lee-Díaz et al., 2021) (Figure 1).

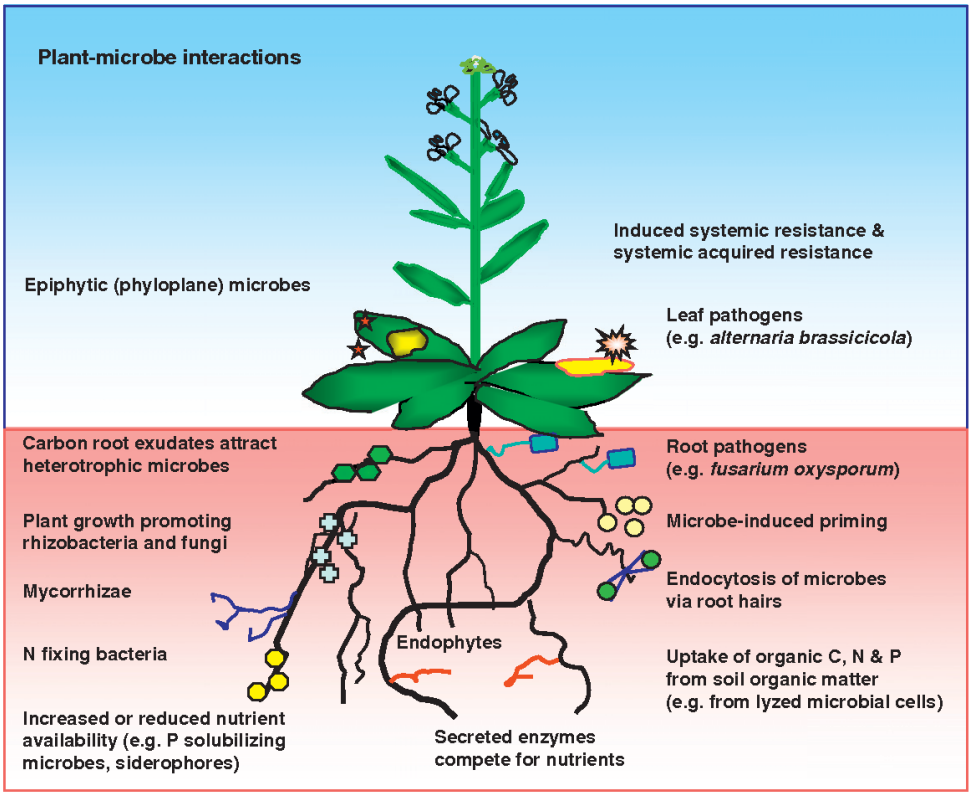

FIGURE 1. General overview of interactions between plants and microorganisms (Schenk et al., 2012).

The list of well-recognized beneficial microorganisms includes arbuscular mycorrhizal fungi (AMF), phosphate-solubilizing microorganisms, nitrogen-fixing bacteria, plant growth-promoting rhizobacteria, and endophytic bacteria (Calvo et al., 2014). It is well-established their positive effect on plants as **biofertilizers**, providing water, macro and micro-nutrients (such as N, P, K, Mn, Mg, Zn) to the plant; they can also produce phytohormones-like hormones, thus boosting plant growth and development, improving crop nutritional quality and productivity (Mącik et al., 2020; Bennett et al., 2022; Tabacchioni et al., 2021; Stefen et al., 2022). On the other side, some microbial inoculants increase plant tolerance against environmental abiotic stresses (e.g. drought, salinity, high temperatures, heavy metals) and plant resistance against biotic stresses (Kumar et al., 2021; Pozo et al., 2021). Although most of these microorganisms are not registered as **biopesticides**, according to the EU Pesticide Database (https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/active-substances), increasing evidence highlights the beneficial effects of microorganisms on improving direct or indirect defenses against pests and pathogens (Vishwakarma et al., 2022). Competition for nutrients and space and the production of antibiotics or hydrolytic enzymes are the primary reasons for direct protection (Ahmad et al., 2018; Ab Rahman et al., 2018). Indirect mechanisms include the induction of plant metabolites to repel the pest or attract the pest's natural enemies,

and the boosting of the plant's immune system, increasing plant defense responses more efficiently, usually through priming of plant defenses (Rasmann et al., 2017; Pieterse et al., 2014).

Then, beneficial microorganisms are a promising alternative to reduce the unsustainable use of chemical fertilizers and pesticides, improving plant fitness without compromising the available resources, for a more sustainable and balanced agriculture (Mącik et al., 2020; Kalamulla et al., 2022).

2. HERBIVORY

Because of their diversity and abundance, insects are among the most important threats to plant performance, causing severe yield losses (Erb and Reymond, 2019). Globally, around 10,000 distinct insect species are considered crop pests, posing a significant challenge for agriculture. Moreover, with the exacerbation of extreme weather conditions resulting from climate change, these pests have become even more detrimental, leading to substantial losses in agricultural production (Kerchev et al., 2012; Skendžić et al., 2021).

Herbivory can have negative effects on several plant traits, such as growth, survival and sexual and vegetative reproduction, which together severely reduce plant biomass and compromise plant fitness (Delaney and Macedo, 2000; Younginger et al., 2017). However, the effect of herbivory on plant fitness depends on many factors related to the herbivore lifestyle, like their feeding guilds, degree of specialization and timing of infestation (Zhou et al., 2015). Insects may be divided into two broad categories depending on their **feeding guild**: piercing and sucking insects (Hemiptera) and chewing insects (Orthoptera, Coleoptera, and Lepidoptera) (Bonaventure, 2012). Piercing and sucking insects, such as whiteflies, aphids, and psyllids remove phloem sap, typically causing minimal cellular rupture, whereas chewing insects, like caterpillars and beetle larvae lacerate plant tissue when they feed (Leitner et al., 2005; Waterman et al., 2019). Chewers cause more visible damage to crops, whereas sap-suckers may cause more subtle yet significant damage by depriving the plant of nutrients (Smith and Clement, 2012).

The **degree of insect specialization** also influences their impact on plant responses. Indeed, specialist herbivores are generally limited to feeding from plants in a specific family or even genus, and they are typically able to tolerate species-specific defensive metabolites (Beran and Petschenka, 2022). In contrast, generalist herbivores, having a wider host range, typically show a lower tolerance to specialized plant toxins and tend to feed from less-defended parts of the host plant (Ballhorn et al., 2010; Rothwell and Holeski, 2020). Finally, the **time of exposure** to insect infestation and the extension of tissue damage that occurs during feeding significantly affects plant responses due to different plant metabolic reprogramming (Zhou et al., 2015). Indeed, the duration of caterpillar infestation may have a greater effect on plant primary metabolism than the degree of herbivore specialization or even the tissue being assayed (Appel et al., 2014).

All these herbivory features influence the plant's response to the attack. This is the result of a long co-evolutionary battle between both parties (plants and insects), with plants evolving strategies to fight herbivores to minimize insect damage and their harmful effects, while insects have evolved strategies to counter plant defenses to ensure their survival (War et al., 2018; Beran and Petschenka, 2022).

2.1 Plant defense responses to herbivory

Throughout their co-evolution with pests, plants have developed sophisticated defense strategies that can be classified as constitutive and inducible (Mithöfer and Maffei, 2017). The **constitutive defenses** represent physical and chemical plant barriers that are present regardless of the herbivore attack. For example, physical barriers, including spines, waxy cuticles, trichomes, thorns and lignified cell walls, are the first line of defense that reduces the accessibility of insect pests to the plant (Ziv et al., 2018; Lee et al., 2019; Liu et al., 2017; Belete, 2018). Chemical barriers are typically stored in inactive forms, comprising toxic compounds such as alkaloids, phenols, anthocyanins, terpenoids and quinones, and their synthesis depends on the plant specialization (War et al., 2012; Speed et al., 2015; Divekar et al., 2022). Conversely, **inducible defenses** are manufactured only after the perception of the attacker. These inducible defenses are activated through hormone-mediated defense signaling and impact herbivore performance and fitness by increasing mechanical protective structures or toxic compounds (Erb and Kliebenstein, 2020; Nawaz et al., 2022). These compounds may kill, retard or reduce the development or reproduction of the pest herbivores (Hanley et al. 2007). Among the inducible defenses are the production of defensive proteins such as proteinase inhibitors, toxic secondary metabolites (see section 2.3), and volatiles that affects the pest in a direct or indirect manner (Dicke et al., 2009; Gebreziher, 2018; Mostafa et al., 2022; Nawaz et al., 2022) (Figure 2).

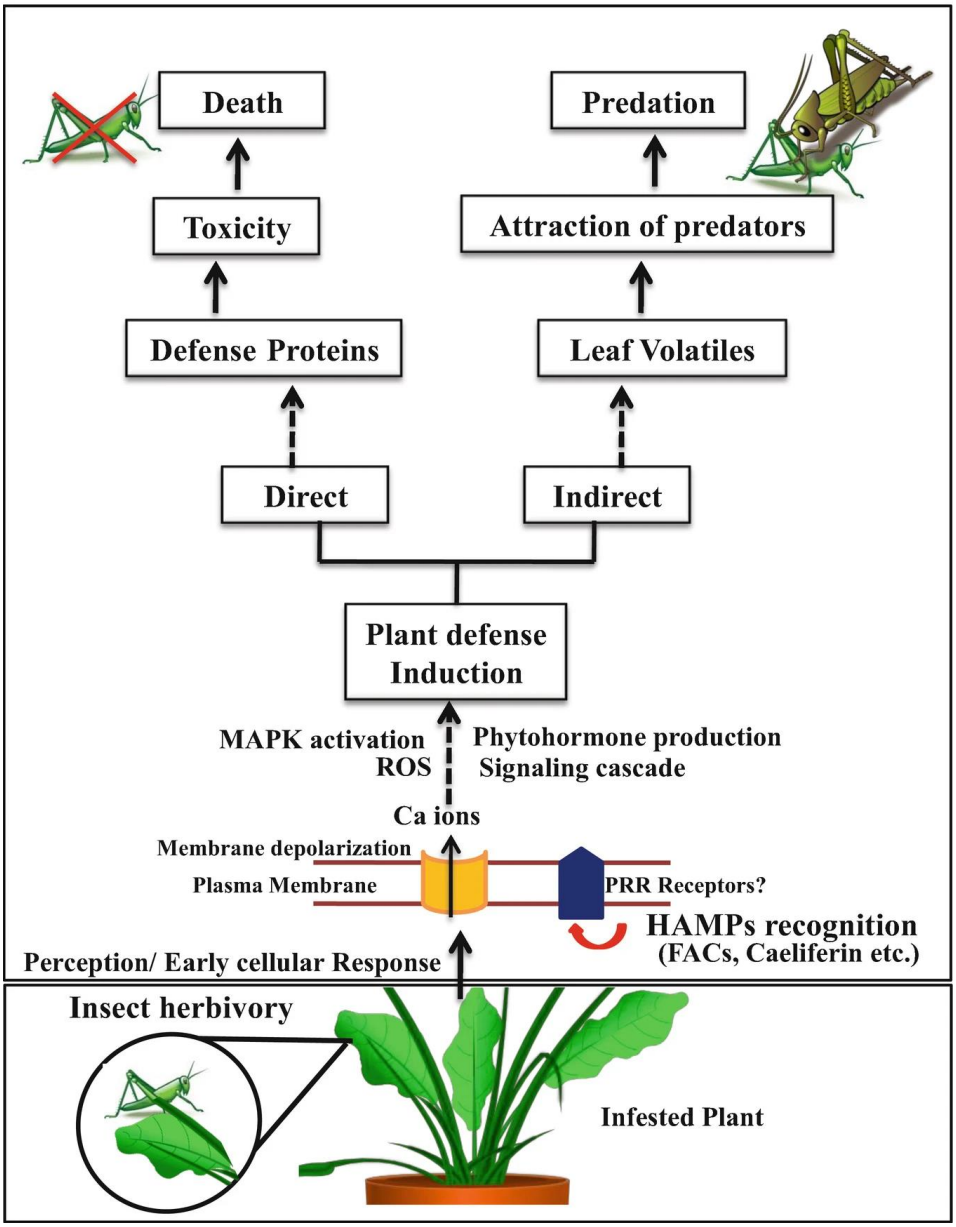

FIGURE 2. Plant direct and indirect defenses induced by herbivore attack. Mechanical damage and exposure to oral secretions from herbivores lead plants to activate direct (such as defensive proteins like protease or trypsin inhibitors) and indirect (such as aromatic compounds, terpenes, and green-leaf volatiles) defense responses (Malik et al., 2021).

The synthesis of **proteinase inhibitors (PIs)** is an effective **direct** mechanism for plants to defend themselves against insect attacks. These inhibitors target the gut proteases of the insect, playing a crucial role in the plant's defense response against herbivores (Singh et al., 2018). Insects possess digestive proteases to break proteins into free amino acids, necessary for their growth and development (Zhu-Salzman and Zeng, 2015). Plant's PIs constitutively produced or accumulated after herbivory, are proteolytic enzymes that cleave specific peptide bonds to protect plants against phytophagous insects (Nawaz et al., 2022). Plants' PIs have the ability to target the digestive proteases involved in food digestion and nutrient absorption of the

majority of insects, binding their digestive enzymes and inhibiting larval development by developing stable complexes that block active sites of their corresponding proteases (Volpicella et al. 2011; Stevens et al. 2013). For example, protease inhibitor II (PINII) impairs the growth and development of lepidopteran insects due to the inhibition of serine protease-like enzymes in the larval gut (Saikhedkar et al., 2018). Similarly, trypsin inhibitors reduce the protease activity in the midgut of Lepidoptera leading to weight reduction, malformation and an increased cost of metabolism (Machado et al., 2017). Thus, PIs provoke a direct negative effect on herbivore fitness, inactivating intestinal proteolysis that disturbs the protein digestion, reducing plant nutritional values and restricting the availability of critical amino acids needed for insects' developmental process and survival (Nawaz et al., 2022). Other inducible direct defense mechanism relies on the production of toxic secondary metabolites that directly inhibits pest development (see section 2.3).

Besides direct defenses, plants can induce **indirect** defenses by realizing volatiles (secondary metabolites) such as aromatic compounds, terpenes, and green-leaf volatiles after pest attack (Erb and Kliebenstein, 2020). The identity of these compounds varies depending on the species and plant cultivar, including terpenoids, volatile fatty-acid derivatives, benzenoids, phenylpropanoids, and volatile amino-acid derivatives (Yactayo-Chang et al., 2020). Plants have the extraordinary capacity to recognize specific compounds from insects' oral secretions or oviposition fluids (Zunjarrao et al., 2020). This recognition triggers the production of defensive compounds to repel the herbivore or attract natural enemies, such as insect predators or parasitoids to control the pest population (Fürstenberg-Hägg, 2013). For example, the release of indolic volatiles induces MAP kinase phosphorylation and primes systemic tissues of the plant to release terpenes in response to herbivory (Erb et al., 2015; Ye et al., 2019). The release of plant terpenes (such as linalool, β-ocimene, (+)-R-limonene, etc.) induces defenses against insect pests, for instance, attracting parasitoids or parasitic insect (such as parasitic wasps) that attack the larvae of a wide range of lepidopterans hosts, promoting the oviposition of pest predators, attracting pest females or acting as a deterrent for the pest (Boncan et al., 2020).

Resistance traits are costly, and their up-regulation after attack requires energy, and as such, may require reductions in growth, reproduction or storage, and/or increases in assimilation to meet their metabolic demands (Schwachtje and Baldwin, 2008). Thus, rearrangement of the plant primary metabolism is important for a cost-efficient management of resources. In fact, the plants' ability to differentiate between herbivory and other challenges is of great importance, allowing them to allocate defense resources more efficiently by producing and releasing compounds that are specifically effective against herbivores

(Fürstenberg-Hägg et al., 2013). During an herbivore attack, plants need to reconfigure their metabolic profile not only to support defense responses, but also to support physiological adjustments to tolerate herbivory and reduce the negative consequences of pest attack in plant fitness (Erb and Kliebenstein, 2020). Indeed, during an attack, plant reallocate primary metabolites to move them between damaged and storage tissues, use them as defense signals or precursors of secondary metabolites (Zhou et al., 2015).

Thus, despite plant's resistance to herbivore attack is thought to be principally determined by its secondary metabolism, plants responses to herbivory drastically modify the metabolic profile involving changes in both primary and secondary metabolisms to be able to tolerate and/or resist to herbivore attack.

2.2 Modulation of primary metabolism upon herbivory

2.2.1 Introduction to CH metabolism

Plant carbohydrate metabolism is formed by a complex and finely regulated network of enzymatic reactions that catabolize the biosynthesis, hydrolysis and transport of sugars. Carbohydrates produced from photosynthesis control biological processes in growth and development and, at the same time, they can also act as signal molecules in response to abiotic and biotic stresses (Ciereszko, 2018; Avila-Sakar 2020; Keller et al., 2021). **Sucrose**, the main product of photosynthesis, can be stored in compartments such as vacuoles, and is transported through the phloem from source to sink organs to promote plant growth and development or be stored as a reserve (Eom et al., 2012; Griffiths et al., 2016; Yoon et al., 2021) (Figure 3). The biosynthesis of sucrose starts with the **sucrose phosphate synthase** (SPS), converting fructose-6-phosphate and uridine diphosphate-glucose (UDP-glucose) into sucrose-6-phosphate, a substrate for sucrose (Stein and Granot, 2019). Different enzymatic transporters control the sucrose distribution. Among them, the SUTs family regulates the loading and unloading of sucrose in various tissues (Chen et al., 2015). For example, in tomato, the low-affinity sucrose transporter SUT2, located in phloem sieve elements, has a crucial role unloading sucrose (Hackel et al., 2006; Barker et al., 2000; Marco et al., 2021).

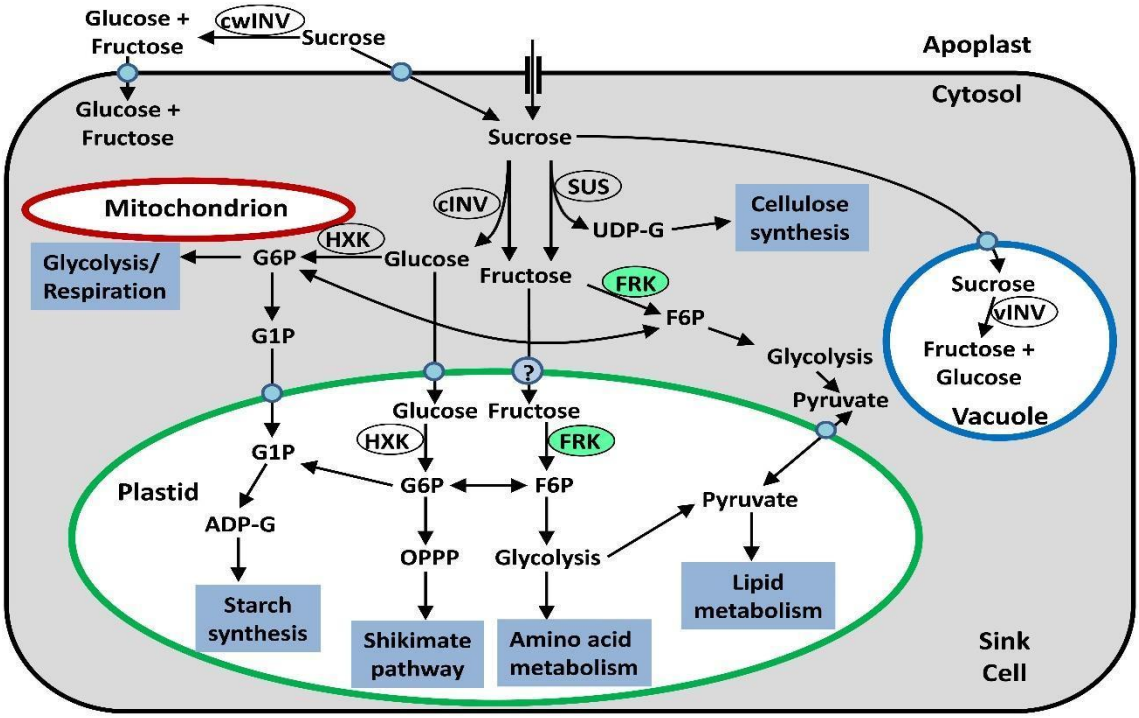

FIGURE 3. Simplified schematic presentation of sugar metabolism in sink tissue cells. Sucrose may be hydrolyzed in the apoplast by cell-wall invertase (cwINV) to yield glucose and fructose, which can be brought into the cell by a monosaccharide transporter. Alternatively, sucrose can be brought into the sink cell by a sucrose transporter or enter through plasmodesmata. Inside the cell, the sucrose can be stored in the vacuole or hydrolyzed by vacuolar invertase (vINV). In the cytosol, sucrose can be hydrolyzed by cytosolic invertase (cINV) to yield glucose and fructose, or cleaved by sucrose synthase (SUS) to yield fructose and uridine diphosphate-glucose (UDP-G). Glucose can be phosphorylated by mitochondria-associated hexokinase (HXK) so that it can be used for glycolysis and then respiration, or be brought into the plastids by a plastidic glucose transporter and then phosphorylated by plastidic hexokinase so that it can be fed into plastidic metabolic pathways. Fructose can be phosphorylated by cytosolic fructokinases (FRK; marked in light green) and then used for cytosolic glycolysis or be brought into the plastid by an unknown transporter, phosphorylated by plastidic FRK (marked in light green) and then fed into plastidic metabolic pathways. Abbreviations: glucose 1-phosphate (G1P), glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), oxidative pentose phosphate pathway (OPPP), adenosine diphosphate-glucose (ADP-G) (Stein and Granot, 2018).

A set of metabolic enzymes utilize sucrose as an energy source, signaling molecule or as a building block (Braun et al. 2022). The sucrose synthase family encoded by *SUS* genes and invertases are essential enzymes for **sucrose cleavage** (Yoon et al., 2021; Wang et al., 2022). The sucrose synthase catalyzes the reversible cleavage of sucrose, using UDP to yield fructose and UDP-glucose, while the invertases catalyze the irreversible hydrolysis of sucrose, producing the hexoses glucose and fructose (Stein and Granot, 2019). Up to now, three **invertase enzymes** have been described: the cytosolic invertases (CytInv), localized in the cytosol; the acidic or vacuolar invertases (VacInv), located in the vacuole, where sucrose is stored; and finally, the cell wall-bound invertases (CwInv), located in the apoplast of sink tissues to transport and unload sucrose (Roitsch and Gonzalez, 2004; Wang et al., 2022). **Invertases** play a crucial role in carbohydrate partitioning, controlling the differential sink strength of plant tissues, but additional CH-related enzymes are also crucial for plant physiology (Proels and Hückelhoven,

2014; Schultz et al., 2013; Jammer et al. 2020). Functional analyses show that enzymatic activities related to cell-wall formation (UDP-glucose pyrophosphorylase, UGPase), sucrose biosynthesis (fructokinase, FK), starch biosynthesis (ADP-glucose pyrophosphorylase and phosphoglucomutase; AGPase and PGM, respectively), oxidative pentose phosphate pathway (glucose-6-phosphate dehydrogenase, G6PDH) and glycolysis (hexokinase, phosphoglucoisomerase, aldolase and Phosphofructokinase; HXK, PGI, Ald and PFK, respectively) are essential for plant growth and development as well as for stress responses (Jammer et al. 2022).

Finally, the **tricarboxylic acid (TCA) cycle**, also known as the Krebs or citric acid cycle, regulates the energetic status of the cell and the carbon skeleton supply and is an essential part of aerobic respiration (O'Leary and Plaxton, 2016; Liu et al., 2019). It consists of 8 enzymes that catalyze mostly reversible reactions to produce TCA cycle intermediates such as citrate, malate and succinate (Liu et al., 2019). Noteworthy, many metabolites derived from glycolysis and the TCA cycle often serve as precursors for the synthesis of secondary metabolites (Pott et al., 2019). Thus, carbohydrate metabolism needs a precise activation of metabolic pathways, leading to biosynthesis, cleavage and allocation of sugar products to support plant growth, development and response to stresses.

2.2.2 Changes in CH metabolism upon herbivory

Upon herbivore attack, plants must allocate energy to activate stress signaling networks and initiate adaptive responses to the new stress condition (Mithöfer and Boland, 2012; Keller et al., 2021). Carbohydrates produced by photosynthesis are the highest source of stored energy for both plants and herbivores. Therefore, different hypotheses have been developed to understand how plants alter photosynthesis to optimize defenses (Zhou et al., 2015).

Although in literature it is described that herbivory can increase or decrease photosynthetic activity, the most common case is the **reduction of photosynthesis** to spare energy for the production of secondary metabolites or reduce available carbohydrates to the insect (Giri et al., 2006; Gutsche et al., 2009; Wei et al., 2009; Bilgin et al., 2010; Coppola et al., 2013; Appel et al., 2014; Kumar et al., 2020). Plants decrease the expression of photosynthesis-related genes when fed by chewing, sap-feeders or phloem-sucking insects (Reymond et al., 2004; Lawrence et al., 2008; Zhu-Salzman et al., 2004; Botha et al., 2006; Kozlov and Zvereva, 2018). However, plants also need energy to produce inducible defenses (Cipollini et al., 2018). For instance, many plants promote the catabolism of energy storage compounds at the site of

insect feeding (e.g. sucrose or starch,) or promote a systemic carbon reallocation in undamaged tissue (Zhou et al., 2015). This rapid **reallocation**, from damaged leaves to storage tissues, is known as induced sequestration (Orians et al., 2011; Osorio et al., 2014). This phenomenon is driven by the differential sink strength of the tissues, which is strongly dependent on CWInv (Sturm and Tang, 1999; Schultz et al., 2013; Li et al., 2021). These enzymes play a crucial role in herbivory breaking down sucrose into glucose and fructose (Siddappaji et al., 2015; Kundu et al., 2018), and work as signal molecules inducing defense-related hormonal pathways and reinforcing cell walls (Veillet 2016; Tauzin and Giardina, 2014). For example, fructose is involved in ABA- and ET-signaling (Cho and Yoo, 2011), and glucose in the hexokinase signaling pathway (Cho et al., 2009). Glucose levels regulate gene expression, primary and secondary metabolism as well as growth and developmental programs (Sheen, 2014; Siddiqui et al., 2020). Hence, this molecule can be crucial for leaf-to-leaf systemic defense activation upon herbivory, indicating an active role for sugars in plant defense (Kundu et al., 2018).

Several studies -with different plant-insect foliar pest models- have shown increased activity of enzymes involved in carbohydrate hydrolysis, such as **invertases.** For instance, in grain amaranth and Arabidopsis plants attacked by different insect herbivores, the CWInv, CytInv and other enzymes involved in the degradation of complex carbohydrates increase their activity in leaves, driving herbivore-induced carbohydrate depletion (Castrillón-Arbeláez et al., 2012; Appel et al., 2014; Ferrieri et al., 2015). Similarly, tomato plants attacked by a chewing herbivore showed a drastic reduction of sucrose, glucose, and fructose in local leaves. Interestingly, the glucose levels increase in systemic leaves, indicating a possible carbon reallocation from local to systemic tissues (Kundu et al., 2018). These observations suggest that the products of the primary metabolism are distributed from injured to uninjured tissues as a strategy to hamper herbivore performance, reducing plant tissue's nutritive value, preserving resources for later regrowth or being incorporated into defense-related compounds (Zhou et al., 2015; Kundu et al., 2018). For instance, Ferrieri et al. (2012) demonstrated by radioactively labeling CH that –in wounded or Me-JA treated plants– the CH are exported from injured to systemic leaves and incorporated in defensive compounds (such as cinnamic acid and phenolic glycosides). Thus, plant response to herbivory involves different changes in primary metabolism that, in part, promote the production of secondary metabolites (War et al., 2020) (Figure 4).

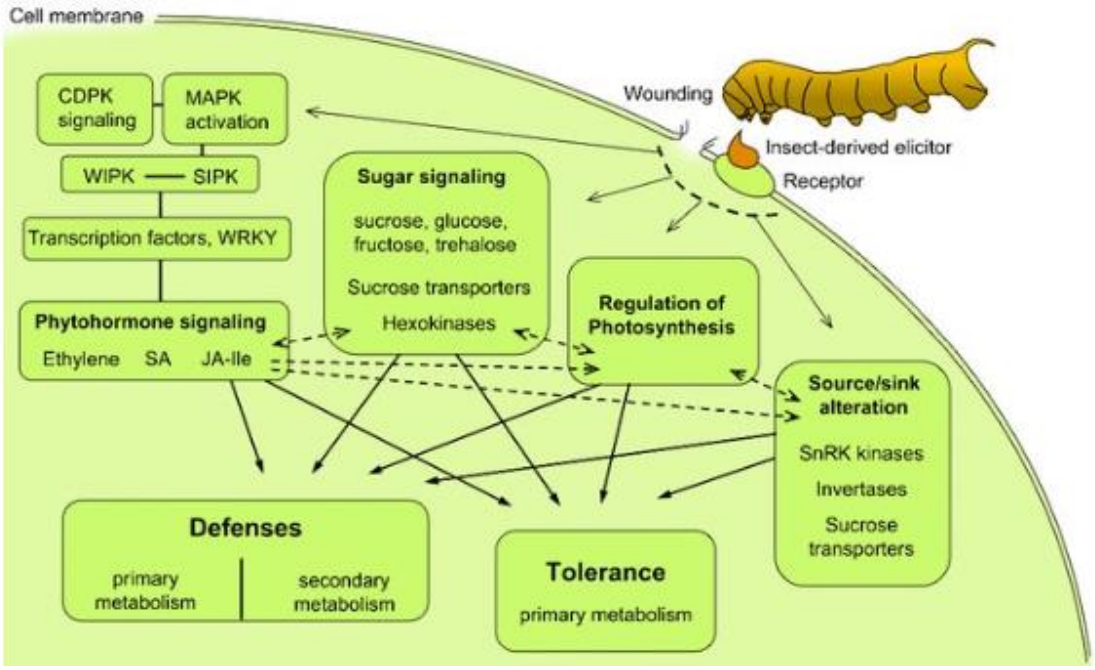

FIGURE 4. Plant physiological reconfiguration after herbivore attack. Resistance signaling is elicited differently from simple wounding when herbivore-specific elicitors are introduced into wounds during caterpillar feeding. Signaling depends on primary metabolites. Herbivory induces a large reorganization of primary metabolism, including altered photosynthesis and altered sink/source relations that are coordinated by a signaling network. The expression of defense and tolerance traits requires changes both in primary and secondary metabolism (Schwachtje and Baldwin, 2008).

2.3 Activation of secondary metabolism

Secondary metabolism is defined as all metabolic pathways connecting small molecular products that are non-essential for the growth and reproduction of the organism (Yang et al., 2018). Although their definition suggests that secondary metabolites are not indispensable for plant survival, these molecules play a variety of crucial functions for plant life that improve plant fitness and increase plant tolerance and resistance to environmental abiotic and biotic stresses (Piasecka et al., 2015; Yang et al., 2018; Ashraf et al., 2018; Pang et al., 2021). Plants produce hundreds of thousands of specialized metabolites from either primary metabolites or intermediates of these primary metabolites, many functioning as defenses by reducing the digestibility of plants (Fürstenberg-Hägg et al., 2013; Piasecka et al., 2015; War et al., 2020).

According to their biosynthetic pathways and their chemical structures, secondary metabolites are classified into three large groups: **nitrogen-** and **sulfur-containing compounds** (as alkaloids, cyanogenic glycosides, benzoxazinoids and glucosinolates), **terpenes** and **phenolic compounds** (as lignin, coumarins, furanocoumarins, tannins and flavonoids) (Ashraf et al., 2018) (Figure 5).

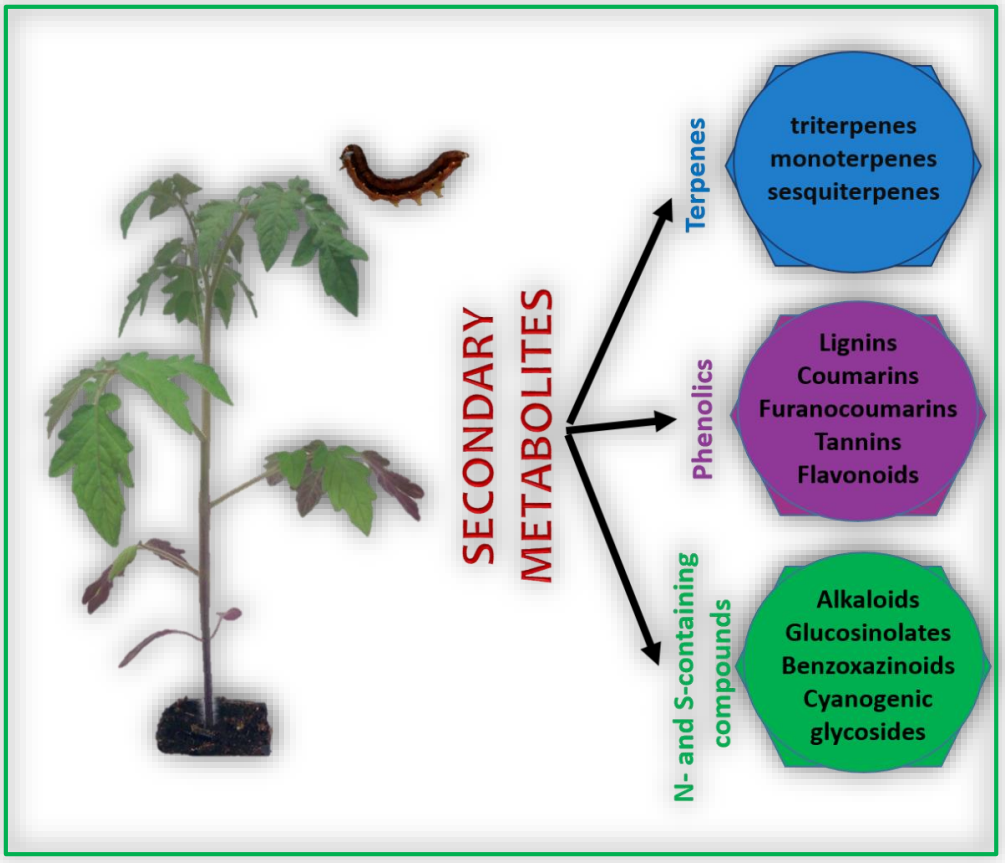

FIGURE 5. Plant secondary metabolites. The main plant secondary metabolites produced by plants as a defense mechanism against herbivores are typically categorized into phenolics, terpenes, and compounds containing sulfur (S) or nitrogen (N).

Among **N-containing compounds, alkaloids** are widely distributed bioactive natural products (>15.000 different alkaloids found in 20% of all vascular plants) that are prevalently found in a plant belonging to the *Berberidaceae, Amaryllidaceae, Liliaceae, Leguminaceae, Papaveraceae, Ranunculaceae*, and *Solanaceae* (Matsuura and Fett-Neto, 2017). These metabolites are biosynthesized mostly from amino acids in various parts of the plants (such as the leaves, roots, seeds, etc.) and are known for their effects in mammals (e.g., caffeine, nicotine, morphine, strychnine, and cocaine) (Bhambhani et al., 2021). In addition, they have an antiherbivore effect in insects due to disruption of neuronal signal transduction, interference in DNA replication, protein synthesis and enzyme activity (Züst and Agrawal, 2016; Ali et al., 2019). **Cyanogenic glycosides** are also nitrogen-containing secondary metabolites, derived from aminoacids, involved in defense that deter herbivores by releasing toxic hydrogen cyanide upon tissue disruption (Nahrstedt, 2007). **Glucosinolates** and **benzoxazinoids**, depending on plant species, may elicit callose deposition and regulate the accumulation of other secondary metabolites, such as phenylpropanoids, after a plant attack (Li et al., 2018; Kim et al., 2015).

Terpenes are the largest group of secondary plant metabolites and the most diverse (>25.000 known structures) (Divekar et al., 2022). These metabolites serve as attractants of pollinating insects and play a role in plant defense, acting as antifeedants, repellents, toxins, oviposition deterrence or modifiers of insect development (Aharoni et al., 2005). As mentioned before, terpene volatiles indirectly protect plants by increasing the efficacy of attracting natural herbivore enemies or directly repelling pest herbivores (Mumm et al., 2008; Boncan et al., 2020). Some examples of terpenes that play an active role in plant defense are iridoids, and volatile compounds, such as mono and sesquiterpenes, α-bisabolene and β-caryophyllene (Wouters et al., 2016).

Phenolic compounds may be produced after plant attack and play a significant role in host plant resistance against herbivores, acting as defensive metabolites by repelling pest insects and inhibiting herbivores' enzymes (War et al., 2012; Gantner et al., 2019). **Lignins** are phenolic derivatives providing a physical barrier against herbivory, hardening plant tissue, reducing the leaf's nutritional content and making it undigestible for insects (Jan et al., 2021). **Coumarins** inhibit herbivores- such as aphids and caterpillars- thus playing a role in plant protection (Stringlis et al., 2019; Barrero et al., 2013). **Furanocoumarins** are toxic compounds released by a few plant species that protect the plant against insect feeding (Jan et al., 2021; Harvey et al., 2020). **Tannins,** anthocyanin-derived polyphenol compounds, are also induced after herbivore attack and reduce the nutritional quality of plant tissues, forming complexes that reduce the amount of nitrogen, and prevents protein hydrolysis by the insect digestive enzymes (Perkovich and Ward, 2022; Kariñho-Betancourt et al., 2018).

Flavonoids play a central role in various aspects of the plant cycle, but mainly in the plant-environment interaction, defending plants against biotic and abiotic stresses. Thanks to their structural differences, flavonoids are divided into seven subclasses: flavonols, flavones, isoflavones, flavanones, flavanols, anthocyanidins (including anthocyanins) and chalcones (Shen et al., 2022). Some are highly produced and accumulated in plants after herbivore attacks, influencing pest behavior, growth and development (Bos et al., 2010; Shen et al., 2022; Zhao et al., 2020). Other phenols with antifeedant properties are metabolites derived from phenylalanine, such as caffeic, ferulic acid and benzoic acid derivatives (Singh et al., 2021).

As previously mentioned, secondary metabolites can be present constitutively in plants and stored in an inactive form (phytoanticipins) or can be induced in response to insect or pathogen attack (phytoalexins) (Yactayo-Chang et al., 2020). The constitutive synthesis and storage of toxic compounds can be costly, as it involves a major reallocation of resources from growth to defense (Mithöfer and Maffei, 2017; Keller et al., 2021). Therefore, in environments

where the risk of attack is not excessively severe, defense mechanisms have evolved toward their **inducibility** (Bekaert et al., 2012). In addition, inducible defenses difficult the chances for the attackers to adapt to induced metabolites reducing pest performance (Howe and Jander, 2008; Agrawal, 2011). Usually, toxic compounds intoxicate generalist herbivores that are less tolerant to specialized plant toxins, while specialists have developed mechanisms to detoxify harmful compounds produced by plants, but at the cost of their growth and development (Kessler and Baldwin, 2002; Steinbrenner et al., 2011). Thus, **inducibility relies on efficient perception and recognition of the aggressor** to trigger the most effective defense response signaling and reprogramming cellular functions.

3. REGULATION OF PLANT DEFENSE RESPONSES

Plants have evolved finely tuned strategies to counteract a multitude of environmental abiotic and biotic challenges. These strategies involve short-term responses to prevent severe damage and long-term adaptations to acquire stress tolerance at the whole plant level (Takahashi et al., 2019). An early perturbation in a cellular component can activate stress-sensing mechanisms if the perturbation is recognized and amplified by molecular machinery in the cell (Zhu, 2016). For example, plants are able to detect insect herbivores through extracellular receptors. They are responsible for sensing damage and identifying specific molecules released by the insects, such as Herbivore-Associated Molecular Patterns (HAMPs) or Damage-Associated Molecular Patterns (DAMPs) (Erb and Reymond, 2019) (see section 3.2).

The stress-response mechanisms in plants are highly intricate and require several integrated pathways to be activated in response to external stresses (Lamers et al., 2020). For example, environmental stresses and stress-related signals modify the plasma membrane potential and modulate the ion fluxes altering H+ and calcium intracellular levels (Kudla et al., 2018). Together with other molecules as reactive oxygen or nitrogen species (ROS and RNS, respectively), they act as secondary messengers, activating signaling cascades and regulating downstream responses to activate gene expression (Tyagi et al., 2022). For instance, ROS rapidly increases in response to stress and activates mitogen-activated protein kinase (MAPK) phosphorylation cascades and the subsequent upregulation of specific stress-related genes (Jalmi and Sinha, 2015). Furthermore, MAPK pathways show cross-talk with ROS by a feedback mechanism, but also with different **phytohormones** (Zhang M. et al., 2018). Phytohormones regulate a wide range of physiological and defensive processes, and they are critical endogenous factors in mediating plant stress responses (Verma et al., 2016).

All the early signaling events, such as calcium oscillation, ROS and MAPK phosphorylation, are rapidly triggered in response to stress conditions, while others, such as phytohormones accumulation, are slower signals (Verma 2016; Takahashi et al., 2019). Phytohormones are induced under stress conditions and are transported at different speeds, mainly through the vasculature, to respond to different environmental stresses, mediating long-distance signaling to maintain stress adaptations (Takahashi et al., 2019).

All these changes coordinate plant defense responses, occurring within seconds-minutes after an herbivory attack until hours or days (See Figure 6) (Zebelo and Maffei, 2015; Mostafa et al., 2022).

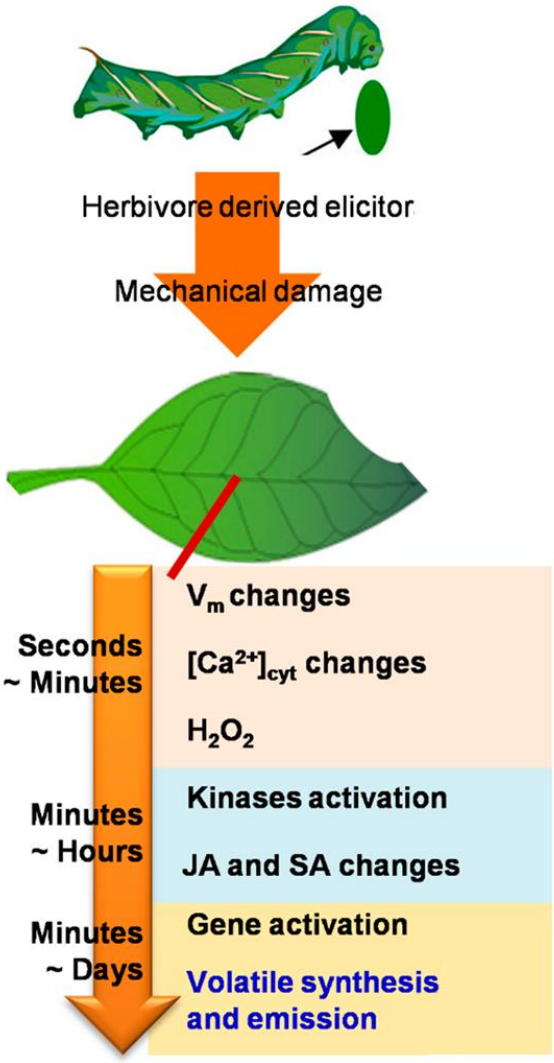

FIGURE 6. Events occurring during plant defense responses upon herbivore attack. Mechanical damage and exposure to HAMPs due to herbivore attacks modify plasma membrane potential, modulate the ion fluxes (such as calcium; Ca^2) and elicit signaling cascades (H_2O_2, ROS, RNS) leading to rapid accumulation of jasmonates (JAs) and activation of defense-related gene expression. Jasmonate also stimulates an increased accumulation of secondary metabolites. Modified from Dong et al. (2016).

3.1 Phytohormones in the regulation of plant defense response

Phytohormones are small molecules acting at low concentrations (Fahad et al., 2015). Hormonal networks connect perception and signaling, activating signaling cascades, transcriptional changes and metabolic (Pozo et al., 2015; Erb and Reymond, 2019). Noteworthy, **phytohormone-dependent signaling** regulates the induction of plant defenses and the activation of the plant immune system during herbivory (Divekar et al., 2022).

Phytohormones have a major role in plant defensive responses against biotic factors. Depending on the type of attacker, specific hormonal pathways are activated, triggering phytohormones accumulation and increasing plant immunity (Aerts et al., 2021). Among the

defense-related phytohormones, salicylic acid (SA) and **jasmonic acid (JA)** form the core of the immune system, and both hormones generally antagonize each other's pathways (Wasternack and Song, 2017; Zhang and Li, 2019). SA regulates plant defenses against biotrophic pathogens, reducing their penetration and spread, while JA orchestrates defense responses against chewing herbivores as well as necrotrophic pathogens and wounding (Ding and Ding, 2020; Diezel et al., 2009; Bruessow et al., 2010; Inbar and Gerling, 2008; Wasternack & Hause, 2013; Conrath et al., 2015; Erb and Reymond, 2019).

The **JA signaling** pathway includes repression and activation modes (Wang et al., 2021). During normal conditions, the level of JA-Ile in the cytoplasm is low, and genes involved in JA synthesis are inactivated (repression) (Ali and Baek, 2020). In this state, JASMONATE-ZIM DOMAIN (JAZ) proteins act as transcriptional repressors of JA-responsive genes by interacting with transcriptional factors and blocking their activity (Liu and Timko, 2021). However, under stress, JA-Ile is accumulated at high levels and binds its core co-receptor forming a complex that leads to JAZ proteins degradation, releasing the previously bound transcriptional factors and initiating the JA response (Chini et al., 2017) (Figure 7).

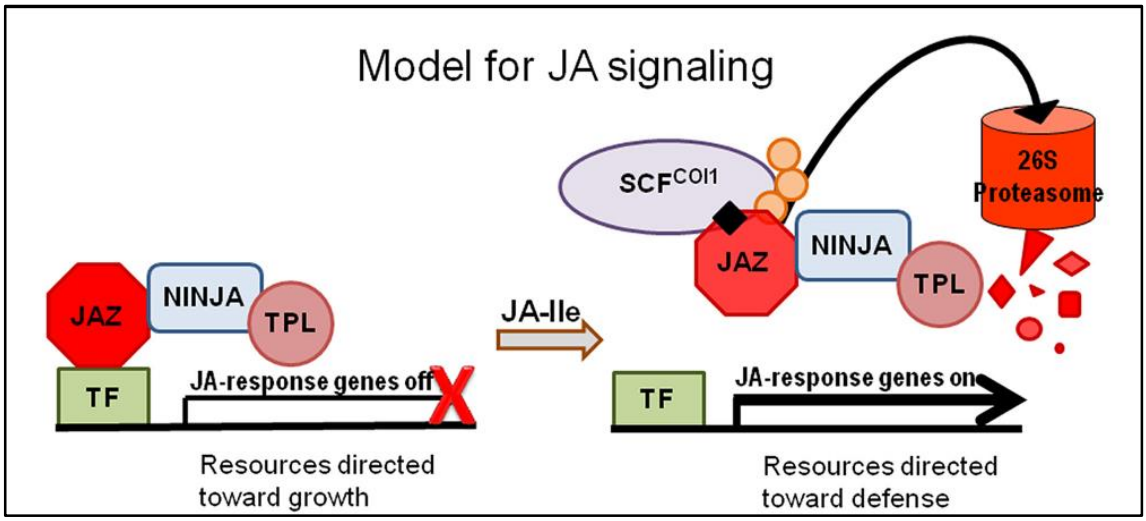

FIGURE 7. JASMONATE-ZIM DOMAIN (JAZ) proteins bind transcription factors (TFs) and recruit co-repressors, including NINJA and TOPLESS (TPL), to repress gene transcription in the absence of the active form of jasmonate (JA), JA–Ile (black diamond). In the presence of JA–Ile, the hormone facilitates interaction between JAZ proteins (Wager and Browse, 2012).

Upon herbivore attack, JA signaling coordinates plant defenses, inducing transcriptional changes that lead to the accumulation of secondary metabolites with toxic, antifeedant or antinutritional properties, such as glucosinolates, benzoxazinoids and alkaloids (Divekar et al., 2022). JA also triggers the production of inducible proteins like polyphenol oxidase, arginase,

threonine deaminase, leucine amino peptidase and proteinase inhibitors to reduce plant digestibility (Orozco-Cardenas et al., 1993; Constabel et al., 1995; Felton et al., 1994; Lisón et al., 2006; Howe and Jander, 2008; Schweizer et al., 2013; Dafoe et al., 2011; Wang et al., 2015; Wang et al., 2021). Many of these proteins are highly accumulated in insect-damaged leaves and are also present in the insect gut (Chen et al., 2005; Chen et al., 2007; Nawaz et al., 2022). Thus, the induced expression of JA-related genes increases the effectiveness of defense, and the fine-tuned regulation of JA signaling reduces the high allocation costs associated with the production of defenses (Howe and Jander, 2008). Additionally, JA, methyl jasmonate (MeJA), and jasmonoyl-L-isoleucine (JA-Ile; the bioactive form of JA) can be transported through both phloem and xylem from wounded tissues to distal unwounded tissues to activate systemic responses in the plant (Thorpe et al., 2007; Farmer et al., 2014; Zhang H. et al., 2020; Schulze et al., 2019).

In addition to JA, other stress-related phytohormones, such as **abscisic acid (ABA)** and **ethylene (ET),** modulate plant defense responses against herbivores (Aerts et al., 2021). Although **ABA** regulates plant responses against abiotic stresses, it is an important modulator of the plant immune system, coordinating with JA the expression of the central positive regulator MYC2 to enhance protection against chewing insects (Adie et al., 2007; Pieterse et al., 2012; Wang et al., 2018). Several studies demonstrate that plants impaired in ABA biosynthesis are more susceptible to herbivores -regardless of their feeding guild or specialism- accumulating lower JA and defense metabolites (Bodenhausen and Reymond, 2007; Dinh et al., 2013; Hillwig et al., 2016). On the other hand, it has been described that **ET** antagonizes JA- and ABA-related responses against herbivores in Arabidopsis (Verhage et al., 2011; Song et al., 2014a). However, lower ET levels in rice reduce plant resistance against a chewing herbivore, but increase resistance to a phloem feeder insect (Lu et al., 2014), and ET signaling is required in tomato for the JA burst upon herbivory (Hu et al., 2021). These results indicate that ET can positively or negatively affect plant resistance against insects depending on the plant species and the insect's feeding strategy (Ma et al., 2020), illustrating the complexity of phytohormone signaling during herbivory (Erb and Reymond, 2019)

Similarly, other growth-related hormones modulate antiherbivore defenses, such as **auxin (AUX)**, **cytokinins (CKs)** and **gibberellins (GAs)**. **AUX** regulates a broad range of plant growth and developmental processes, and the most common naturally occurring form of AUX is the indole-3-acetic acid (IAA) (Gallei et al., 2020). This phytohormone generally acts synergistically with JA in response to wounding and herbivore attacks (Hentrich et al., 2013; Pérez-Alonso et al., 2021). For instance, IAA biosynthesis increases in leaves and roots under

caterpillar attack. Furthermore, its accumulation promotes the synthesis of secondary metabolites like phenols and flavonoids that, together with JA, induces changes in the primary metabolism dynamics and allocation (Lulu et al., 2015; Mahdieh et al., 2015; Machado et al., 2013; Machado et al., 2016). Additionally, IAA is involved in anthocyanins biosynthesis and allocation in systemic uninjured tissues (Lev-Yadun et al., 2008; Machado et al., 2016). **CKs** regulate systemic responses against herbivory, inducing the accumulation of JA and increasing the activity of phenolamides and proteinase inhibitors (Schäfer et al., 2015). Additionally, the accumulation of CKs promotes stomatal closure as a pre-invasive defense and, together with SA, induces the plant's immune system as a post-invasive defense, regulating callose deposition, the expression of defense-related genes, and phytoalexin production (Arnaud et al., 2017; Cortleven et al., 2019). Finally, it has been shown that **GAs** can inhibit JA-related responses. This hormone regulates plant growth through DELLA repressors (Blázquez et al., 2020). DELLA represses GA signaling, thus, repressing growth, and these repressors modulate the JA pathway by physically interacting with JAZs, thereby preventing the negative effect of JAZs on MYC2-related defense expression (Song et al., 2014b). Thus, activation of the GA pathway leads to DELLA degradation, and therefore, JAZ release and inhibition of JA responses (Erb and Reymond, 2019). **Hormone crosstalk** is a complex network of synergistic, antagonistic and additive interactions among the different hormone-regulated signaling pathways. Thus, its coordination provides an efficient regulatory system to shape and fine-tune the appropriate response to specific stresses (Aerts et al., 2021; Nambara and Wees, 2021) (Figure 8). In nature, plants constantly face a wide range of stresses, frequently simultaneous, so they need to optimize the use of their resources between growth and defense (He et al., 2022). Therefore, plants finely orchestrate specific signaling pathways to efficiently prioritize developmental or defense processes that require a remarkable degree of signaling plasticity and control (Karasov et al., 2017). These plant **growth–defense trade-offs'** existence underscores the need for plants to maintain a delicate balance between growth and defense to successfully develop, reproduce and fight against challenges (Albrecht and Argueso, 2017). The precise coordination between phytohormones is crucial to maintain plant homeostasis and counteract any negative external challenge (Bostock et al., 2014). Then, the perception and recognition of these external stresses are fundamental for the correct development of plant defense responses.

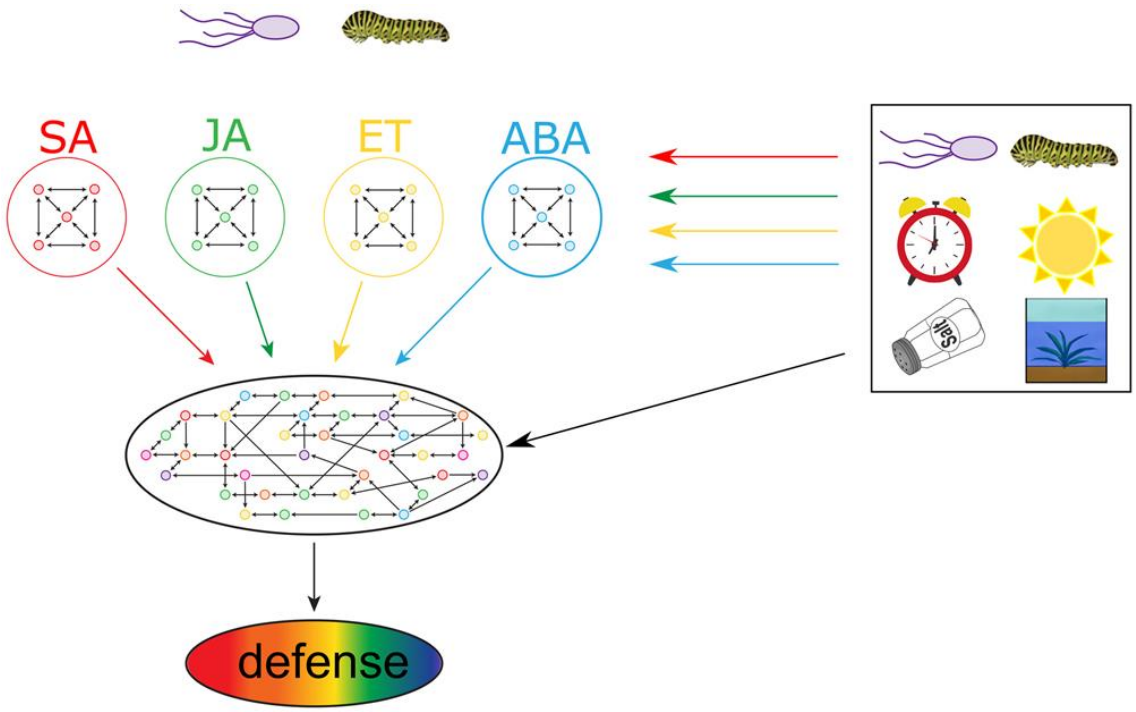

FIGURE 8. Schematic overview of integration of hormone networks involved in plant defense. Microbes and insects elicit the accumulation of specific blends of hormones. The main hormones involved in the regulation of plant defense responses are salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA). Each hormone regulates its own pathway, but also influences other hormone pathways in a complex mix of synergistic, antagonistic and additional interactions, a phenomenon known as hormone crosstalk. Moreover, the accumulation of these hormones and the responsiveness to them can be further modulated by (i) light quality, (ii) time of day, (iii) abiotic stresses such as drought, flooding and salt stress and (iv) prior or simultaneous interactions with other microbes or insects. Integration of the different hormone networks shapes the defense response leading to the elimination or accommodation of the microbe or insect in diverse environmental and internal contexts (Aerts et al., 2020).

3.2 Damage and aggressor perception

An efficient perception of danger signals and a rapid response are essential for the plant's survival (de Bobadilla et al., 2022). During insect herbivory, plants perceive the pest through extracellular receptors responsible for sensing damage and recognizing specific molecules released directly or indirectly by the insect, such as DAMPs and HAMPs (Erb and Reymond, 2019). The **cell wall** represents the first layer of defense for the plant cell, and it is fundamental for the plant to monitor cell wall integrity (Hamann, 2015). Pectin is the primary component of the plant cell wall matrix, and its integrity is compromised upon insect infestation (Mohnen, 2008). Therefore, plants have evolved specific and sophisticated mechanisms for sensing pectin integrity, monitoring critical structures in the pectin network and alerting the cells in case of danger (De Lorenzo et al., 2011). **DAMPs** are endogenous molecules with an "all-day job" in the cell but are sensed as danger signals when they appear in an abnormal compartment. That includes cell wall fragments, like oligogalacturonides (OGs; see section 3.2.1), extracellular protein fragments, peptides, nucleotides, and amino acids (Gust et al., 2017; Hou et al., 2019).

During chewing herbivory, insects damage plant cells and modify the extracellular space. Hence, the plant recognition of DAMPs induces a plethora of defense responses that trigger the activation of the plant immune system (Louis et al., 2013). **HAMPs** are generally induced by chemical compounds in herbivores' oral secretions or oviposition fluid (Gandhi et al., 2020). For example, volicitin is a peptide from *S. exigua* oral secretions, and glucose oxidase is an enzyme present in the saliva of different caterpillar species, and their perception may provoke the release of leaf volatiles and terpenoids (Reymond, 2021; Truitt et al., 2004; Acevedo et al., 2015).

3.2.1 Oligogalacturonides (OGs)

The plant's ability to perceive the presence of damage or "self" danger signals, referred to as DAMPs, is essential to activate the plant immune system (De Lorenzo et al., 2011). Characterized DAMPs include diverse molecules, such as sugars, oligosaccharides, amino acids, proteins, and nucleotides, and typically appear in the apoplast (Tanaka and Heil, 2021). **Oligogalacturonides (OGs)**, the best-characterized plant DAMPs, are oligomers of α-1,4-galacturonic acid released to the extracellular cell space upon degradation of homogalacturonan, the main component of pectin, by the cleavage activity of polygalacturonases (PG) enzymes (De Lorenzo et al., 2011; Ferrari et al., 2013; Benedetti et al., 2015; Pontiggia et al., 2020).

Different challenges may produce the release of OGs. Pathogen attack provokes the cellular rupture and release of OGs through the activity of pathogen PG (Cervone et al., 1989; Pashkoulov et al., 2002; De Lorenzo and Ferrari, 2002; D'Ovidio et al., 2004; Ferrari et al., 2013). Wounding also induces endogenous PG in leaves of different plant species, including tomato (Bergey et al., 1999; Orozco-Cárdenas et al., 1999). Additionally, some insects possess PG that contributes to herbivory efficiency, while others, such as lepidoptera, do not have these enzymes (Calderón Cortés et al., 2012; Haeger et al., 2020). However, it is possible that chewing insects also produce OGs caused by their feeding guild, triggering wound responses and also inducing endogenous PG (Haeger et al., 2020; Erb and Reymond, 2019). It was demonstrated that endogenous PG activity provokes OGs release and promotes immunity (Benedetti et al., 2015). For instance, transgenic tomato plants, showing elevated levels of endogenous PG activity, are more resistant to the chewing herbivore *Manduca sexta*, suggesting that endogenous OG can elicit defense responses against herbivores (Orozco-Cárdenas et al., 2003; Ferrari et al., 2013; De Lorenzo et al, 2018; Kirsch et al., 2020).

OG fragments with a degree of polymerization between 10 and 15 are highly active elicitors that act as danger signals, triggering a downstream signaling cascade and activating plant immunity (Côté and Hahn, 1994; De Lorenzo et al., 2018; Savatin et al., 2014). In several plant species, OGs trigger a wide range of defense responses, including the production of ROS, nitric oxide, accumulation of defense-related hormones, glucanases, chitinases and secondary metabolites including flavonoids, alkaloids and lignans (Bellincampi et al., 2000; Galletti et al., 2008; Rasul et al., 2012; Davis and Currier, 1986; Davis and Hahlbrock, 1987; Broekaert and Peumans, 1988; Gamir et al., 2020). Furthermore, exogenous OGs applications induce protection in grapevine, Arabidopsis and tomato against *Botrytis cinerea*, triggering JA, SA and ET-related responses and the biosynthesis of antimicrobial enzymes (Bishop et al., 1984; Doares et al., 1995; Ryan and Jagendorf, 1995; Ferrari et al., 2007; Gravino et al., 2015; Aziz et al., 2004; Gamir et al., 2020). Local responses to OGs trigger a rapid and transient hormonal response. In contrast, at the systemic level, the hormonal changes are more robust and prolonged in time, showing that plants allocate their resources in distal parts to defend undamaged tissues (Gamir et al., 2020). Therefore, OGs perception triggers a cascade of reactions leading to early defense responses and activation of different hormonal pathways to tailor an efficient plant defense response.

3.2.2 Wall-associated receptor kinases (WAKs)

The detection of DAMPs depends on membrane-bound pattern recognition receptors (PRRs). However, only a few receptors have been identified so far. Among them, the **wall-associated receptor kinases (WAKs)** can bind pectin (Stephens et al., 2022) and possess an extracellular transmembrane helix and a cytoplasmic Ser/Thr kinase domain that potentially mediates the interaction with the cell wall (Shiu and Bleecker, 2001). Like other extracellular receptors, the WAKs family can monitor and interact with the extracellular environment (Reymond, 2021). This family is widespread in dicots such as Arabidopsis (*Arabidopsis thaliana*), tomato (*Solanum lycopersicum*), cotton (*Gossypium hirsutum*), oil seed rape (*Brassica napus*) and sweet orange (*Citrus sinensis*), and monocots crops such as wheat (*Triticum aestivum*), rice (*Oryza sativa*), maize (*Zea mays*), and barley (*Hordeum vulgare*) (Brutus et al., 2010; Kurt et al., 2020; Yang et al., 2021; Larkan et al., 2020; Dmochowska-Boguta et al., 2020; Li et al., 2020; Li et al., 2009; Zuo et al., 2015; Ameen et al., 2020). Although several studies demonstrate that WAKs have a function in growth, development and abiotic stress tolerance, the most studied function is in plant immunity, recognizing either pathogen- or plant-derived invasion molecules and, among immunity-related WAKs, WAK1 has a prominent role in damage perception and

activation of plant defenses (Wagner and Kohorn, 2001; Yin and Hou, 2017; Zhang et al., 2019; Stephens et al., 2022). For example, *SlWAK1* expression is induced in tomato leaves by the Pathogen-Associated Molecular Patterns (PAMPs) flg22 and flg28, and *SlWAK1* tomato-silenced plants display enhanced disease symptoms against bacterial infection (Rosli et al., 2013; Zhang N. et al., 2020). Additionally, the overexpression of *WAK1* triggers induced resistance against pathogenic fungi in Arabidopsis, rice and maize (Brutus et al., 2010; Li et al., 2009; Hurni et al., 2015). Therefore, this receptor is a cell wall integrity sensor with a high affinity to bind OGs in Arabidopsis, activating a downstream defense signaling cascade (as ROS production, Ca2+ ion influx, MAPK phosphorylation) (Moscatiello et al., 2006; Gravino et al., 2015; Kohorn, 2016; Ferrari et al., 2013) (Figure 9).

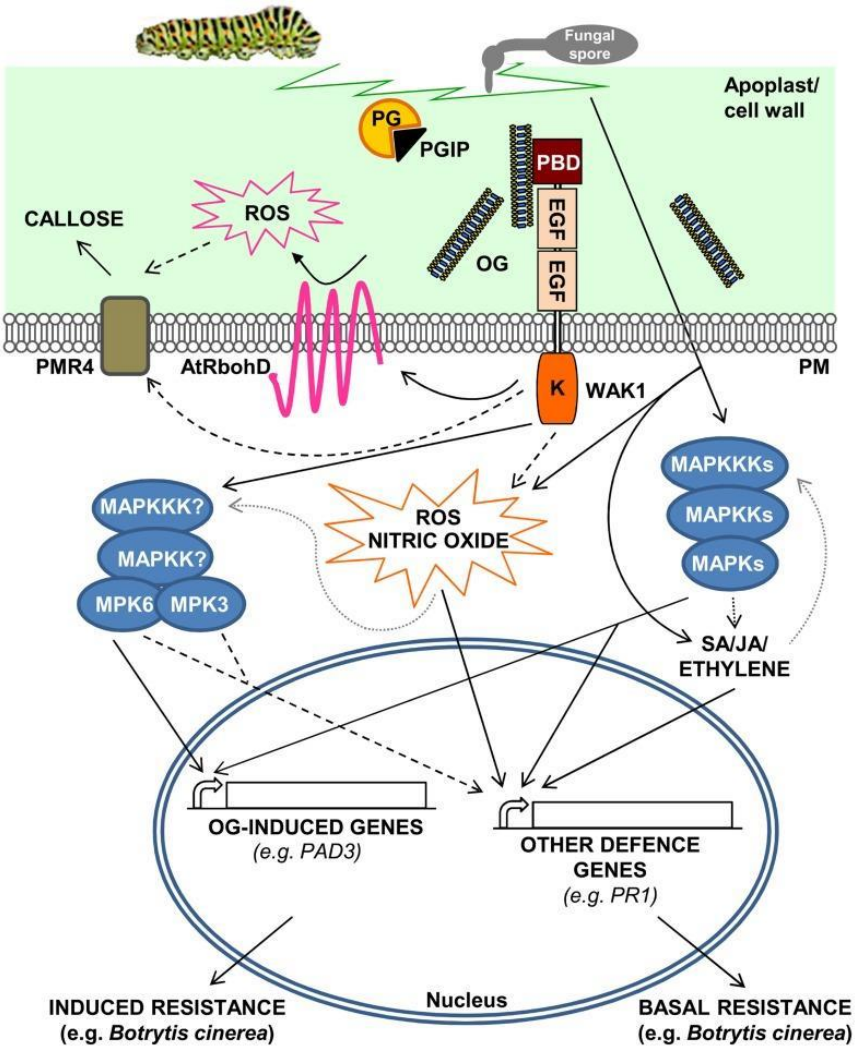

FIGURE 9. Model of defense responses triggered by oligogalacturonides in *Arabidopsis thaliana.* OGs are released from the cell wall after degradation of homogalacturonan by mechanical damage or by the action of hydrolytic enzymes, secreted by pathogens, such as PGs. In the apoplast PGIPs modulate PG activity, favouring the accumulation of elicitor-active OGs. OGs are perceived by the receptor WAK1 (Wall-associated kinase 1) and trigger defense responses such as ROS accumulation through the activation of the NADPH oxidase AtRbohD, nitric oxide production,

callose deposition, and MAPK-mediated activation of defense gene expression. Pathogen invasion or mechanical damage also cause an increase of hormones levels (JA, SA, and ET), mediated by MAPK cascades, triggering defense responses independently of OGs. DAMP- and hormone-mediated defense responses result, respectively, in induced and basal resistance to necrotrophic pathogens, such as *Botrytis cinerea*. Dashed lines indicate hypothetical cascades; dotted gray lines indicate oversimplification of the complex and still partially uncharacterized roles of MAPKs in the regulation of hormone and ROS synthesis/response (Ferrari et al., 2013).

Different studies have demonstrated that hormones can regulate the expression of *WAK1*. For example, *WAK1* expression in Arabidopsis is upregulated under high levels of SA and after exogenous application of SA and MeJA in rice (He et al., 1998; Li et al., 2009). In the same line, WAK1 induces the expression of JA biosynthesis-related genes or JA accumulation in *Arabidopsis* and maize (Moscatiello et al., 2006; Yang et al., 2019). Therefore, the fact that WAKs could induce hormonal responses depending on the pathogen or the lifestyle of the pest would be an intriguing feature of this family of receptors (Bari and Jones, 2009). Hence, the ubiquitous distribution of WAKs across plant families may imply their potential role in plant life processes, conferring benefits to different crop species in growth and development and increasing resistance to abiotic and biotic stress (Stephens et al., 2022).

4. ARBUSCULAR MYCORRHIZAL FUNGI

Arbuscular mycorrhizal fungi (AMF) are obligate symbionts belonging to the subphylum *Glomeromycota* (Schüßler et al., 2001). These fungi are widely distributed in the soil of agroforestry ecosystems and form mutualistic symbioses with ~71% of terrestrial vascular plants, including several crops (Brundrett and Tedersoo, 2018). The origin of the symbiotic relationship between AMF and land plants is dated to 450 million years ago and may be involved in the terrestrial colonisation of plants (Field et al., 2015; Martin et al., 2017). Around 250 AMF species have been described in several ecosystems (Öpik et al., 2013). Their diversity and distribution are affected by several factors, including soil, host plant, environmental conditions and agricultural practices (Hayman, 1982; Li et al., 2007; Lenoir et al., 2016).

The AMF are obligate biotrophs that need the plant to develop and complete their life cycle. It initiates with spore germination, the germinating hyphae grow until contacting a host root, penetrate intercellularly until reaching the root cortex where it spreads and forms symbiosis structures s, developing intra-and extraradical mycelium to finally generate new spores (Koltai and Kapulnik, 2010). In the absence of a host plant, fungal growth stops after around 25–30 days of culture (Goltapeh et al., 2008). Thus, symbiosis requires a fine coordination based on a precise molecular dialogue between both plants and AMF.

4.1 AMF symbiosis establishment and functioning

An active signal exchange between the symbionts leads to the common symbiosis signaling pathway (Kiers et al., 2011). This exchange occurs in the rhizosphere, where root plants release strigolactones (SLs) that are carotenoid-derived phytohormones (belonging to the subfamily of terpenoids), generally produced at low P availability in soil (Mashiguchi et al., 2021). The plant exudates are perceived by AMF and regulate several fungal responses as spore germination, hyphal growth, branching and hyphopodium formation, for the **establishment of symbiosis** (López-Ráez et al., 2017; Waters et al., 2017). At the same time, AMF releases signaling molecules called MYC factors that induce the symbiotic response in the plant. These compounds are a mixture of lipo-chitooligosaccharides that, once perceived by the plant, induce the establishment of symbiosis, triggering extensive transcriptional changes in the host plant to accommodate the fungus (MacLean et al., 2017). Upon recognition, AMF hyphae penetrate the root epidermis to colonize cortical cells and develop little tree-like subcellular invaginations called arbuscules (Figure 10). These structures are composed of highly branched hyphae

surrounded by a modified form of the cortical cell plasma membrane defined as a periarbuscular membrane, avoiding direct contact of the fungus with the plant cytoplasm (Parniske, 2008; Wipf et al., 2019). The cortical cell reorganization forms a new membrane-bound apoplastic compartment in which the arbuscules reside and exchange nutrients and information between AMF and host plants (Park et al., 2015). Here, the nutrient exchange between the partners takes place. Outside the roots, the AMF develop an extensive hyphal network (extraradical hyphae) that can reach far from the root area, thus increasing the capacity of plants to access water and mineral nutrients (in particular phosphate). In return, the host plant provides physical support and supplies **carbohydrates** and **lipids** to AMF (Bago et al., 2000; Keymer et al., 2017; Bedini et al., 2018).

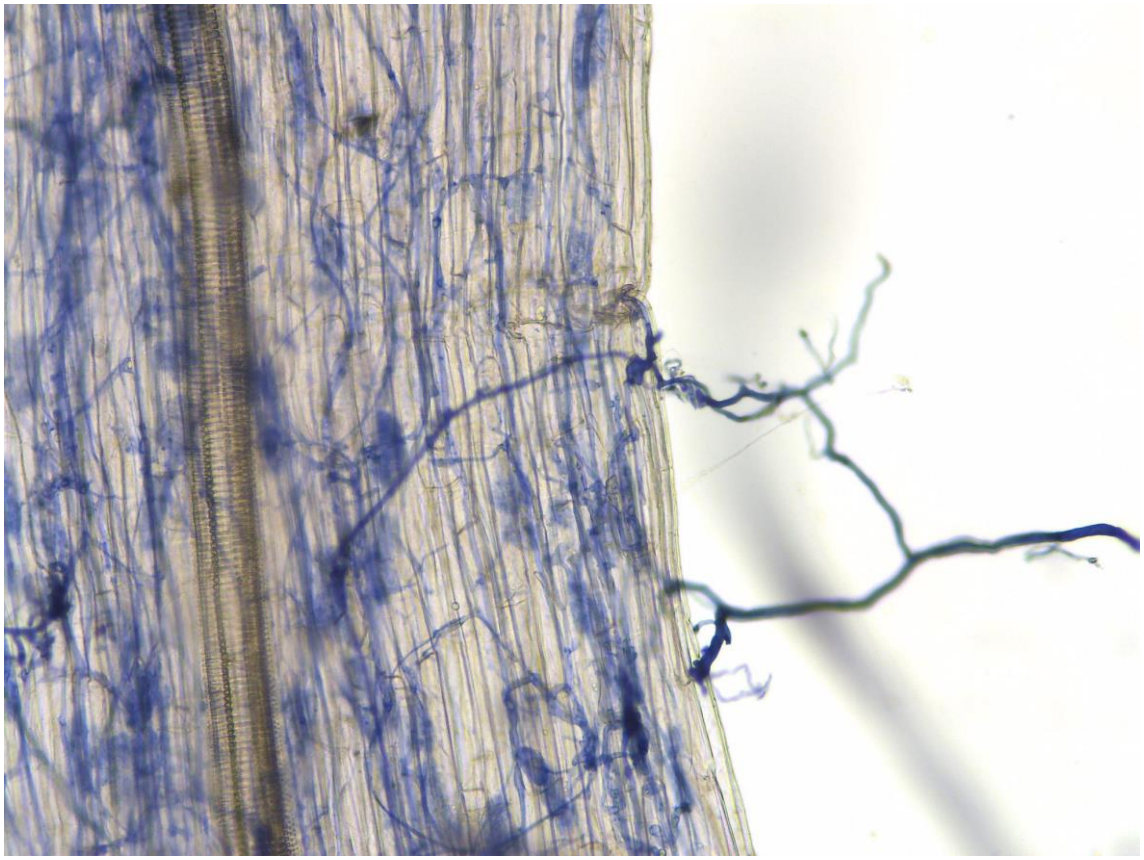

FIGURE 10. Tomato root colonized by Arbuscular Mycorrhiza Fungi (AMF). The hypae and arbuscular structures are visible in the image. The picture was kindly provided by Estefania Berrio Pozo.

The establishment of AM symbiosis increases the energy demand of the plant to maintain the symbiosis, producing profound changes in the plant metabolism and influencing their transcriptome, proteome and metabolome (Mhlongo et al., 2018; Sivaprakasam Padmanaban et al., 2022). Generally, mycorrhizal plants increase their photosynthetic rates. Indeed, plants invest almost 20% of photoassimilates in maintaining symbiosis (Valentine et al., 2013), providing sugars and lipids to the AMF (Bago et al., 2000; Keymer et al., 2017). The

molecular machinery activated during the symbiosis alters carbon partitioning and induces key enzymes related to sucrose biosynthesis, transport, and catabolism (Salmerón-Santiago et al., 2021). Although some responses are specific to AMF strains and plant lineages (Korenblum and Aharoni, 2019), this mechanism seems to be conserved among plant species (Kaur and Suseela, 2020). In general, the levels of hexoses, mainly glucose, are altered in the roots of mycorrhizal plants, because of their demand by AMF structures. For example, in soybean roots, glucose and fructose concentrations are higher in AM plants at the beginning of symbiosis, while successively, their content decreases, indicating a possible metabolization (Schubert et al., 2004; Goddard et al., 2021). However, both hexoses are transported in mycorrhizal roots across the periarbuscular and fungal plasma membranes in arbusculated cells to reach the fungal partner (Parniske, 2008). Furthermore, the establishment of AM symbiosis induces the production of several **secondary metabolites** (Fiorilli et al., 2019). Elevated levels of flavonoids have been reported in AM plants to control AMF colonization (Mierziak et al., 2014; Schweiger et al., 2015; Hill et al., 2018). Other metabolites such as carotenoids, phenylpropanoids and antioxidants are also accumulated in plants after AM establishment (Hill et al., 2018; Chen et al., 2013). In addition, some compounds from the blumenols family have been found only in roots and shoots of different species of mycorrhizal plants, including tomato, potato and barley, making these compounds great candidates as shoot biomarkers of AMF colonization in many plants (Wang et al., 2018).

The successful **development** and **functioning** of the AM symbiosis require a high degree of coordination between both partners that is finely regulated by phytohormones. As mentioned, **SLs** initiate the AMF-plant symbiosis stimulating spore germination, hyphal elongation, and the formation of hyphopodia (Akiyama et al., 2005). Once the AM symbiosis is well established in the root, SLs biosynthesis is reduced, probably due to autoregulation mechanisms to avoid over-colonization (López-Ráez et al., 2015). **ABA** is another apocarotenoid hormone with a regulatory role during mycorrhizal colonization. In the first stage, ABA promotes mycorrhizal development, but once the roots reach sufficient colonization, ABA accumulation may inhibit the progression of the fungi (Charpentier et al., 2014). Similarly, low concentrations of **GA** may be required for correct mycorrhizal development, while high concentrations inhibit AM colonization and arbuscule formation (Takeda et al., 2015). Generally, auxins positively regulate arbuscule development and functionality. **IAA** is responsible for plant development and root elongation, and auxin levels positively correlate with higher AM colonization (Chen et al., 2022; Zou et al., 2017). Finally, jasmonic acid showed positive and negative effects on mycorrhization, favoring arbuscule formation and controlling fungal spread in roots (Wasternack

and Hause, 2013). In contrast, **SA**, **ET** and **CKs** prevent AM fungal penetration and colonization (Foo et al., 2013). Thus, the fine-tuned dialogue between AMF and plant is linked to hormone signaling in all AM symbiosis stages (Pozo et al., 2015; Ho-Plágaro et al., 2022). All in all, the symbiosis alters plant primary and secondary metabolism while providing several benefits to the plant (Kaur and Suseela, 2020) (Figure 11).

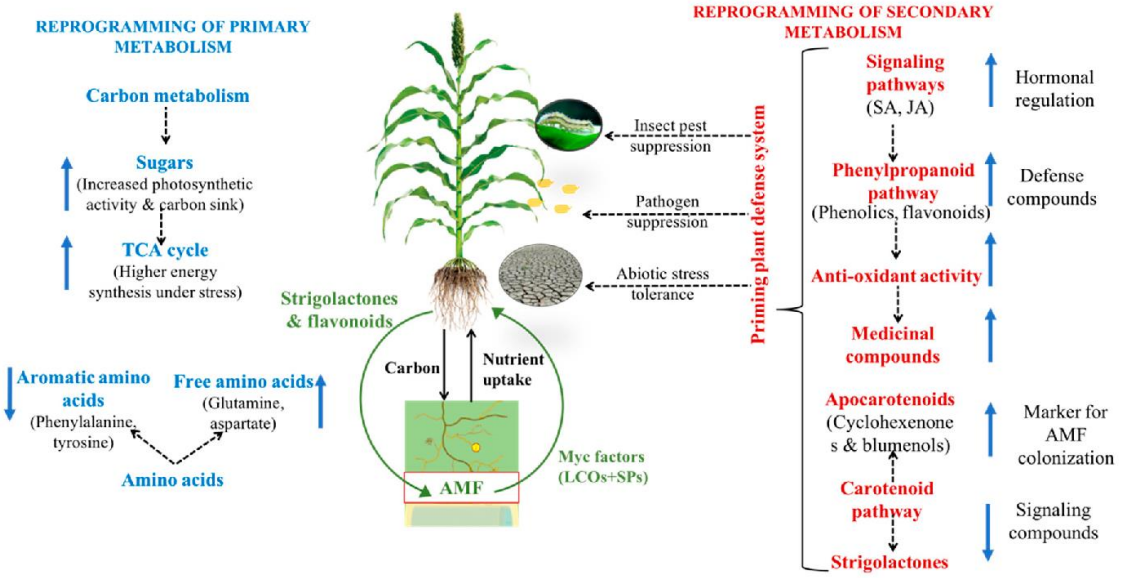

FIGURE 11. Schematic representation of the potential pathways through which arbuscular mycorrhizal fungi (AMF) reprograms the plant metabolome. Plant–AMF symbiosis reprograms the primary and secondary metabolites in plants. Reprogramming of secondary metabolites autoregulates the AMF colonization in the plant by modulating the production of signaling compounds. Interaction of plants with AMF primes plant defense via changes in the secondary metabolic pathways, which increase plant tolerance to biotic and abiotic stresses. Key: LCOs-lipochitooligosaccharides; SPs-secreted proteins (Kaur and Suseela, 2020).

4.2 AM symbiosis benefits on plant health

AMF increase the fitness of their host plants in multiple aspects. The fungal symbiont improves the acquisition of water and nutrients, in particular, the inorganic phosphate (acting as biofertilizers). Additionally, the symbiosis enhances plant tolerance and resistance against environmental abiotic and biotic stresses, commonly through priming of plant defenses.

4.2.1 Nutritional plant benefit of AM symbiosis

AMF play a crucial role in improving the host plant nutrient uptake. Because of their highly extended network of branching hyphae, AMF increase the host plant's acquisition of essential nutrients, including phosphorus (P), nitrogen, potassium, sulfate and trace minerals such as manganese, magnesium and zinc (Bennett and Groten, 2022) (Figure 12).

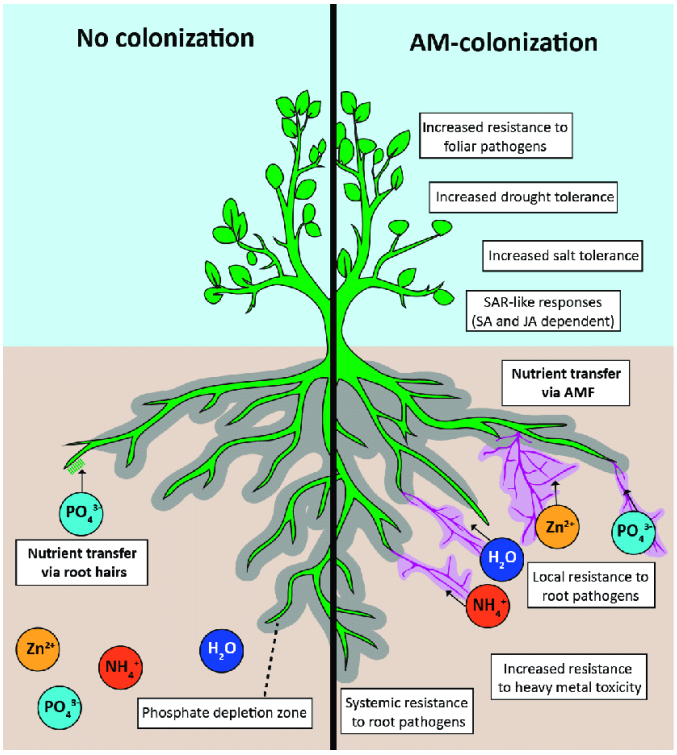

FIGURE 12. Positive effects of arbuscular mycorrhizal (AM) colonization. The hyphal network of arbuscular mycorrhizal fungi (AMF) extends beyond the depletion zone (grey), accessing a greater area of soil for phosphate uptake. A mycorrhizal-phosphate depletion zone will also eventually form around AM hyphae (purple). Other nutrients that have enhanced assimilation in AM-roots include nitrogen (ammonium) and zinc. Benefits from colonization include tolerances to many abiotic and biotic stresses through induction of systemic acquired resistance (SAR) (Jacott et al., 2017).

The **improvement of P** uptake is one of the most important benefits of the mycorrhizal symbiosis for the host plants that receive more than 90% of their P from AMF (Smith and Read, 2008). Generally, low P concentrations in the soil increase the production of plant root exudates containing strigolactones, phenolic compounds, amino acids and sugars with a positive effect on AM symbiosis (López-Ráez et al., 2010; Nagahashi et al., 2010; Tawaraya et al., 2013; Nadal et al., 2017; Bedini 2018). On the contrary, high P levels inhibit plant exudation and reduce the percentage of plant root length colonized by AMF (Russo et al., 2013; Smith et al., 2011; Carbonnel and Gutjahr, 2014). Plants uptake soluble orthophosphate through specific phosphate transporter proteins in roots, through the 'direct P pathway' (Strawn, 2018). However, P is mainly present in the soil as poorly soluble phosphate salts bound to organic molecules or minerals, thus, inaccessible to plants (Smith and Smith, 2011; Wipf et al., 2019). The low mobility of P in soil results in a P-depletion zone around the root surface, and the extensive network of extraradical AMF hyphae allows exploring further improving P uptake (Thirkell et al., 2017). The acquisition and transfer of P from soil to plant roots via the mycelium is known as the 'mycorrhizal P pathway', by which P enters the plant cell through specialized

symporters in the periarbuscular membrane at the arbuscule branch domain (Smith and Smith, 2011; Młodzińska and Zboińska, 2016; Bedini et al., 2018). The P is incorporated as polyphosphate in the arbuscules and subsequently hydrolysed and translocated to the plant cell cytosol (Hijikata et al., 2010; Ezawa and Saito, 2018).

In environments with mineral deficiency, mycorrhizal plants show better nutritional status than non-mycorrhizal plants, improving plant development and stress tolerance. Besides plant nutritional status, AMF symbiosis improves other plant processes, such as photosynthesis, water uptake, osmotic potential, cell membrane integrity and reprogramming plant primary and secondary metabolism (Amani Machiani et al., 2022).

4.2.2 AMF increase plant tolerance to abiotic stress

Environmental changes affect plant growth and development, perturbing the nutritional status and physiological parameters and altering agricultural production (Nadeem et al. 2014; Begum et al., 2019; Latef et al., 2016). Several studies have demonstrated that AM symbioses mitigate the harmful effects of abiotic stresses, such as nutrient deficiency, drought, salinity, temperature change and heavy metals in plants (Marro et al., 2020; Nguyen et al., 2019; Bahadur et al., 2019; Saxena et al., 2017; Romero-Munar et al., 2019; Zhu et al., 2017; Miransari, 2017).

Under non-stress conditions, mycorrhizal plants control the water and nutrient status better than non-mycorrhizal plants (Mitra et al., 2021). Additionally, the energy distribution is more efficient in AM plants, increasing chlorophyll content and photosynthesis rates (Huang et al. 2020; Quiroga et al. 2019). For example, mycorrhizal plants are more tolerant to **oxidative stress,** displaying higher protection of the photosynthetic system than non-mycorrhizal plants against ROS, increasing the production of antioxidant enzymes to maintain photosynthesis levels in an optimal range (Behrooz et al. 2019; Nadeem et al. 2014). On the other hand, mycorrhizal plants showed a more efficient water transport from the soil through an extracytoplasmic hyphal pathway, increasing plant tolerance to **drought** (Kakouridis et al., 2022). For instance, AM plants accumulate more proline and soluble sugars, improving osmoregulation in roots and leaves and increasing the tolerance in drought conditions through a better osmotic adjustment in underground and epigeous plant tissues (Porcel and Ruiz-Lozano 2004; Zhang et al. 2010; Huang et al. 2020).

AM plants also minimize **salt stress** by reducing Na^+ absorption and enhancing photosynthesis and gas exchange (Fadaei et al., 2020). Furthermore, several studies showed increased tolerance of AM plants at low and high **temperatures**, being able to regulate

membrane permeability and improving photosynthesis, respiration, nutrients absorption, osmolytes accumulation and antioxidant activity (Zhu et al. 2010; Maya and Matsubara 2013; Devi et al. 2019; Xian-can et al. 2010). In addition, mycorrhizal symbiosis regulates metal homeostasis in plants through the immobilization of **metals** in soils (phytostabilization) and their transport to shoots (phytoextraction) (Krishnamoorthy et al. 2019). Finally, AM plants display increased sugar levels in leaves -regardless of plant or AMF genotypes- under different abiotic conditions (such as salt, drought, flooding, P starvation or arsenic contamination), showing a conserved response mechanism against different abiotic stresses (Sheng et al., 2011; Liu et al., 2020; Yooyongwech et al., 2016; Gupta et al., 2021; Wu et al., 2007), and thus, modulating an efficient response adapted to each specific challenge.

4.2.3 AMF increase plant tolerance to biotic stress: MIR

Although AMF are not considered biopesticides in the EU (https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/active-substances), as they do not display direct antagonism against aggressors, they have been proposed as biological control agents - microorganisms, natural or genetically manipulated that can reduce the frequency or intensity of diseases and damage caused by plant pathogens or pests (Vishwakarma et al., 2022). The beneficial effect of the mycorrhizal symbiosis on plant resistance against a wide spectrum of pests and diseases is known as **Mycorrhizal Induced Resistance (MIR)** (Pozo and Azcón-Aguilar, 2007; Jung et al., 2012; Kadam et al., 2020; Rivero et al., 2021).

The enhanced resistance of AM plants is not restricted to colonized roots, but it is also applies to other parts of the root system and aboveground tissues (Pozo and Azcón-Aguilar, 2012; Jung et al., 2012). In fact, mycorrhizal plants have been shown to up-regulate the expression of defense-related genes and accumulate toxic secondary metabolites in roots, making them more resistant to **soil-borne pathogens, nematodes**, **root-chewing insects** (Currie et al., 2011; Jung et al., 2012; Olowe et al., 2018; Schouteden et al., 2015; Nair et al., 2015a), and in leaves, enhancing resistance against **aboveground pathogens** such as *Alternaria solani*, *Botrytis cinerea, Plasmopara viticola, Xanthomonas translucens* or *A. alternata* (Song et al., 2015; Bruisson et al., 2016; Fiorilli et al., 2018; Sánchez-Bel et al., 2016; Sanmartín et al., 2020; Fritz et al., 2006; Nair et al., 2015b) and **chewing herbivores** as *Helicoverpa arimigera*, *H. punctigera, Spodoptera exigua*, *S. littoralis, S. litura, Trichoplusia ni* Hübner (Song et al., 2013; Rivero et al., 2021; Formenti and Rasmann, 2019; Frew and Wilson, 2021; Selvaraj et al., 2020; Schoenherr et al., 2019). Generally, MIR is effective against necrotrophic pathogens and chewing

leaf insects, which are both affected by **JA-dependent defenses** (Jung et al., 2012; Song et al., 2013; Song et al., 2015; Sánchez-Bel et al., 2016; He et al., 2017).

4.3 Priming of plant defenses in mycorrhizal plants

Defence priming is defined as a physiological state in which a plant is conditioned for a more efficient activation of defenses upon diverse challenges (Martínez-Medina et al., 2016). Experimental evidence has demonstrated that MIR is usually associated with priming of plant defenses (Pozo and Azcón-Aguilar, 2007; Jung et al., 2012; Pieterse et al., 2014; Mauch-Mani et al., 2017). Beyond AMF, other beneficial microbes, but also previous exposure to biotic (avirulent pathogens or herbivores), and abiotic stresses, or certain chemical compounds may induce defense priming (Conrath et al. 2015; Mauch-Mani et al., 2017; Hilker et al. 2019; Schillheim et al., 2020).

Defense priming implies different stages. First, upon application of the priming stimulus, there is an onset of plant defenses, usually transient, followed by a maintenance phase (Mauch-Mani et al., 2017). Later, despite the lack of notorious defense responses activated, primed plants display a faster and stronger activation of the plant defenses when exposed to a challenging stress, for example, a pathogen or pest attack (Hilker et al., 2015) (Martínez-Medina et al., 2016) (Figure 13).

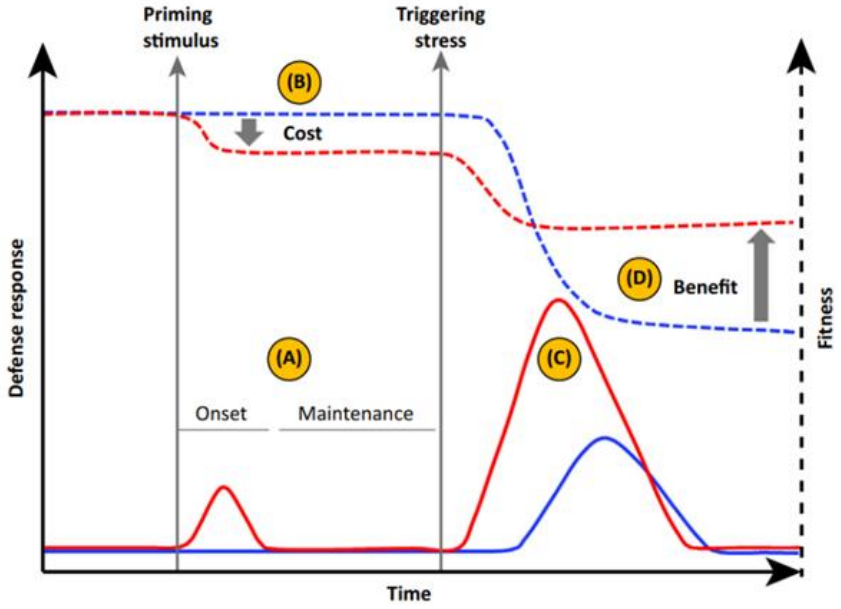

FIGURE 13. Scheme of the Relation between Defense Responses (Solid Lines) and Fitness (Dashed Lines) in Primed (Red) versus Unprimed (Blue) Plants. Analysis of defense priming requires a set of steps encompassing both the assessment of plant defenses and the associated cost–benefit balance. Here, we suggest some criteria that may help in deciding whether defense priming is present: (A) Memory: two sequential environmental events are required for asserting memory in the absence of molecular markers: the priming stimulus and the triggering stress. During priming

and in the primed state (before the triggering stress), plant defenses are expected to be only transiently and generally faintly induced. (B) Low fitness costs: the maintenance of the primed state (before the triggering stress) has low fitness costs compared with the direct activation of defense. (C) A more robust defense response: in response to the triggering stress, primed plants mobilize cellular defenses in a faster, earlier, stronger, and/or more sustained manner than do unprimed plants. (D) Better performance: primed plants are expected to defend better against a given stressor than unprimed plants. Therefore, priming enhances plant fitness in hostile environments (Martínez-Medina et al., 2016).

The enhanced ability of primed plants to respond more efficiently to challenges usually relies in enhanced sensitivity to immune regulatory signals, chromatin modifications, accumulation of inactive mitogen-activated protein kinase and transcriptional factors and increasing levels of stress receptors (Mauch-Mani et al., 2017; Conrath et al., 2015). Then, priming involves changes in primary and secondary metabolism that may mobilize energy and defensive resources in a more efficient way under attack (Rivero et al., 2021; Sanmartín et al., 2020; Kaur and Suseela, 2020). The advantage of this plastic immune response is the low energy cost, which maintains the plant in a state of alertness leading to increase tolerance and resistance to different stresses only when it is required (Martínez-Medina et al., 2016; Mauch-Mani et al., 2017; Pastor et al., 2013).

It has been proposed that induced resistance by beneficial microorganisms is usually associated with priming of JA-dependent defenses (Pieterse et al., 2014). Indeed, MIR is mediated by JA signaling and accordingly, is more effective against chewing herbivores and necrotrophic pathogens (Pieterse et al., 2014; Mora-Romero et al., 2015; Gruden et al., 2020) (Figure 14). Mycorrhizal colonization of plant roots implies different physiological, transcriptional and metabolic changes. Transcriptional changes occur, including changes in signaling, transport, and hormone-related genes (Jung et al., 2012; Pozo et al., 2015). Upregulation of the synthesis of JA and other oxylipins have been shown in mycorrhizal roots (López-Ráez et al., 2010; Ho-Plágaro & Garcia, 2022). In shoots, transcriptional regulation associated with mycorrhization is very moderate. However, upon challenge, JA-dependent defenses are usually upregulated (Jung et al., 2012; Mora-Romero et al., 2014). For example, mycorrhizal tomato plants showed primed accumulation of defensive metabolites and upregulation of the expression of several JA-regulated defense-related genes, like proteinase inhibitors, upon attack by the chewing herbivores *Helicoverpa arimigera* and *S. exigua.* These primed responses led to a lower performance of the larvae that fed on mycorrhizal plants (Song et al., 2013; Rivero et al., 2021).

The effects may be not restricted to chewing herbivores, but can also apply to other feeding guilds. Mycorrhizal citrus plants attacked by the piercing´ sacking insect pest

Tetranychus urticae showed reduced damage levels in leaves and lower mite oviposition rates than non-mycorrhizal plants. These mycorrhizal plants displayed primed expression of oxylipin-related genes and accumulation of flavonoids after spider mite infestation (Manresa-Grao et al., 2022).

Finally, mycorrhiza-induced resistance primes not only direct defenses against pests, but can also prime indirect defenses. For example, mycorrhizal *Phaseolus vulgaris* (bean) plants can increase the attraction of *Phytoseiulus persimilis,* the natural predator of *T. urticae*, increasing the population of the predator and reducing the pest (Hoffmann et al., 2011). The pattern fits with the priming of indirect defenses through JA signaling, as the production of volatile compounds upon herbivore attack, mediating predator attraction, is also dependent on JA signaling (Mauch-Mani et al., 2017).

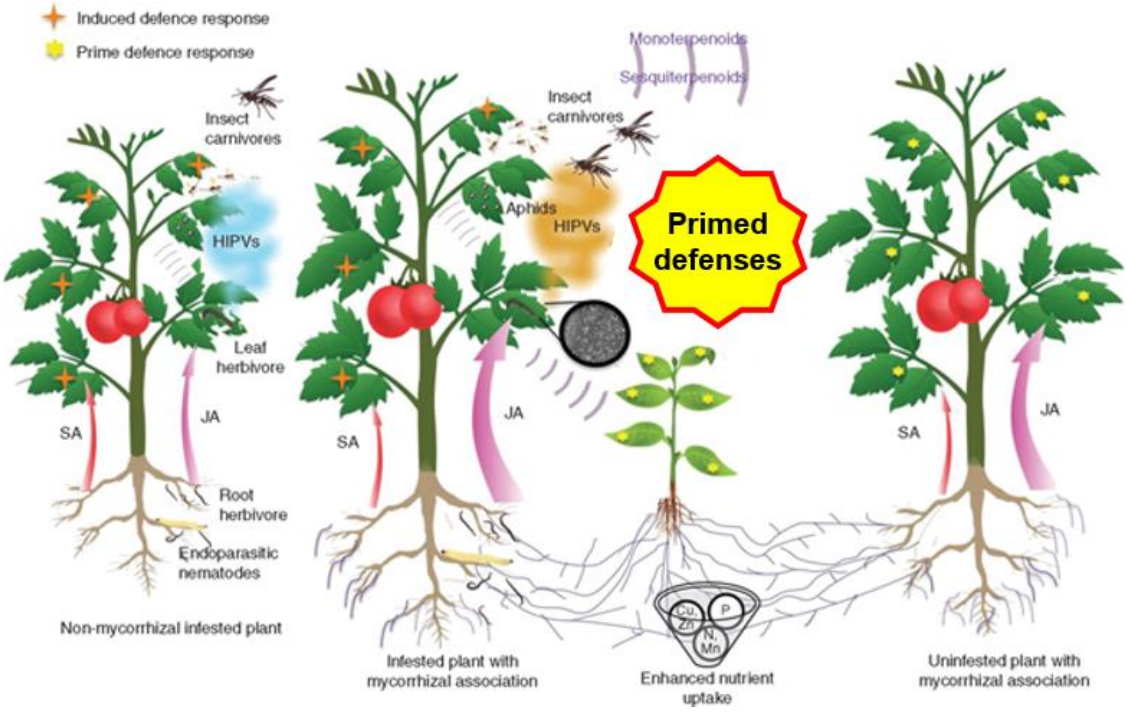

FIGURE 14. Overview of arbuscular mycorrhiza (AM)-reinforced defense against herbivorous insects. Mycorrhizal association improves plant acquisition of soil nutrients through the AM fungal hyphal network that often leads to growth plant promotion. Upon pest attack, priming of plant defenses leads to a reduction in the severity of damage caused by different soil-borne pathogens, nematodes, and chewing insects. In above-ground tissues, the activation of defense mechanisms regulated by JA reduces the proliferation of necrotrophic pathogens and the performance of phytophagous insects. Moreover, indirect defense mechanisms, such as the production of volatiles, are heightened by AM symbiosis, leading to the attraction of natural enemies of the pests. Modified from Sharma E. et al. (2017).

4.4 Context dependency of MIR

Although mycorrhiza colonization has usually positive effects on plant growth, development, and stress tolerance, the outcome of this symbiosis is not always positive (Chaudhary et al., 2016). Indeed, depending on the context, the outcome of a plant-beneficial microbe interaction can range from positive to neutral or even negative for the plant if the costs of the symbiosis outweigh the benefits (Pozo et al., 2020). This context dependency is usually associated with the biotic and abiotic conditions, thus environmental conditions and crop management can strongly influence the outcome (Frederickson, 2017; Lee-Díaz et al., 2021). Additionally, the outcome of MIR also depends on the plant and AMF genotypes (Mustafa et al., 2016; Mora-Romero et al., 2015). For example, the performance of *S. littoralis* in 7 plants (*Poa pratensis, Festuca rubra, Agrostis capillaris, Deschampsia flexuosa, Senecio jacobaea, Plantago lanceolata* and *Artemisia vulgaris*) inoculated with *Glomus intraradices,* was differentially impaired depending on the plant species (Kempel et al., 2010). It indicates that the plant's genetic background is an important aspect to consider MIR.

Similarly, the beneficial effect of the mycorrhizal symbiosis depends on the AMF species, indicating a functional diversity in MIR. For example, *Funneliformis mosseae* provides higher protection against *Spodoptera exigua* than *Rhizoglomus irregularis* and *Glomus versiforme*; and even different isolates from the same species modulate plant resistance in a different manner (Roger et al., 2013; He et al., 2017). Interestingly, the induction of secondary metabolites also differs between AMF species, some being more efficient than others in boosting the accumulation of defensive metabolites, as phenolics and flavonoids among others, and antioxidant activity (Mandal et al., 2010; Kaur and Sauseela, 2020; Rivero et al., 2018; Avio et al., 2020; Silva et al., 2019).

The surrounding environment is also a very important factor for the effectiveness of MIR. A wide variety of **abiotic factors,** most of them predicted to change in a drastic manner by the end of the century, affect the output of MIR against stresses, including nutrient availability, atmospheric CO_2 concentration, soil moisture, water, pH and organic matter content soil, temperature and light (quality and intensity) (Miransari, 2013; Oldroyd, et al., 2020; Schnecker, et al., 2014; Del Valle et al., 2020; Augé et al., 2001; Juniper and Abbott, 2006; Ulrich, et al., 2019, Aciego et al., 2008; Nagata, et al., 2015; Konvalinková and Jansa, 2016; Goicoechea, 2020; Frew and Price, 2019). Indeed, nutrient availability is a key aspect, as it may affect the symbiosis itself, the interaction of the plant with the herbivore, or the three-way interaction (Pozo et al., 2021). For example, nitrogen availability has been shown to impact the effect of AM symbiosis on plant and herbivore performance (Ramírez-Serrano et al., 2022). Even transient changes may

affect, for example, plants exposed to double stresses, as transient nitrogen depletion and pathogen attack, prioritize abiotic stress tolerance (Sánchez-Bel et al., 2016), demonstrating the complexity of understanding MIR.

Then, the complex interaction plant-AMF-insect should be considered in the context of a multi-trophic system that is the result of eco-evolutionary forces that may have a negative or positive impact on insect herbivores (Meiners, 2015). In conclusion, the outcome of MIR is highly context-dependent, as it is often only activated when a number of specific conditions occur and it is conditioned by environmental factors that may alter the outcome of plant-AMF interactions (Lee-Díaz et al., 2021).

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Interest of the study

INTEREST OF THE STUDY

Plants face numerous challenges that can compromise their growth and productivity. Historically, humans have attempted to mitigate yield losses due to environmental stresses by using excessive fertilizers and pesticides. However, these practices have resulted in undesirable consequences such as soil degradation, pollution of water and the environment, and negative impacts on beneficial organisms and on human health.

The current awareness of these risks increases the need to match environmental protection while maintaining high productivity levels. Then, particular interest is focused on finding sustainable and environmentally friendly strategies for agriculture practices. One prominent strategy is using natural resources, such as soil-beneficial microbes, as inoculants to improve crop yield without compromising the surrounding environment. Among them, soil-borne Arbuscular Mycorrhiza Fungi (AMF) have biofertilizer and bioprotective properties, making them an optimal alternative to the excessive use of chemical fertilizers and pesticides in sustainable agriculture.

The present doctoral thesis investigates how the interaction between beneficial AMF and tomato plants increases the resistance and tolerance to different stresses (abiotic and biotic), focusing on the underlying molecular mechanisms. I focused on analyzing the effect of AM symbiosis on pest insects and the related changes in the metabolic profile that exhibited a resistance profile against pests. Transcriptional, metabolic, and enzyme activity changes related to primary and/or secondary metabolism were analyzed to identify potential key elements for plant protection.

To unlock the full potential of AMF and improve their biotechnological applications in agriculture, understanding the mechanisms behind the beneficial plant-AMF interaction that promotes enhanced stress resistance is essential.

Aim & Objectives

AIM & OBJECTIVES

This PhD thesis aims **to investigate the main defense mechanisms and signaling components mediating the impact of the beneficial arbuscular mycorrhizal fungi on plant resistance to insect pests and how the abiotic context -in particular nutrient availability- may shape the effects of the interaction.** For that, I explore the reprogramming of primary and secondary metabolism associated with mycorrhiza-induced resistance (MIR) under different P fertilization levels and in a tomato genotype altered in damage perception.

The following specific objectives were defined and addressed to achieve this main objective:

1. To determine how phosphorous availability modulates MIR against the generalist herbivore *Spodoptera exigua* and the foliar pathogen *Botrytis cinerea* in plants colonized by the AMF *Funneliformis mosseae* (Chapter 1).
2. To elucidate the contribution of SlWAK1 receptor in the regulation of the plant primary and secondary metabolism in the response to *S. exigua* and its contribution to plant resistance (Chapter 2).
3. To evaluate the potential role of SlWAK1 and the regulation of carbohydrate metabolism on plant resistance against *S. exigua* in plants colonized by different mycorrhizal fungi (*F. mosseae* and *Clareidoglomus etunicatum*) (Chapter 3).

General Material & Methods

GENERAL MATERIAL & METHODS

This section will cover the broadest aspects of the materials and methods used in this thesis. However, to obtain more detailed information, it may be necessary to refer to the specific materials and methods outlined in different Chapters.

1. BIOLOGICAL MATERIAL AND GROWING CONDITIONS

1.1 Plant material

For bioassays, tomato plants *Solanum lycopersicum* L. cv. Moneymaker were used (**Chapter 1-2-3**). In **Chapters 2** and **3** the mutant line *Slwak1,* with a single T-DNA insertion localized in the promoter region of SlWAK1 (Solyc09g014720), and its corresponding genetic background as control (cv. Moneymaker) were used (Meco et al., 2020).

Seeds were surface disinfected by immersion in 4% NaClO (10 min), rinsed thoroughly with sterile water and incubated for 10 days in an open container with sterile vermiculite at 25°C. Plantlets were transferred to 350 mL pots containing sterile sand:vermiculite (1:1) mixture and randomly distributed under greenhouse conditions (24/16°C with a 16/8 h diurnal photoperiod and 70% humidity). Plants were watered twice a week with half-strength Hewitt nutrient solution (Hewitt, 1966) modified in the P content (0.3, 0.7 and 1.0 mM **Chapter 1**; 0.7 mM **Chapters 2-3**), and water was supplied as needed.

1.2 Arbuscular mycorrhizal fungi (AMF)

All AMF were continuously maintained in an open-pot culture of *Trifolium repens* mixed with *Sorghum vulgare* plants using vermiculite-sepiolite substrate at EEZ-CSIC greenhouse. The inoculum consisted of the substrate containing colonized root fragments, fungal mycelia and spores. The following AMF species have been used:

- *Funneliformis mosseae* (Fm BEG12) obtained from the International Bank of Glomeromycota (http://www.i-beg.eu/cultures/BEG12.htm) **(Chapters 1-3).**

- *Claroideoglomus etunicatum* (Ce, EEZ163), AMF isolated from a highly salinized soil from Cabo de Gata Natural Park (Almería, Spain) (Estrada et al., 2013) **(Chapter 3)**.

Pots for mycorrhizal treatments were inoculated by adding 10% (v/v) AMF inoculum. All plants, including non-inoculated ones, received a 3 ml aliquot of a filtrate (<20 mm) of the inoculum to provide the general microbial population but free of AMF spores.

1.3 Herbivore rearing and performance bioassays

Spodoptera exigua (Lepidoptera: *Noctuidae*) eggs were provided by Dr. S. Herrero lab (ERI-BIOTECMED, Universitat de Valencia, Spain). Larvae were reared on an artificial diet (Greene et al., 1976) at 25°C with 16:8h light: dark regime and 70% relative humidity until L2-L3 larval stage for their application. Larval weight, mortality (**Chapters 1-2-3**) and pupation (**Chapters 2-3**) were monitored for insect performance.

Insect were placed on leaves in different ways (Figure 1):

- (A) placing 2 larvae *per* leaf using detached leaves (see **Chapter 1**)

- (B) placing 2 larvae *per* leaflet using whole plant (see **Chapter 1**)

- (C) placing 1 larva *per* leaflet (in two different leaflet *per* plant) using whole plant (see **Chapter 2-3**)

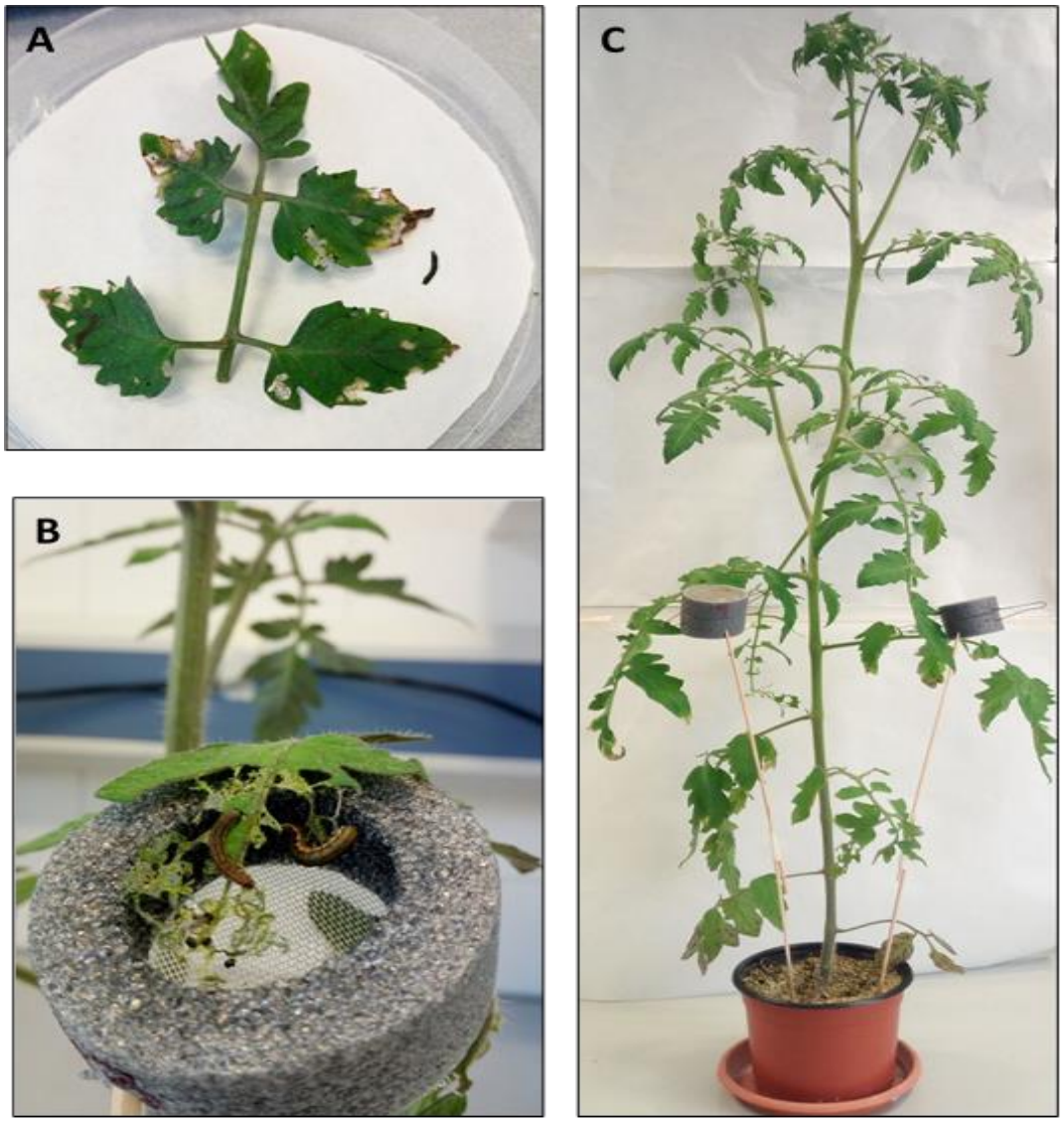

FIGURE 1. Two *Spodoptera exigua* in (A) Petri dish, (B) clip-cage, (C) two different clip-cages (one for each larva).

1.4 *Botrytis cinerea* cultivation and infection

The necrotrophic pathogen *Botrytis cinerea* was cultivated in potato dextrose agar plates supplemented with freeze-dried tomato leaves. Three weeks later, *B. cinerea* spores were collected from plates in 0.5X potato dextrose broth, as previously described by Sanmartín et al. (2020). Tomato plant leaves were detached, and each leaf was inoculated with two drops per leaflet containing a conidia suspension of *B. cinerea* (1×10^6 spores/ml).

Then, the infected leaves were kept in Petri dishes on wet filter paper and incubated in a phytotron chamber at 80% humidity and 22°C until the development of necrotic lesions was measured (Figure 2).

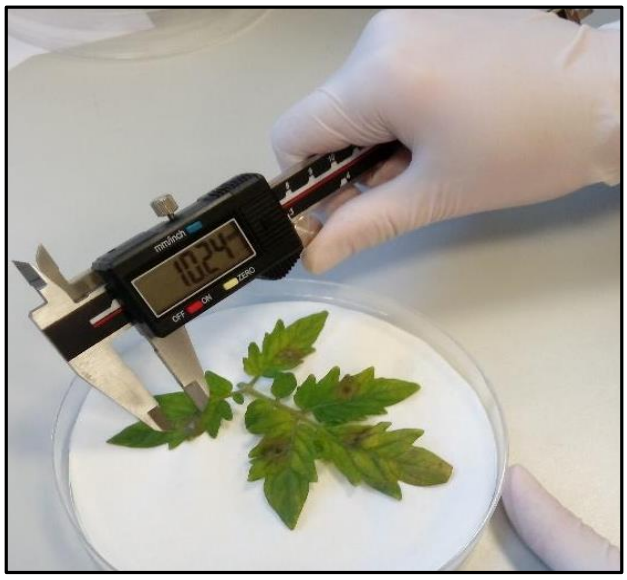

FIGURE 2. Measurement of *Botritis cinera* infection area through a digital caliper.

2. TREATMENT

2.1 Plant treatment with oligogalacturonides

The oligogalacturonides (OGs) were used to elicit plant defense responses. The OGs (DP 10-15) were provided by Dr. De Lorenzo lab (Department of Biology and Biotechnology "Charles Darwin" BBCD, La Sapienza University, Rome, Italy). Tomato leaves were treated with an aqueous solution of OGs (50 mg/ml in milliQ water), or milliQ water as control and collected 6 hours after the application to analyze early plant defense responses as described in Gamir et al. (2020) (**Chapter 1**).

3. ANALYSIS

3.1 Determination of mycorrhizal colonization

A representative sample of the root was harvested. Root samples were cleared and digested in KOH (10%) for 24h at room temperature (RT; 20 – 25°C). After that, root samples were rinsed thoroughly with tap water and acidified with acetic acid (2%) solution. The fungal root structures were stained with a 5% black ink (Lamy, Germany) in acetic acid (2%) solution for 24h at RT (Vierheilig et al., 2005). Finally, the ink solution was washed with tap water (**Chapters 1-3)**.

The percentage of total root length colonized by *F. mosseae* (**Chapters 1-3**) and *C. etunicatum* (**Chapter 3**) was estimated according to the grid-line intersection method (Giovannetti and Mosse, 1980) using a BOECO zoom stereo microscope Model BST-606 (Figure 3). We evaluated the percentage of total root length colonised to assess the extent of AMF colonization in the root system. Specifically, we placed the stained root in a Petri dish marked with grid-lines, ensuring that the roots were adequately spaced apart from each other, following the methodology described by Garcia et al. (2020). To ensure a representative percentage of colonization, we counted at least 200 intersects between the roots and grid-lines for each root sample.

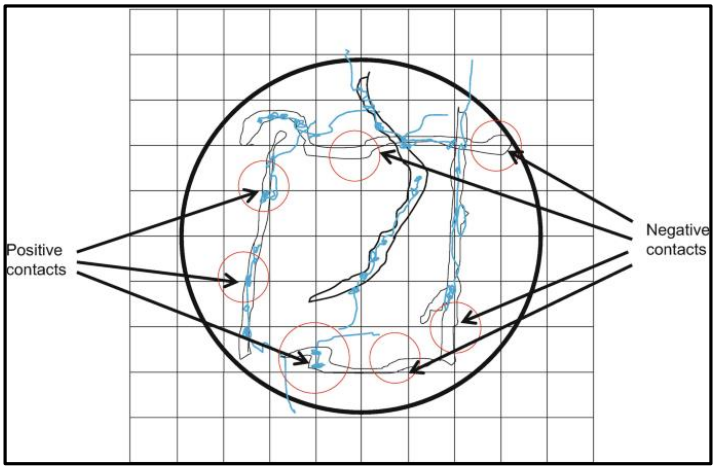

FIGURE 3. Quantification of AM fungal colonization in mycorrhizal roots using the gridline intersect method developed by Giovannetti and Mosse (1980). The source of the image is Garcia et al. (2020).

3.2 Determination of mineral nutrients in plant tissues

Nutrient analyses were performed at the Technical Services of the Estación Experimental del Zaidín (CSIC). P and micronutrients concentrations were determined after acid digestion of samples by inductively coupled plasma optical emission spectrometry (ICP-OES;

Varian ICP 720-ES). Total C and N contents were analyzed using an Elemental Analyzer (LECO TruSpec CN) according to standard procedures (**Chapters 1-2-3**).

3.3 Determination of anthocyanin content

Anthocyanin content was evaluated by adapting the protocol described by Rabino and Mancinelli (1986). Pigments were extracted from the freeze-dried leaf powder in acidic methanol. The amount of anthocyanin was determined by measuring the absorbance at 530 nm and 657 nm of the crude extract. To compensate for the contribution of chlorophyll and its degradation products to the absorption at 530 nm, the formula A530 -0.25 A657 was used (**Chapter 1**).

FIGURE 4. Leaves of plant watered with different P concentrations. The anthocyanin pigments are already evident by eyes**.**

3.4 Targeted hormonal extraction and quantification

Hormone extraction was performed as described in Sanchez-Bel et al. (2016). Leaves samples were extracted with a solution of H2O:MeOH (9:1) containing HCOOH (0.01%) and internal standards (100 ng/ml). Following several centrifugations and resuspensions, an extract aliquot was injected into an Acquity Ultra Performance Liquid Chromatography system (UPLC) (Waters located in Mildford, MA, USA). Hormones were separated chromatographically utilizing an HPLC Kinetex C18 analytical column from Phenomenex and connected to a triple quadrupole mass spectrometer (TQD) from Waters, Manchester, UK. The chromatography and mass spectrometry conditions were consistent with those Gamir et al. (2012). Hormone quantification of abscisic acid (ABA), indolacetic acid (IAA), jasmonic acid (JA), its precursor (+)-12-oxo-phytodienoic acid (OPDA), and salicylic acid (SA) were determined by implementing calibration curves with each pure chemical standard (**Chapter 1**).

3.5 Untargeted metabolomic profiling by liquid chromatography and electrospray ionization (LC-ESI) mass spectrometry (Q-TOF instrument)

Leaves samples were homogenized in a MeOH:H_2O (1:9) solution containing HCOOH (0.01%). The homogenate was centrifuged and the supernatant was recovered and filtered through cellulose filters (0.2 µm). Subsequently, 20 µl aliquot of the filtered was injected into an Acquity ultra-performance liquid chromatography system (UPLC; Waters, Mildford, MA, USA), that was interfaced with a hybrid quadrupole time-of-flight equipment (QTOF-MS Premier). Analytes were eluted with an aqueous methanol gradient containing HCOOH (0.01%). Solvent gradients and other chromatographic conditions were performed as previously described by Gamir et al. (2020). The LC separation was performed with a C18 analytical column, 5 µm particle size, 2.1 × 100 mm (Phenomenex, Madrid, Spain) using ultra-performance liquid chromatography (UPLC). For a correct and accurate identification of the detected signals, a second fragmentation function was introduced into the TOF analyzer. This function was programmed in a t-wave ranging from 5 to 45 eV to obtain a fragmentation spectrum of each analyte (Gamir *et al.*, 2014).

3.6 Full scan data analysis

Data was acquired in centroid mode and subsequently transformed into .cdf files using the Databridge package from MassLynx 4.1 software (MassLynx 4.1, Waters). Chromatographic signals were processed using the software *R* for statistical purposes. Signals from positive and negative electrospray ionization (ESI+; ESI-) were processed separately. Peak peaking, grouping and signal corrections were performed using the XCMS algorithm (Smith et al., 2006). Adduct and isotope correction was also performed by using associated software packages MarVis Filter and MarVis Cluster. Metabolite amounts were analyzed on the basis of normalized peak area units relative to leaf weight. Statistical analysis was performed using the MarVis Suit 2.0 software tool for clustering and visualization of metabolic biomarkers (Kaever et al., 2015). The Kruskal–Wallis test was applied to analyze the metabolomic differences between treatments. Data were normalized by sum, transformed by cube root, and scaled by Pareto method to obtain the principal component analysis (PCA), sparse partial least-squares discriminant analysis (sPLSDA) and heat map plots which were generated using MetaboAnalyst 4.0 software (www.metaboanalyst.ca), a comprehensive web-based package for a range of metabolomics applications (Chong et al., 2019).

The signals obtained in the untargeted metabolomic analysis were confirmed by contrasting the fragmentation spectrum in the Massbank or Metlin (www.massbank.jp; www.masspec.scripps.edu) to identify metabolites precisely. For compound identification, at least two of the following three criteria were needed: matching the exact mass; matching the retention time between the standard and experimental samples; and contrasting the obtained fragmentation spectrum with those available databases (**Chapters 2-3**).

3.7 Quantification of soluble carbohydrates and starch

The extraction of sugars from leaves and roots was performed as described in De Rocchis et al. (2022). The plant tissue was suspended in ultrapure water with mannitol as the internal standard, centrifuged twice and the supernatant was used for glucose, fructose, and sucrose analysis. The remaining pellet was pooled, resuspended in mannitol (0.5 mM), treated with amyloglucosidase (1.5 U; Sigma-Aldrich, Saint Louis, USA), centrifuged twice and the final supernatant was used for starch analysis. Starch and sugars were quantified using HPLC DIONEX ICS 3000 (Thermo Fisher Scientific, Waltham, USA) with a CarboPacTMPA200 AnalyticI (3 × 150 mm) column (Thermo Fisher Scientific, Waltham, USA) (**Chapters 2-3**).

3.8 Enzymatic activity assay

Plant tissues were used for extractions in order to analyse the activity of 12 key enzymes of primary carbohydrate metabolism: fructokinase (FK), hexokinase (HXK), phosphoglucoisomerase (PGI), phosphofructokinase (PFK), aldolase (Ald), ADP-glucose pyrophosphorylase (AGPase), phosphoglucomutase (PGM), UDP-glucose pyrophosphorylase (UGPase) and glucose-6-phosphate dehydrogenase (G6PDH), cell wall invertase (CwInv), vacuolar invertase (VacInv) and cytoplasmic invertase (CytInv); as described by Jammer et al. (2015) with few adjustments. To extract proteins from plant material, an extraction buffer containing TRIS-HCl pH 7.6, EDTA, PMSF, benzamidine, β-mercaptoethanol and NADP was used. Cell wall-bound proteins were extracted from the remaining pellet using a high-salt buffer with TRIS-HCl pH 7.6, NaCl, EDTA and MgCl2. The activities were measured photometrically using kinetic assays in a 96-well plate format and normalized to protein content determined using the Bradford method (Bradford, 1976), with Bovine serum albumin (BSA) as a standard protein (**Chapters 2-3**).

3.9 RNA extraction, cDNA synthesis and gene expression by qPCR

Total RNA from tomato leaves was extracted and treated with DNase using the Direct-zol RNA MiniPrep kit (Zymo Research). The RNA was then purified through a column using the RNA Clean & Concentrator-5 kit (Zymo Research), and stored at -80°C until further use. Gel electrophoresis and measurement of 260/230 and 260/280 ratios in NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) were performed to assess RNA quality and integrity. To synthesize first-strand cDNA, 1µg of purified total RNA was used, using the iScript cDNA Synthesis kit (Bio-Rad). All kits were used according to the manufacturer's recommended protocols.

Quantitative PCR reactions were performed using SYBR® *Premix Ex Taq*™ (TaKaRa) and an iCycler 5 device (Bio-Rad) according to the manufacturer suggested protocols. The relative quantification of mRNA levels was assessed with a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The expression of three different housekeeping genes, actin (Solyc03g078400), elongation factor 1-α (Solyc06g005060) and β-tubulin (Solyc04g081490) was measured to find the optimal normalization gene, using the Normfinder software (https://moma.dk/normfinder-software) (Andersen et al., 2004). According to the results, expression values of each marker gene (see Chapters for more details) were normalized using the housekeeping gene β-tubulin (**Chapter 1**) or actin (**Chapters 2-3**).

3.10 Statistical analyses

Besides the methods and software for analysis already described, all statistical analyses (one- and two-way ANOVA, PERMANOVA, log-rank, binomial test, t-test and post hoc tests applied when appropriate, as indicated in the corresponding figure legends) were conducted using STATGRAPHICS PLUS 3.1 (Rockville, MD, USA) and *R* software v.3.5.2 (R Development Core Team). Survival distribution comparison between treatments was performed using the non-parametric log-rank test (Mantel-Cox). Survival analyses were performed using *survival* and *survminer* R packages. Model validations were performed using Shapiro-Wilk and Levene's tests.

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Chapter 1

Phosphorus Availability Drives Mycorrhiza Induced Resistance in Tomato

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Phosphorus Availability Drives Mycorrhiza Induced Resistance in Tomato

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ABSTRACT

Arbuscular mycorrhizal (AM) symbiosis can provide multiple benefits to the host plant, including improved nutrition and protection against biotic stress. Mycorrhiza induced resistance (MIR) against pathogens and insect herbivores has been reported in different plant systems, but nutrient availability may influence the outcome of the interaction. Phosphorus (P) is a key nutrient for plants and insects, but also a regulatory factor for AM establishment and functioning. However, little is known about how AM symbiosis and P interact to regulate plant resistance to pests. Here, using the tomato-*Funneliformis mosseae* mycorrhizal system, we analyzed the effect of moderate differences in P fertilization on plant and pest performance, and on MIR against biotic stressors including the fungal pathogen *Botrytis cinerea* and the insect herbivore *Spodoperta exigua.* P fertilization impacted plant nutritional value, plant defenses, disease development and caterpillar survival, but these effects were modulated by the mycorrhizal status of the plant. Enhanced resistance of *F. mosseae*-inoculated plants against *B. cinerea* and *S. exigua* depended on P availability, as no protection was observed under the most P-limiting conditions. MIR was not directly explained by changes in the plant nutritional status nor to basal differences in defense-related phytohormones. Analysis of early plant defense responses to the damage associated molecules oligogalacturonides showed primed transcriptional activation of plant defenses occurring at intermediate P levels, but not under severe P limitation. The results show that P influences mycorrhizal priming of plant defenses and the resulting induced-resistance is dependent on P availability, and suggest that mycorrhiza fine-tunes the plant growth vs defense prioritization depending on P availability.

INTRODUCTION

The development of sustainable technologies for crop management aiming to reduce the use of fertilizers and pesticides is an ongoing global challenge in agriculture (Pretty et al., 2018). In this scenario, the potential of beneficial symbiotic microbes to improve plant nutrition and stress resistance/tolerance is well established (Berendsen et al., 2012; Pieterse et al., 2014; Pozo, 2020). However, their performance under agronomic conditions is not optimal, as the outcomes of plant-microbe interactions are highly context-dependent and may vary according to the plant growing conditions (van der Heyde et al., 2017; Lee Díaz et al., 2021). Among the beneficial organisms used as inoculants in agriculture, fungi, and particularly arbuscular mycorrhizal fungi (AMF), are receiving increasing interest (Pozo et al., 2021). These soil-borne microorganisms, belonging to the phylum *Glomeromycota*, are widespread in natural and agricultural ecosystems and colonize the roots of more than 80% of terrestrial plant species, including major crops (Smith and Smith, 2011). This mutualistic association, known as mycorrhiza, has major benefits for both partners: AMF receive plant organic carbon in the form of carbohydrates and lipids (Keymer et al., 2017; Salmeron-Santiago et al., 2022). In return, AMF improve water and nutrients uptake by the plant, especially phosphorus (P) (Chen et al., 2018; Ferrol et al., 2019). In addition, they also increase plant phenotypic and metabolic plasticity to cope with biotic and abiotic stressors (Mitra et al., 2021; Rivero et al., 2018, 2021; Sánchez-Bel et al., 2018, Campo 2020; Pozo de la Hoz et al., 2021).

AM symbiosis usually renders the plant more resistant to certain soil-borne and aboveground pathogens and chewing herbivores, as shown in multiple systems (Jung et al., 2012; Song et al., 2013, 2015; Selvaraj et al., 2021; Dowarah et al., 2021). This Mycorrhiza-Induced Resistance (MIR) is related to an improved ability of mycorrhizal plants to trigger defense responses upon challenge, a cost-efficient strategy known as defense priming (Pozo and Azcón-Aguilar, 2007; Martinez-Medina et al., 2016; Mauch-Mani et al., 2017). Priming is a common mechanism during induced systemic resistance triggered by beneficial microbes, consisting of a stronger and faster activation of plant defense mechanisms, usually dependent on jasmonate signaling (Pieterse et al., 2014; Mora-Romero et al., 2014; Gruden et al., 2020). Despite priming of plant defenses in response to pathogens and herbivores in mycorrhizal plants is well documented (Campos-Soriano et al., 2012; Song et al., 2013, 2015; Sanchez-Bel et al., 2016; Fiorilli et al., 2018; Schoenherr et al., 2019; Sanmartín et al., 2020; Rivero et al., 2021; Manresa-Grao et al., 2022) consequences of AM symbiosis for insect herbivores are complex to predict. Indeed, the nutritional benefits of the symbiosis may increase food availability or improve the diet quality for the pest, depending on the fungal and insect identity and growing conditions (Koricheva et al., 2009; Frew and Price, 2019). Thus, the interaction outcome results from the interplay

of opposite effects: nutritional improvement, benefiting the herbivore, and defense priming, potentially reducing herbivore performance (Pozo et al., 2020). Indeed, plant-microbe-insect interactions are very complex, with plants coordinating their responses according to multiple external and internal cues through a precise regulation orchestrated by phytohormone networks (Gruden et al., 2020; Pozo et al., 2015).

Many biotic and abiotic factors influence mycorrhizal colonization (Diagne et al., 2020; Mitra et al., 2021). Indeed, the plant-AMF interaction is finely regulated by several factors, including the plant and fungal genotypes and environmental conditions, with nutrients availability as one of the most influential aspects (Pozo et al., 2015; Lee Díaz et al., 2021). P is a central regulator of mycorrhizal establishment, root colonization and fungal growth within the host plant. Thus, the abuse of P fertilizers inhibits the establishment of mycorrhizal symbiosis in different plants (Smith and Smith, 2011; Smith et al., 2011; Higo et al., 2020). P, an essential nutrient for plants, is a non-renewable resource and poorly available in the field, being a limiting factor for plant growth (Wang et al., 2021). This element is taken up from the soil in the form of inorganic P by the roots (Rouached et al., 2010), and its deficiency triggers important changes in plant physiology and biochemistry including changes in plant growth, metabolism, hormonal balance, gene expression and root architecture. P deficiency further promotes the production of the phytohormones strigolactones, accumulation of anthocyanins and other phenolic compounds (Ha and Tran, 2014; Marro et al., 2022; Vysotskaya et al., 2016).

In agriculture, chemical and organic fertilizers are strongly and widely applied to solve P soil deficiency, causing contamination of aquifers and altering plant interactions with beneficial microbes, including AMF (Smith and Smith, 2011; Cordell and White, 2015). P levels also impact plant interactions with other organisms by altering plant tissues' nutritional value for organisms feeding on them (pathogens and herbivores) and by modulating plant defenses (Breullin et al., 2010; Castillo et al., 2017; Chan et al., 2021). The role of P in plant immunity is currently under intense scrutiny, and the data reveal a very complex scenario (Chan et al., 2021; Qu et al., 2021; Val-Torregrosa et al., 2022). The phosphate starvation response in plants has been suggested to increase plant resistance to necrotrophic pathogens and leaf chewing herbivores in several plant species (Arabidopsis, tomato, tobacco) pointing to a positive cross-talk between the JA- and P- starvation signaling pathways that activates plant immunity (Khan et al., 2016). However, opposite results have been also found in different plant species facing different aggressors (Campo et al., 2020; Val-Torregrosa et al., 2022). It should be noted that most basic studies dealing with the molecular mechanisms mediating P effects on plant immunity compare very contrasting P levels, usually far from those used in agricultural settings.

Despite the increasing number of studies addressing P influence on plant defenses, only a few have investigated the relationship between P and MIR, suggesting a complex interaction with contrasting results (Wang et al., 2020; Qu et al., 2021). While evidence of the role of P in regulating mycorrhizal colonization, plant growth and immunity responses exist, the interplay among the different effects and mechanisms behind this interplay remain poorly studied (Wang et al., 2020; Qu et al., 2021). Our study aims to elucidate whether the mycorrhizal effect on tomato resistance to biotic stressors is regulated by P availability. For that, we evaluated the impact of different P fertilization regimes on tomato growth, mycorrhizal colonization and herbivore resistance using the tomato/*Funneliformis mosseae* system and the necrotrophic pathogen *Botrytis cinerea* and the generalist chewing herbivore *Spodoptera exigua*. We found that P availability strongly influences plant growth and resistance to biotic stresses, and that these effects are modulated by the AM symbiosis, showing that MIR is indeed dependent on P availability. By analyzing plant nutrients, defense-related phytohormones and transcriptional regulation of defenses in response to the damage-associated signals oligogalacturonides (OGs), we aimed to uncover the mechanistic basis of the impact of P availability on MIR. This knowledge will contribute to improve AMF application and management for sustainable crop protection.

MATERIAL & METHODS

Biological material and mycorrhizal inoculation

The AMF *Funneliformis mosseae* BEG12 (Young, 2015) obtained from the International Bank of Glomeromycota (http://www.i-beg.eu), was maintained at the EEZ-CSIC greenhouse as open-pot cultures of *Trifolium repens* mixed with *Sorghum vulgare* plants using vermiculite-sepiolite substrate. The inoculum consisted in the substrate containing colonized root fragments, fungal mycelia and spores.

Botrytis cinerea was cultivated in potato dextrose agar plates, supplemented with freeze-dried tomato leaves. Three weeks later, *B. cinerea* spores were collected from plates in 0.5X potato dextrose broth as previously described (Sanmartín et al., 2020).

Spodoptera exigua (Lepidoptera: Noctuidae) eggs were provided by Dr. S. Herrero lab (ERI-BIOTECMED, Universitat de Valencia, Spain). Larvae were reared on artificial diet (Greene et al., 1976) at 25°C with 16:8h light:dark regime and 70% relative humidity until L2-L3 larval stage, for their application.

Tomato seeds (*Solanum lycopersicum* L. cv. Moneymaker) were surface disinfected by immersion in 4% NaClO (10 min), rinsed thoroughly with sterile water and incubated for 10 days in an open container with sterile vermiculite at 25°C. Plantlets were transferred to 100 mL pots containing a sterile sand:vermiculite (1:1) mixture. Pots for mycorrhizal treatments were inoculated by adding 10% (v/v) *F. mosseae* inoculum. All plants, including non-inoculated ones, received a 3 ml aliquot of a filtrate (<20 µm) of the inoculum, in order to provide the general microbial population but free of AMF spores.

Experimental design

Different experiments were carried out to elucidate the effect of P nutrition on plant performance and herbivore resistance in mycorrhizal and non-mycorrhizal plants. All experiments included tomato plants inoculated with *F. mosseae* (Fm) or not (Nm). We performed a first screening using three P concentrations: 0.3 mM, 0.6 mM and 1.0 mM (10 plants per treatment). For this, plants were watered with Hewitt nutrient solution (Hewitt, 1966), modified in the P (H_2NaPO4) concentration as described in Table S1. Plants were harvested after 8 weeks of growth and the performance of *S. exigua* fed on detached leaves was evaluated (see herbivore bioassays below). Plant biomass, mycorrhizal colonization, plant nutrients, anthocyanin content and phytohormone levels were also determined.

Based on the results obtained, 0.3 or 0.7 mM P concentrations were selected for follow-up experiments. In a second experiment, we assessed P effects on *Botrytis cinerea* lesion development through a detached leaf assay as described below, and we evaluated the performance of *S. exigua* larvae directly fed on Fm and Nm plants (whole plant bioassay). Six-weeks-old plants were infested with *S. exigua* larvae and caterpillar performance (weight, survival and pupation) was steadily monitored for 3 weeks (9 plants per treatment). Finally, early plant defense responses were compared in mycorrhizal and non-mycorrhizal plants growing at these two P levels (0.3 and 0.7 mM; 6 plants per treatment). For that, we assessed the plant response to damage by using oligogalacturonides (OGs), well characterized damage-associated molecular patterns (DAMPs) (see Plant treatment with oligogalacturonides below). The levels of defense-related phytohormones and defense-related gene expression were determined 6 hours post treatment (hpt) as described below. Thus, the factors considered in this study were M: mycorrhizal inoculation (levels: Nm, Fm), P: P fertilization regimes (levels 0.3, 0.7 and 1.0 mM), and OG: Oligogalacturonide treatment (levels: -OG, +OG).

Plant growing conditions

For the different experiments, plants were randomly distributed and grown in a greenhouse at 24/16°C with a 16/8 h diurnal photoperiod and 70% humidity. Plants were watered twice a week with half strength Hewitt nutrient solution (Hewitt, 1966) modified in the P content as described above, and water was supplied as needed. Upon harvesting shoots and roots, fresh weight was determined and the material was immediately frozen in liquid N and stored at -80°C for further analyses. An aliquot of each individual root system was preserved for mycorrhizal quantification.

***Botrytis cinerea* infection**

The fourth leaf of tomato plants was detached for pathogen bioassays. Pathogen infection was performed by applying 10 µL drops containing a conidia suspension (1×10^6 spores/ml) to the detached leaves. One leaf per plant was inoculated by adding two drops per leaflet, five leaflets per leaf, with a total of 90 lesions. The infected leaves were maintained in 15cm Petri dishes on wet filter paper and incubated in a phytotron chamber at 80% of humidity and 22ºC. Necrotic lesions were measured 3 days post-inoculation.

Herbivore performance bioassays

Insect performance was evaluated in two different bioassays, using detached leaves or whole plants. For the detached leaves assay, two second-instar *S. exigua* larvae were placed on one detached leaf of each tomato plant, placed on wet filter paper in 15 cm Petri dishes. Plates were then incubated in a phytotron at 26-24ºC, 16:8h day/night and 60% relative humidity. Larval mortality and weight was evaluated every 2 days for 8 days.

For whole plants assay, two second-instar larvae were placed in a leaflet of the fourth true leaf of each tomato plant, using clip-cages to avoid their escape, as described in Rivero et al. (2021). Every two days, clip-cages were moved to new fresh leaflets and caterpillar biomass, mortality and pupation were monitored.

Plant treatment with oligogalacturonides

For the analysis of early plant responses, the damage associated molecules oligogalacturonides (OGs) were used to elicit plant defense responses. The OGs (DP 10-15) were prepared as described in Benedetti et al. (2017) and provided by Dr. De Lorenzo lab (Department of Biology and Biotechnology "Charles Darwin" BBCD, La Sapienza University, Rome, Italy).

Six weeks post AMF inoculation tomato plants grown at 0.3 or 0.7 mM P fertilization regimes were treated with an aqueous solution of OGs (50 µg/ml in milliQ water) as described in Gamir et al. (2020). The fourth true leaf of each plant was sprayed with the OG solution or milliQ water for control plants using an aerograph until running off. Treated leaves were harvested after 6 hours to study early plant defense responses, as this time was the most appropriate to identify changes in hormone contents and in the expression levels of OG responsive, defense related (Gamir et al., 2020).

Determination of mycorrhizal colonization

AM colonization was measured after clearing washed roots in KOH (10%) and staining fungal structures with 5% ink in 2% acetic acid (Vierheilig et al., 2005). The percentage of total root length colonized by *F. mosseae* was estimated according to the gridline intersection method (Giovannetti and Mosse, 1980) using a BOECO zoom stereo microscope Model BST-606.

Anthocyanin content

Anthocyanin content, as P starvation indicator, was evaluated by adapting the protocol established in Rabino and Mancinelli (1986). Frozen leaves were grinded and lyophilized, and 5 mg aliquot of dry tissue was used per sample. The pigments were extracted by shaking the plant freeze-

dried leaf powder in acidic (1% HCl, w/v) methanol in dark overnight. Extracts were centrifuged for 10 min at 13000 rpm. The amount of anthocyanin was calculated measuring absorbance at 530 and 657 nm of crude extract, using the formula A530 - 0.25 A657 to compensate for the contribution of chlorophyll and its degradation products to the absorption at 530 nm. Six independent biological replicates were analyzed per treatment.

Determination of mineral nutrients in leaves

Nutrient analyses were performed at the Technical Services of the Estación Experimental del Zaidín (CSIC). Frozen leaves were grinded and lyophilized, and 50-100 mg aliquot of dry tissue was used per sample. The concentration of P and micronutrients were determined after acid digestion of samples, by inductively coupled plasma optical emission spectrometry (ICP-OES; Varian ICP 720-ES). Total C and N contents were analyzed using an Elemental Analyzer (LECO TruSpec CN), according to standard procedures. Six independent biological replicates were analyzed per treatment.

Targeted hormonal extraction and quantification

Hormone extraction was performed from freeze-dried powdered plant leaves as described in Sanchez-Bel et al. (2016). Six independent biological replicates per treatment were analyzed. Briefly, 30 mg of plant dry tissue was extracted with 1 ml of H_2O:MeOH (9:1) containing 0.001% of HCOOH and 100 ng/ml of internal standards. After different centrifugations and resuspensions, an aliquot of the extract was injected into an Acquity Ultra Performance Liquid Chromatography system (UPLC) (Waters, Mildford, MA, USA). Hormones were chromatographically separated using an HPLC Kinetex C18 analytical column (Phenomenex) connected to a triple quadrupole mass spectrometer (TQD, Waters, Manchester, UK). The chromatographic and mass spectrometry conditions were those used by Gamir et al. (2012). Hormone quantification (ng/g dry weight) was performed using calibration curves with each pure chemical standard. The plant hormones abscisic acid (ABA), indolacetic acid (IAA), jasmonic acid (JA), its precursor (+)-12-oxo-phytodienoic acid (OPDA) and salicylic acid (SA) were determined.

Analysis of gene expression by qPCR

The expression of marker genes from different metabolic pathways was analyzed by real time quantitative PCR (qPCR). Six independent biological replicates per treatment were used. Total RNA from tomato leaves was extracted and treated with DNase using the Direct-zol RNA MiniPrep kit (Zymo Research). Subsequently, the RNA was purified through a column using the RNA Clean and Concentrator-5 kit (Zymo Research), and stored at -80°C until use. The first-strand cDNA was

synthesized with 1 µg of purified total RNA using the iScript cDNA Synthesis kit (Bio-Rad). All kits were used according to the manufacturer's suggested protocols.

The expression of three different housekeeping genes, actin (Solyc03g078400), elongation factor 1-α (Solyc06g005060) and β-tubulin (Solyc04g081490) was measured to find the optimal normalization gene, using the Normfinder software (https://moma.dk/normfinder-software) (Andersen et al., 2004). According to the results, expression values were normalized using the housekeeping gene β-tubulin and relative quantification of specific mRNA levels was performed using the comparative 2–Δ (ΔCt) method (Livak and Schmittgen, 2001). The sequences of the specific primers used are shown in Table S2.

Statistical analyses

All statistical analyses (multi-way ANOVAs and post hoc tests applied when appropriated, as indicated in the corresponding figure legends) were conducted using Statgraphics Plus 3.1 (Rockville, MD, USA) or 'R' software v.3.5.2 (R Development Core Team). Figures were obtained using *ggplot2* R package (Wickham, 2011). Treatment effects on larval survival were assessed by comparing the survival curves using the Kaplan-Meier estimator (Kaplan and Meier, 1958). Survival distribution comparison between treatments was performed using the non-parametric Logrank test (Mantel-Cox). Survival analyses were performed using *survival* and *survminer* R packages. Model validations were performed using Shapiro-Wilk and Levene's tests.

RESULTS

P fertilization levels impact mycorrhizal colonization, plant growth and herbivore performance in tomato

To explore how mycorrhizal development and its effects on plant and caterpillar performance are affected by P fertilization, we compared 3 fertilization regimes differing only in the P content, ranging from limiting to sufficient P (0.3, 0.7 and 1.0 mM). Analysis of the plant biomass confirmed that P levels had a significant impact on plant growth (p<0.001) (Figures 1A and B). Plants grown at 0.3 mM P showed about 50% reduced root and shoot weights. Significant differences between 0.7 and 1.0 mM P were also observed; however, these were mild compared to the most P limiting conditions (Figures 1A and B). Interestingly, mycorrhization did not have a global significant effect on plant fresh weight, but there was a significant interaction between the P and mycorrhizal treatments, with mycorrhiza promoting plant growth only at the intermediate (0.7 mM) P level and repressing root biomass at the highest P level (Two way ANOVA, Figure1A and 1B). The evaluation of anthocyanin accumulation in leaves, as an indicator of plant P-starvation response, also confirmed the dose-dependent effects of P fertilization on the plants. Plants grown under low P (0.3 mM) showed the highest anthocyanin levels, while the levels in plants growing at 0.7 and 1.0 mM were not significantly different (Figure 1C). Mycorrhization also had a significant effect reducing the anthocyanin levels (two-way ANOVA; p<0.05). Mycorrhizal colonization was also significantly impacted by P fertilization, with increasing P concentrations leading to a reduction in colonization (Figure 1D). Differences were significant already after 4 weeks of growth, with mycorrhizal colonization in the moderate and high P conditions being half of those at low P. The effect was more pronounced at the later time point (final harvest, 8 weeks), as root colonization continued to increase in plants fertilized with the lowest P concentration (0.3 mM), but not with the other P levels (Figure 1D).

The effect of plant P fertilization on the performance of *S. exigua* larvae fed on leaves of those plants was also evaluated by using detached leaves. Larval weight was influenced by P levels, being significantly lower at 0.3 mM P (Figure 1E).

No differences between larvae fed in 0.7 or 1.0 mM were found, regardless of the mycorrhizal status of the plant (Figure 1E). P levels also had a significant impact on *S. exigua* mortality, showing the highest mortality at the low 0.3 P level (ranging between 50-60%) (Figure 1F). At higher P levels (0.7 and 1.0 mM) larvae mortality ranged from 6 to 21% (Figure 1F). Although higher mortality was found in Fm compared to Nm plants at these medium and high P levels (21% Fm vs 6% Nm for 0.7mM, and 16%Fm vs 6%Nm for 1.0 mM), the differences were not significant. Noteworthy, when analyzing the data separately according to the mycorrhizal status, P effect on larval survival was more pronounced

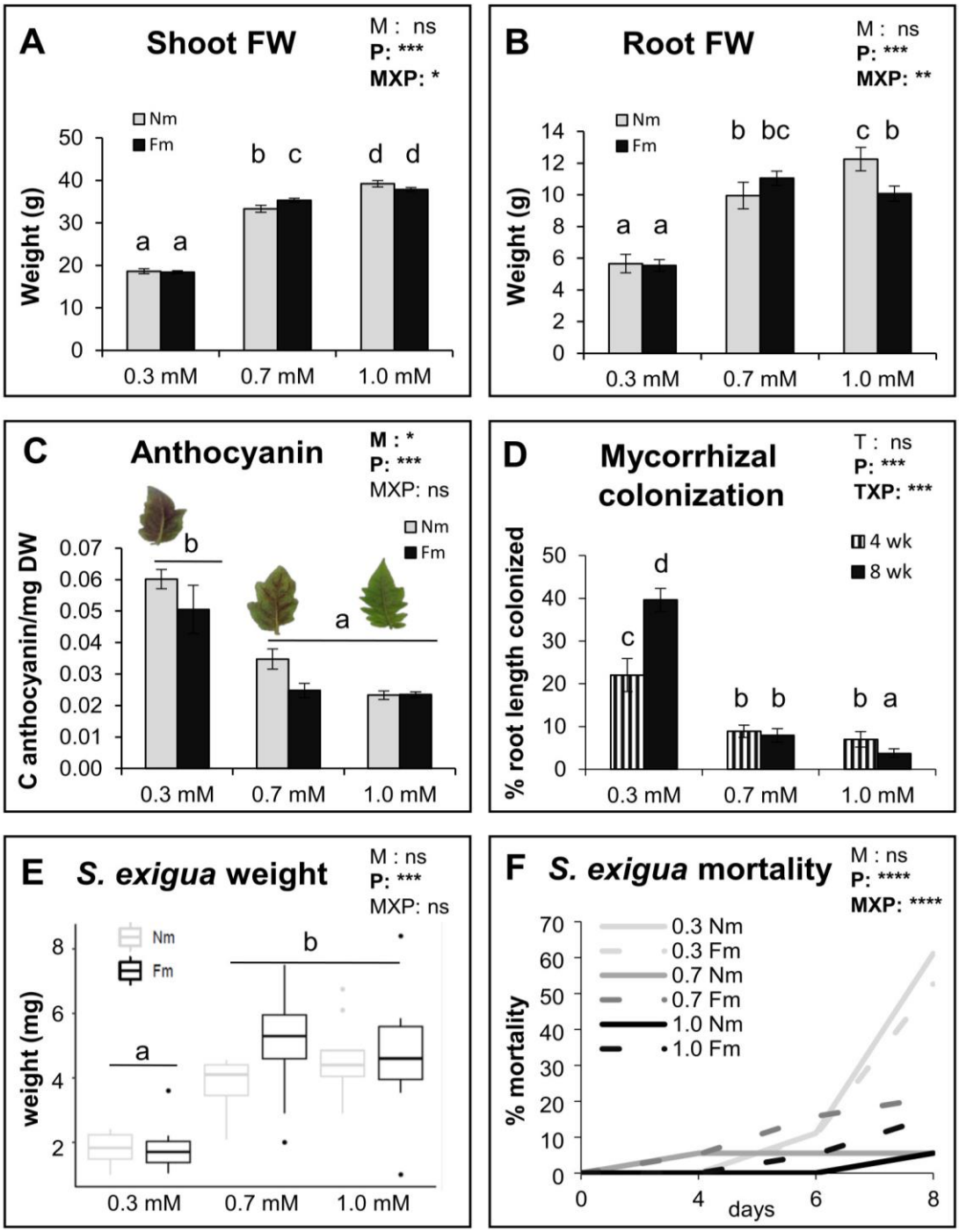

FIGURE 1. Impact of P levels on plant growth, AM colonization and herbivore performance. Plant growth parameters, fungal colonization and herbivore performance on non-mycorrhizal (Nm) and mycorrhizal tomato plants colonized by *Funneliformis mosseae* (Fm). Plants were fertilized with different P concentrations: 0.3 mM, 0.7 mM and 1mM of H_2NaPO_4. **(A)** Shoot and **(B)** root fresh weight (n=10), and **(C)** anthocyanin content (n=6) were determined in tomato plants at harvest, 8 weeks post mycorrhizal inoculation (pmi). **(D)** Percentage of root length colonized by the mycorrhizal fungi at 4 and 8 weeks pmi (n=10). One leaf per plant was detached 8 weeks pmi and infested with 2 second instar *S. exigua* larvae (n=20), and **(E)** weight of the larvae was determined after 6 days of feeding. **(F)** Larval mortality was monitored during 8 days of continuous feeding on the detached tomato leaves. Data from A to E represent means of the n independent biological replicates ± SD. Two-way factorial ANOVA **(A-C, E, F)** using AM symbiosis (M) and P treatments (P) as factors, or **(D)** using time (T) and P treatments (P) as factors were performed, and significance values of each factor and their interactions are indicated in the upper right corner of each graph. Asterisks denote significant effect of a factor and their interaction. ns: no significant; *: p<0.5; **: p<0.01; ***: p<0.001; ****: p<0.0001. Different letters represent statistically significant differences (ANOVA, Fisher's Least Significant Difference (LSD) test; p<0.05). For **(F)**, data represent the percentage of mortality at the different time points, and the differences in the survival distribution according to P, M and MxP were performed using the non-parametric Log-rank (Mantel-Cox) test.

in Nm than in Fm plants: P levels had a very significant impact on larval survival when feeding on Nm plants (p<0.0001), but not on those feeding in mycorrhizal plants (p=0.052) (Figure S1A).

P effects on herbivore performance depends on the mycorrhizal status of the plant and determine MIR

Overall, the results of the P dose screening revealed that most differences occur between the low (0.3mM) and the higher P regimes (0.7 and 1.0 mM) that showed similar values for most parameters. Accordingly, 0.3 and 0.7 mM levels were selected for further experiments as the closest doses with contrasting effects. Bioassays on detached leaves are useful for quick screenings of major effects, but these effects are usually weaker than those using whole plant bioassays. The latter allow a more realistic set up and longer evaluation periods. Therefore, we performed a second experiment focused on the selected P levels (0.3 and 0.7 mM) to better address the effect of mycorrhization on larval performance under different P fertilization. Mycorrhizal colonization by *F. mosseae* was 14% and 6% for the 0.3 and 0.7 mM P levels, respectively. Again, P effect on *S. exigua* mortality was significant for larvae feeding in Nm plants (p<0.0001), but not for those feeding on mycorrhizal (Fm) ones (p=0.27) (Figure S1B). Thus, mycorrhizal colonization seems to buffer the strong effect of P on plant resistance to the pest. As in the previous experiment using detached leaves, larvae performed worst in the lowest P fertilized plants, showing higher mortality levels (Figures 2A and B), lower weight (Figures 2C and D) and worst development –evaluated as the percentage of individuals reaching the pupal stage- (Figures 2E and F). Regarding the effect of mycorrhization on *S. exigua* performance, under low P no significant changes were found in mortality nor development between Fm and Nm plants (Figures 2A and E), and larval weight was even higher at some time points in mycorrhizal plants (Figure 2C).

In contrast, under moderate P levels (0.7 mM), larvae fed on mycorrhizal Fm plants performed worse than those fed on Nm, showing higher mortality, lower weight and impaired development (Figures 2B, D and F). The results reveal that the effect of mycorrhization on larval performance depends on P availability, as MIR was observed at the moderate (0.7 mM) P levels, but not at the low (0.3 mM) one. Remarkably, a similar pattern was observed in the interaction with *B. cinerea*. Again, MIR was only observed at 0.7 mM P, but not at 0.3mM P, and while the effect of P was significant in Nm plants (Nm 0.3 vs Nm 0.7 t-test, p<0.0001), it was not significant in Fm plants (Fm 0.3 vs Fm 0.7 t-test, p= 0.24) (Figure 3A).

Differences in MIR are not directly related to changes in the nutritional status of the plant

To address whether the P-dependent effects of mycorrhiza on larval performance were due to nutritional aspects, we evaluated plant biomass, anthocyanin and nutrient contents. Shoot biomass

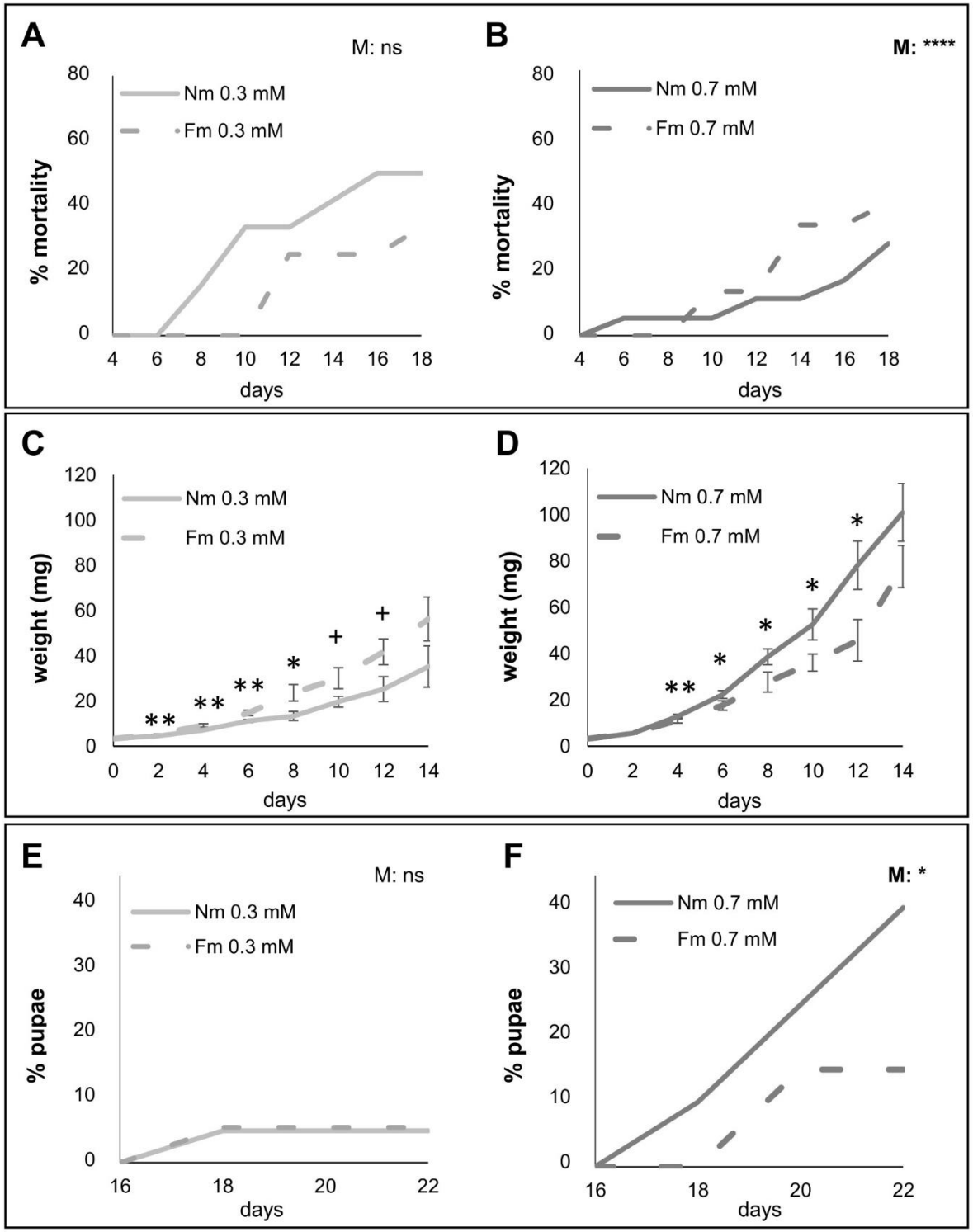

FIGURE 2. Impact of mycorrhiza on herbivore performance under different P availability. *S. exigua* performance of larvae fed on leaves of mycorrhizal (Fm, dotted line) and non-mycorrhizal (Nm, continuous line) plants fertilized with 0.3 or 0.7 mM of P, light and dark grey, respectively; (n=9). Six-weeks post-inoculation with *F. mosseae* (Fm), plants were infested with second instar *S. exigua* larvae (two per plant, n=18 per treatment) using a clip-cage to confine the larvae to a leaflet. Infestation was maintained for three weeks by moving the clip-cage every two days. **(A, B)** *S. exigua* mortality **(C, D)** weight and **(E, F)** individuals reaching pupa stage. Statistical analyses were performed independently for each P fertilization level: **(A, E)** 0.3 mM and **(B, F)** 0.7 mM. **(A, B, E, F)** P values in the upper right corner of each graph indicate statistically differences in mortality and pupation between Nm and Fm, according to Log-rank (Mantel-Cox) test. For larval biomass **(C, D)**, values are the weight mean of survived larvae ± SD. Asterisks indicate significant differences between Nm and Fm treatments at given time point according to t-test. +: p<0.1; *: p<0.05; **: p<0.01; ***: p<0.001.

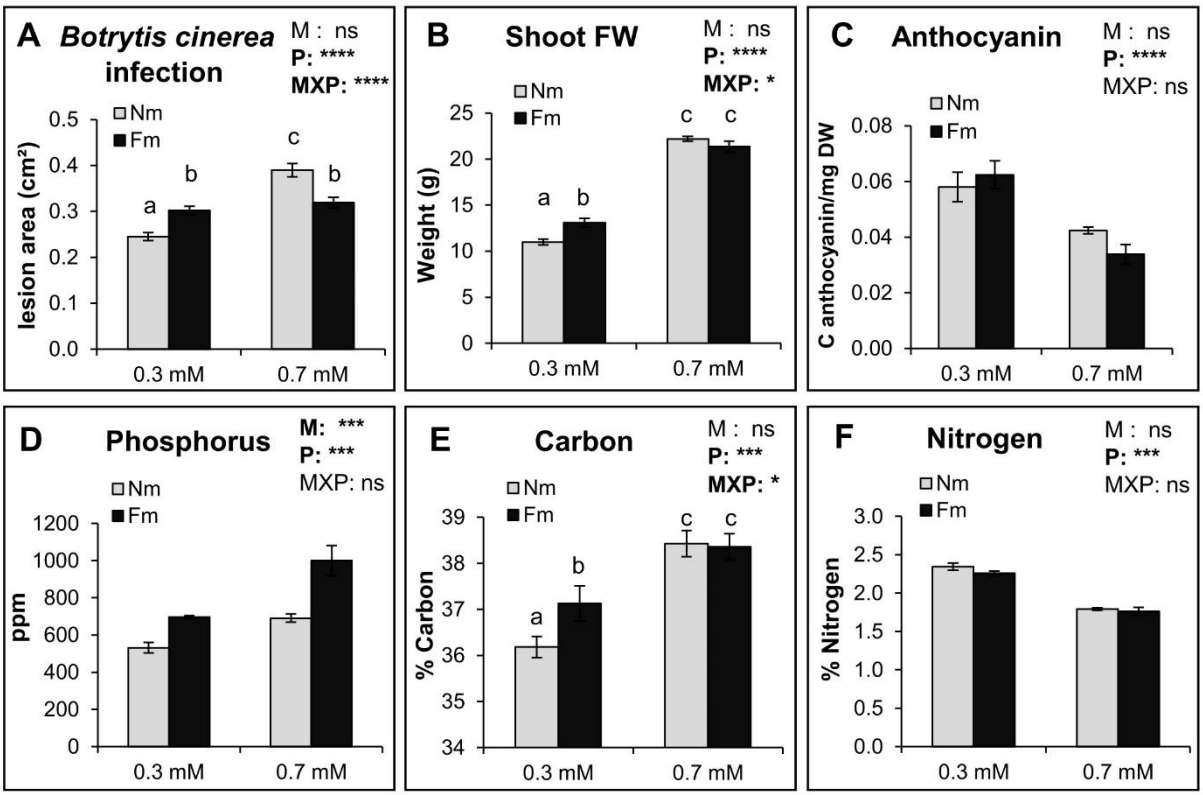

FIGURE 3. Impact of P fertilization and mycorrhization on the nutritional value of tomato leaves. *Botrytis cinerea* infection, shoot biomass, anthocyanin, and nutrient contents in tomato leaves of non-mycorrhizal (Nm) and mycorrhizal tomato plants colonized by *F. mosseae* (Fm). Plants were fertilized by two P concentrations: 0.3 mM and 0.7 mM P. **(A)** Diameter of necrotic lesions 3 days post inoculation with *B. cinerea* in detached adult leaves from tomato (n=9). **(B)** Shoot fresh weight (n=9), **(C)** anthocyanin (n=6), **(D)** phosphorus (n=6), (**E)** carbon (n=6) and **(F)** nitrogen (n=6) content were measured in tomato plants 6 weeks post mycorrhizal inoculation. Data represent the means of n independent biological replicates ±SD. Two-way ANOVA with mycorrhizal (M) and P treatments (P) as factors, was performed, and the significance of the factors and their interaction is indicated in the upper right corner of each graph. Different letters represent statistically significant differences (ANOVA, Fisher's Least Significant Difference (LSD) test; $p<0.05$) where the interaction between factors was observed. Otherwise, asterisks denote significant effect of a factor and their interaction. ns: no significant; *: p<0.05; **: p<0.01; ***: p<0.001.

was dependent on P fertilization, and growth promotion by mycorrhiza depended on P levels: Fm promoted plant growth at low P, while no plant growth promotion was observed at 0.7 mM P (Figure 3B). Anthocyanins were only influenced by P levels (Figure 3C). Regarding the nutritional value of the leaves, P content in leaves increased with P fertilization, and it was significantly higher in mycorrhizal plants (Figure 3D). P fertilization also significantly influenced carbon (C) and nitrogen (N) levels in leaves, increasing C and reducing N concentration at 0.7mM as compared to 0.3 mM. While mycorrhization did not have a global effect on their content, the interaction between P and M was significant for C content, with mycorrhiza displaying higher C levels than Nm plants under the low P fertilization (Figures 3E and F), while the interaction between P and M was significant in C content. As mycorrhization had the same effect on the nutrient content of leaves at the highest P level (higher P values, no changes in C nor N as compared to Nm plants), mycorrhiza-related changes in the nutritional

value of leaves do not seem to explain the differential impact of mycorrhization on larval performance under the different P fertilization levels.

Most micronutrients were significantly influenced by P (Ca, Cr, Cu, Fe, Mg, Mn, Mo, Na, Ni, S, Sr and Zn), while M influenced only some of them (as Cu, Fe, K, Li, Mn, Sr, Zn) (Table S3). Only K was significantly regulated by the interaction of the two factors (Table S3).

P availability impacts the levels of defense-related phytohormones and gene expression

We explored whether the impact of P levels on MIR was related to differential activation of plant defense responses. In order to monitor early defense responses, and aiming to reduce the variability associated with pathogen development or differential feeding by the larvae, we used OGs as elicitors. Plants grown in parallel to those in the whole plant herbivory assay (thus same age, growing conditions and mycorrhizal colonization levels) were challenged by spraying a fully expanded leaf with an OG solution, as described in Gamir et al. (2020). Treated leaves were analyzed for hormone content and defense-related gene expression 6 hours after OG application. The levels of jasmonic acid (JA), its precursor OPDA, auxin (IAA) and abscisic acid (ABA), as the major hormones involved in plant responses to chewing herbivores and necrotrophic pathogens, and described to be regulated by OGs, were evaluated (Figure 4). Multiway ANOVA confirmed a significant impact of P fertilization on the OPDA, ABA and IAA levels, but not on the JA content. AM symbiosis only impacted the IAA levels, while OG treatment significantly affected all hormone levels except for ABA. Noteworthy, no significant interaction between the different factors was observed for hormones except for ABA levels. Overall, P deficiency significantly reduced OPDA and IAA levels, but increased ABA. OGs enhanced JA, OPDA and IAA levels regardless of the mycorrhizal status of the plant (Figure 4). Mycorrhization only had a significant effect on IAA levels, although it modulated the impact of P and OG on ABA levels (Figure 4). Salicylic acid (SA) levels were also determined, showing a reduction by increasing P or OG treatment, but no significant effect of mycorrhiza or factors interaction was observed (Figure S2).

As hormone levels were not different in leaves of mycorrhizal plants, we hypothesized that mycorrhizal plants could prime downstream defense responses, but that this effect was dependent on P levels. We analyzed the gene expression of well characterized JA regulated anti-herbivore defense markers, including the *Leucyl aminopeptidase A (LapA*), *Proteinase inhibitor II* (*PinII*), *Threonine deaminase* (*TD*) and *Multicystatin* (*MC*) (Yan et al, 2013; Uppalapati et al. 2005). Surprisingly, in non-challenged plants (-OG) all these defense genes (except MC) were up-regulated in Nm plants grown under low P levels as compared to Nm grown under moderate P levels, but this upregulation was not observed in mycorrhizal Fm plants (Figures 5A-E; Tables S4 and S5).

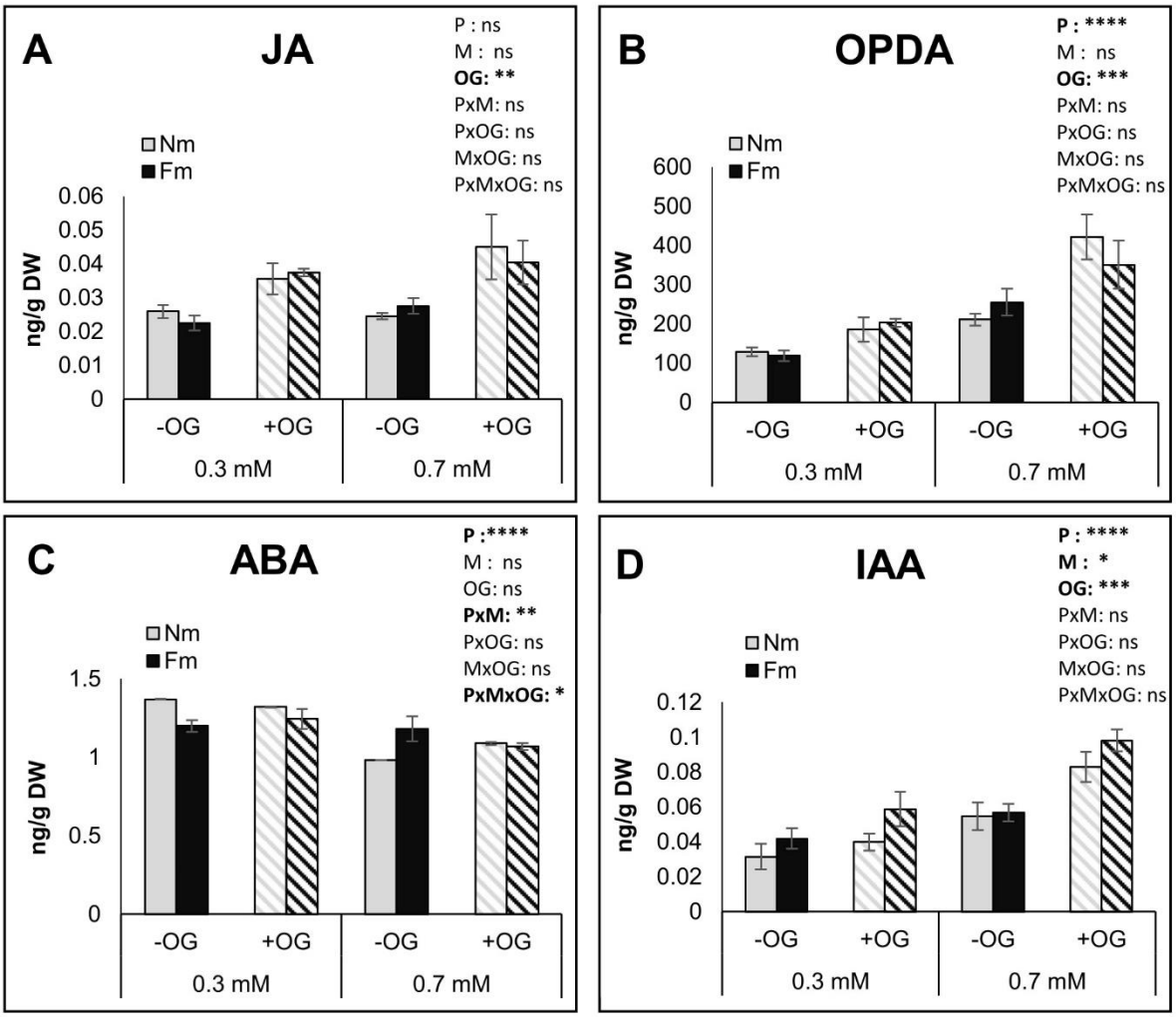

FIGURE 4. Impact of OG treatment on the content of defense-related phytohormones in tomato leaves. Targeted analysis of phytohormones in non-mycorrhizal (Nm) and mycorrhizal tomato plants colonized by *F. mosseae* (Fm) grown under 0.3 mM or 0.7 mM P fertilization. The fourth true leaf of each plant was sprayed with 50 µg/ml OG solution (+OG; stripped bars) or water for the control (-OG; filled bars) and harvested at 6h post elicitation. (A) JA, (B) OPDA, (C) ABA and (D) IAA were quantified by UPLC-MS/MS in tomato leaves. Data represent the means of 6 independent biological replicates ± SD. Three-way ANOVA using P fertilization (P) mycorrhizal (M) and OG treatments (OG) as factors was performed and significance is indicated in the upper right corner of each graph. Asterisks denote significant effect of a factor or their interaction. ns: no significant; *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001.

OG treatment in plants grown under low P resulted in a reduced expression of these genes in Nm plants, while they showed a slight induction in Fm plants (see fold changes in Table S5). In contrast, under moderate (0.7 mM) P levels, these genes showed similar expression levels in mycorrhizal and non-mycorrhizal plants in the absence of challenge; however, they were significantly induced by OG treatment only in Fm plants. Thus, the expression analyses confirmed a primed response of mycorrhizal plants to the OG treatment under sufficient P (Figures 5 A-D; Table S5). A similar primed response was found for the gene encoding a defense polygalacturonase inhibiting protein (*LePGIP*), also related to defense responses (Baroncelli et al., 2016) (Figure 5E).

The reduced levels of defense genes in non-challenged (-OG) mycorrhizal plants under P starvation, led us to explore the expression of regulators of JA-dependent defense responses.

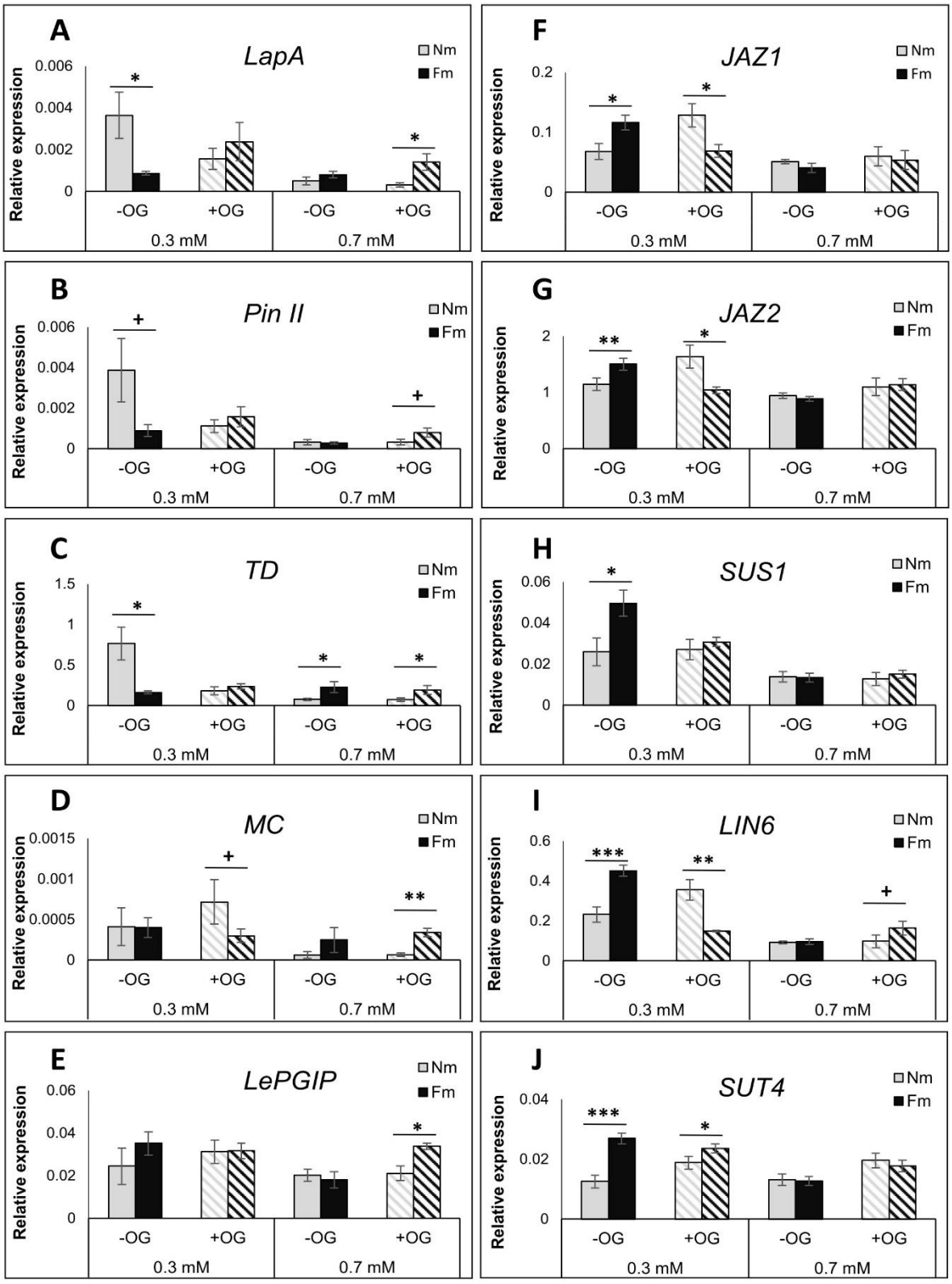

FIGURE 5. Transcriptional regulation of defense- and growth-related genes. Gene expression levels in OG-treated (+OG; stripped bars) or water-treated (-OG; filled bars) leaves of non-mycorrhizal (Nm) and mycorrhizal (Fm) plants grown under 0.3 mM and 0.7 mM P fertilization regimes. Real-time quantitative RT-qPCR analysis of genes coding for the leucyl aminopeptidase A (*LapA*), proteinase inhibitor II (*PinII*), threonine deaminase (*TD*), polygalacturonase inhibiting protein (*LePGIP*), multicystatin (*MC*) and sucrose synthase-1 (*SUS1*), sucrose transporter 4 (*SUT4*), cell wall invertase 6 (*LIN6*), and the transcriptional regulators JASMONATE ZIM DOMAIN 1 (*JAZ1*) and JASMONATE ZIM DOMAIN 2 (*JAZ2*) are shown. Values were normalized to the tomato housekeeping gene β-tubulin. Bars represent mean ± SD from 6 biological replicates. Asterisks indicate significant differences between Nm and Fm within the same treatment according to t-test. +: $p<0.1$; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

JAZ proteins are key negative regulators of JA signaling, repressing JA-regulated defenses and promoting plant growth (Guo et al., 2018). We checked the genes coding for *JAZ1* and *JAZ2*, both described to be JA-responsive in tomato leaves (Chini et al., 2017). These genes were indeed differentially regulated in mycorrhizal plants under low P, while no differences were found at sufficient P levels (Figures 5F and G; Table S5). Remarkably, in the absence of challenge (-OG), mycorrhizal plants showed higher expression levels of these negative regulators than Nm plants, in agreement with the repressed expression of the defense genes. However, when plants were elicited with OGs, *JAZ* expression was reduced in mycorrhizal plants, thus releasing the suppression of JA defenses (Figures 5F and G; Table S5). This effect was illustrated by the increased expression of the JA-regulated defense genes *LapA, PINII* and *TD* in Fm plants when comparing elicited (+OG) with non-elicited (-OG) plants (Figures 5 A-D, Table S5). Since JAZs negatively regulate JA-dependent defenses and modulate the growth-defense balance (Guo et al., 2018), we explored whether the differential *JAZ* expression was associated with transcriptional differences related to primary metabolism. For that, we analyzed genes involved in carbohydrate metabolism and transport: sucrose synthase 1 (*SUS1*), cell wall invertase 6 (*LIN6*) and sucrose transporter 4 (*SUT4*) (Sanmartín et al., 2020). Under low P, they all showed a very strong up-regulation in non-elicited mycorrhizal plants, while the difference was absent (or even reversed as for *LIN6*) upon OG elicitation (Figures 5H-J). In contrast, these genes were unaltered by mycorrhizal colonization at high P levels.

The multiway ANOVA of the expression data confirmed the very significant impact of P levels in all genes analyzed, while significant global effects of OG treatment and mycorrhizal status were restricted to specific genes. However, the interaction between mycorrhization and P levels, and between mycorrhization and OG treatment was significant for most genes, confirming that changes in gene expression triggered by both P and OG are modulated by the mycorrhizal status (Table S6). Indeed, a strong increase of JA-dependent defenses occurs in Nm plants under P deficiency in the absence of challenge, while they were down-regulated in mycorrhizal Fm plants as compared to Nm plants in these conditions. This defense suppression in Fm plants is likely related to the highest expression of the negative regulators *JAZ1* and *2*, and a transcriptional up-regulation of the carbohydrate metabolism, while no differences were found under sufficient P (Figure 5; Table S5). In contrast, upon OG treatment, Nm plants did not elicit defenses at any of the P doses (Table S5). Plant defenses were only induced in Fm plants, leading to a clear primed defense pattern in mycorrhizal plants under sufficient P fertilization. The transcriptional data suggest a prioritization of the primary metabolism (growth) over defense under P limiting conditions in mycorrhizal plants in the absence of challenge. In contrast, defense responses are prioritized upon challenge, leading to a clear primed response only under sufficient P levels (Figure 5; Tables S4, S5, S6).

DISCUSSION

Evidence of the major role of P in regulating plant immunity is increasing in recent years (Campos-Soriano et al., 2020; Chan et al., 2021; Val-Torregrosa et al., 2022). To identify key molecular regulators and their function, studies usually compare very extreme P levels, addressing null P to dissect P starvation responses (Dixon et al., 2020; Higo et al., 2020). However, even moderate differences in P concentration may have an impact on plant physiology, and severe P deficiencies are unlikely to occur in agricultural settings. In the present study, we assess how moderate differences in P fertilization, which are more likely to occur in agricultural conditions than severe ones, affect mycorrhizal colonization, growth, and resistance to *S. exigua* in tomato, using varying P levels that still allow a good plant development. Our results illustrate that while P regulates plant fitness and pest resistance, its effects depend on the mycorrhizal status of the plant. Moreover, they confirm that plant protection by mycorrhiza depends on P availability, highlighting that P fertilization is a key element in the context-dependency of MIR.

We show the dose-dependent effects of P on tomato growth and resistance against *B. cinerea* and *S. exigua*. Changes in most parameters were not linearly related to the P supply (0.3, 0.7 and 1.0 mM). Instead, P-related differences in most parameters (including plant biomass, anthocyanin content, mycorrhizal colonization, pathogen and herbivore performance on plant) were more pronounced when comparing 0.3 and 0.7 mM P, with no significant differences between 0.7 and 1.0 mM P -except for shoot fresh weight. Therefore, we selected 0.3 and 0.7 mM P as limiting and sufficient P conditions, respectively, to address the mechanisms that may underlie the differences observed in the plant resistance to the herbivore and in MIR under different P scenarios. We observed that the moderate P fertilization (0.7 mM) allowed mycorrhizal colonization while maintaining good plant development.

The inhibitory role of P on mycorrhizal colonization is well documented but varies with the plant and AMF species (Smith and Smith, 2011; Chen et al., 2014; Higo et al., 2020). Mycorrhizal colonization in tomato is indeed highly dependent on P levels. In our study, the medium and high P doses allowed mycorrhizal establishment, but prevented the progression of mycorrhizal colonization in time. However, although mycorrhizal colonization was around 10%, this was enough to significantly affect several parameters. Some growth promotion was observed at the moderate P level, and anthocyanin content, a good indicator of P starvation, were significantly reduced by mycorrhiza under these conditions. The relationship between the extension of mycorrhizal colonization in roots and the benefits of symbiosis has been discussed for years (Treseder, 2013). While higher colonization levels can lead to a stronger P uptake by the mycorrhizal pathway, benefits are not proportional to colonization, and even low colonization levels may have important benefits for the plant.

Regarding the attackers performance, a clear effect of P fertilization was observed. P levels affect both *B. cinerea* lesion development and *S. exigua* performance. Caterpillars performed worst on plants growing under low P, displaying very low weight and very high mortality compared to the other fertilization regimes. This is in agreement with P being an essential nutrient also for caterpillars (Perkins et al., 2004), as previously shown in other caterpillar-plant systems as *Mamestra brassicae-Plantago lanceolata* or *Spodoptera littoralis-Nicotiana benthamiana* (Qu et al., 2021; Khan et al., 2016). In our system, mycorrhization *per se* did not affect any of the parameters, but the data analyses revealed a significant interaction between the factors P and mycorrhiza (M). Remarkably, the impact of P levels on larval performance was significant in non-mycorrhizal plants, but not in mycorrhizal plants. A similar pattern was found in the interaction with the pathogen: lesion development was significantly impacted by the P levels in Nm plants, but not in Fm plants. Thus, the mycorrhizal symbiosis buffered the influence of P fertilization on the susceptibility to the attacker. Because of the limitations of performing herbivory tests in detached leaves, we performed more detailed analyses allowing the caterpillars to feed directly in the plant using the low (0.3 mM) and moderate (0.7 mM) P doses, which previously showed contrasting effects on plant and *S. exigua* performance. Using whole plants, not only local, but also systemic plant responses may contribute to the final outcome, as for example roots and distal leaves play a key role in response to damage and to herbivory (Gamir et al., 2020; Kundu et al., 2018; Erb and Reymond, 2019).

Under the lowest P level, there was no effect of the mycorrhizal symbiosis on larval mortality nor on their development to pupae. Noteworthy, caterpillar weight was higher for those feeding on mycorrhizal plants than for those on non-mycorrhizal ones, pointing to a potential benefit of the AM symbiosis at the nutritional level. In contrast, under 0.7 mM P, functional MIR was observed, as the symbiosis with *F. mosseae* reduced *S. exigua* survival, weight and the proportion of individuals reaching the pupa state. The prevalence of MIR at 0.7 but not at 0.3 mM P was also observed in the interaction with *B. cinerea.* Thus, the results show that mycorrhiza effects on plant resistance to pathogens and herbivores performance are P-dependent, and that MIR was impaired under P deficiency. A modulatory role of nitrogen deficiency on MIR against pathogens was also previously shown in tomato (Sanchez-Bel et al., 2016).

The effects of mycorrhization on plant interaction with herbivores are very complex. Enhanced resistance against chewing herbivores had been described in multiple systems (Schoenherr et al., 2019; Selvaraj et al., 2020; Jiang et el., 2021; Shafiei 2022), but opposite effects have also been reported (Bennett et al., 2009; Hoffmann et al., 2009; Bernaola et al., 2018, Zeng et al., 2022), as the improved nutritional status of AM plants may benefit insects (Koricheva et al, 2009; Pozo et al., 2020). To understand the mechanisms underlying the contrasting effects of P availability on AM impact on insect

performance, we investigated several plant nutritional and defensive traits that may contribute to the observed resistance phenotypes. Regarding plant nutrition and growth, mycorrhizal colonization enhanced shoot growth at low P doses and significantly elevated P levels in the plants regardless of the fertilization doses. C and N, recognized as key limiting-nutrients for insect herbivores (Mattson, 1980), were both influenced by P, but mycorrhiza did only change C levels under low P fertilization. The increase by mycorrhiza in C content under low P may have contributed to the better performance of the larvae in mycorrhizal plants under the lowest fertilization conditions. However, the contribution of changes in these nutrients to the worst performance of the herbivore in mycorrhizal plants under the 0.7 mM P is unlikely, since P is increased -and this would be positive for the pest- and C and N did not differ between Nm and Fm plants. Nevertheless, other nutritional aspects, such as the changes in other micronutrients, and potential variations in the content of sugars and amino acids upon herbivory may have contributed to the resistance phenotype and deserve further attention in follow-up studies. In fact, nutrition related changes, including protein and amino acid contents in mycorrhizal plants, have been proposed to contribute to resistance against *S. exigua* in maize plants (Ramirez et al., 2022). In tomato, significant changes in sugar and amino acid contents occurs in response to herbivory in systemic tomato leaves may contribute to defense (Kundu et al., 2018). Whether this systemic regulation is modulated in mycorrhizal plants remain to be determined.

P effects can go beyond nutritional aspects. P starvation has been associated with an increase in JA signaling and expression of JA-related genes in different plant species, potentially leading to increased defense against pathogens and chewing herbivores in Arabidopsis (Khan 2016, Luo et al., 2021). Although we found lower *B. cinerea* lesion development and higher mortality of *S. exigua* when feeding in plants under deficient (0.3 mM) P as compared to the medium 0.7 mM dose, no basal changes in the JA content among these plants were observed. Only the JA precursor OPDA differed among those plants, with higher levels at the higher P doses. Therefore, increased mortality at low P is not likely to be explained by a higher basal JA accumulation under such growing conditions, but rather by poor quality of the leaves (e.g. low nutrients and high anthocyanins) or by a stronger activation of defenses in those leaves as indicated by the transcriptional analysis of marker genes discussed below. The levels of IAA and ABA were also evaluated, as they are also regulated during responses to herbivory (Machado et al., 2016; Vos et al., 2013; Erb and Reymond, 2019). P fertilization impacted ABA, IAA and SA levels in the leaves, with ABA and SA concentrations being higher and IAA lower under low P conditions. Mycorrhizal colonization did not alter hormone basal levels except for a slight increase in auxins at P deficiency. Accordingly, basal differences in phytohormone levels among mycorrhizal and non-mycorrhizal plants are not likely to mediate the observed mycorrhizal effect on pathogen and larval performance under moderate P conditions.

We then addressed whether the differences may be related to a differential plant capacity to activate defenses. In order to analyze early defense responses and avoid variability associated with pathogen and caterpillar preference, we chemically elicited damage responses by application of OGs. OGs are released from the plant cell wall by pathogen polygalacturonases (Cervone et al., 1989; Ferrari et al., 2013), but also by endogenous polygalacturonases induced upon wounding, as shown in multiple plant species including tomato (Bergey et al., 1999, Orozco Cardenas et al., 1999; De Lorenzo et al., 2011). As chewing herbivores elicit wound responses in the plant, they are also thought to trigger OGs release (Aljbory et al., 2018; Erb et al., 2019) although this assumption needs further experimental evidences. Endogenous and exogenously applied OG elicitors activate plant defense responses and enhance resistance to different aggressors (De Lorenzo et al., 2011; Benedetti et al., 2015; Gamir et al., 2020). In the present study, OG application promoted JA, OPDA and IAA accumulation in leaves from plants under both, low and moderate, fertilization levels. Furthermore, OPDA and IAA levels were higher under the higher P fertilization, while JA was not affected by P availability. Although JA, OPDA and IAA increased their content under elicitation, no differences in their levels between Fm and Nm plants were found. We further analyzed well characterized JA regulated, anti-herbivory defense marker genes as those coding for *LapA, Pin II, MC* and *TD* (Yan et al, 2013; Uppalapati et al. 2005). All these marker genes showed a priming profile in mycorrhizal plants under 0.7 mM P, with a significantly higher expression upon OG treatment than in Nm plants. Conversely, under the most limiting P level, most of those genes showed much higher expression in non-elicited Nm plants, suggesting that Nm plants activate JA-related defenses upon P starvation in the absence of damage elicitation. It should be noted that although no differences were found between mycorrhizal and non-mycorrhizal plants elicited with OGs under the lowest P level (0.3 mM), there were differences when considering the fold change between non-elicited and elicited plants within each type of plants. While Nm plants showed a repression of defense-related genes upon OG treatment under P-deficient conditions, mycorrhizal Fm plants showed an increase in their expression. Thus, the main difference is the basal, non-elicited status of these plants: Nm plants are expressing JA-dependent defenses at the basal level, but fail to elicit defenses upon elicitation, in contrast to the behavior of mycorrhizal plants. The enhanced growth promotion of mycorrhizal plants under the lowest P fertilization, higher P content in leaves and lower anthocyanin levels suggest that mycorrhizal plants prioritize the improvement in nutrition under P deficiency, but prioritize defense activation under non-deficient conditions. Priming of plant defenses against herbivores has been shown in different systems (Song et al, 2013; Rivero et al, 2021; Jiang et al., 2021), but a failure of defense priming has been also reported (Qu et al., 2021; Ramirez-Serrano et al., 2022; Zeng et al., 2022). Analysis of chemical defenses, as the accumulation of polyphenols, lignins and other defensive metabolites in future studies will contribute to better characterize the differences in the defense responses under the different nutrition scenarios. Moreover, the potential contribution

of nutrition to the different outcomes in those experiments is difficult to address, as the experimental systems were very different, but it is tempting to speculate that nutrient availability is a key player in defining the final resistance phenotype. The results of the present study support this hypothesis by evidencing contrasting defense-responses in mycorrhizal and non-mycorrhizal plants to elicitation depending on the P nutritional status.

It is well established that when subjected to multiple stresses, plants prioritize their responses to promote growth or defense to optimize their resources according to the specific needs in a given context (Smakowska et al., 2016). JA signaling has been described to be a key player in such prioritization, with negative regulators of JA signaling acting when defenses are not needed (Major et al., 2017; Guo et al., 2018). We explored the regulation of two JAZ transcriptional repressors characterized as JA-inducible in tomato leaves (Chini et al., 2017). We found that while no significant differences related to the mycorrhizal status of the plant were observed at the higher P level, *JAZ* expression was higher in mycorrhizal plants under P limitation, in agreement with the lower expression levels of the defense-related genes *LapA*, *PinII*, *MC* and *TD*. In contrast, upon OG elicitation the expression of *JAZ* genes was suppressed in mycorrhizal plants, likely releasing the repression of the JA signaling pathway to activate plant defenses. Finally, to test whether this role in mediating the defense-growth trade-off reported for JAZ may be leading to differences in the regulation of the primary and secondary metabolism in mycorrhizal plants, we analyzed the expression of carbohydrate-related genes. The genes encoding the sucrose synthase *SUS1*, the invertase *LIN6* and the sucrose transporter *SUT4* (Sanmartin et al., 2020) were all showing higher expression in mycorrhizal plants under P deficiency in the absence of challenge, matching the expression profile found for the *JAZ* genes. These higher expression levels were not maintained upon OG elicitation. Remarkably, no differences were found in these carbohydrate-related genes at moderate P levels. Therefore, mycorrhizal plants showed reduced expression of defense-related genes in the absence of challenge under P-limiting conditions, which correlated with higher expression levels of the genes coding for the JA negative regulators *JAZ1* and *JAZ2* and of genes related to C metabolism. Thus, under P deficiency, mycorrhizal symbiosis drives the plant response towards the activation of primary metabolism, that is, growth-related responses but this effect disappears upon a challenge or pest attack. On the contrary, when P levels are higher, no differences between mycorrhizal and non-mycorrhizal plants are found in the absence of challenge. However, upon damage signaling elicitation defense responses are primed in mycorrhizal plants, thus promoting defenses. Differential regulation of the growth-defense balance in mycorrhizal plants was also shown under N depleted environments (Sanchez-Bel et al., 2018). Thus, experimental evidences support a fine-tuned regulation of the growth-defense balance in mycorrhizal plants according to the nutrients availability.

In summary, our results show that P availability plays a regulatory role in JA-dependent plant defense responses, and the effect is modulated by the mycorrhizal status of the plant. We also show that P availability can be a key element in the reported context dependency of MIR, as mycorrhization effects ranged from moderately positive (increase in larval biomass under the lowest P level) to negative effects on disease and herbivore development (enhanced mortality and reduced biomass and individuals reaching pupa stage) under moderate P levels, thus displaying MIR. Our hormonal and transcriptional analyses point to a differential regulation of the growth-defense balance in mycorrhizal plants, where the symbiosis tailors the plant to prioritize primary metabolism and nutritional-growth related responses under P deficiency, but promotes priming of plant defenses when this key element is not limiting. This fine-tuned regulation of defenses may contribute to the enhanced resilience of mycorrhizal plants under varying conditions and multi-stress contexts. Understanding the underlying molecular mechanisms will contribute to improve the efficacy of mycorrhizal inoculants in crop protection.

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SUPPLEMENTARY DATA

FIGURE S1. Survival probability curves of *S. exigua* fed on leaves of mycorrhizal (Fm, dotted line) and non-mycorrhizal (Nm, continuous line) tomato plants**. (A)** Larvae were feeding on detached leaves from plants fertilized with 0.3 (light grey), 0.7 (grey) or 1.0 (black) mM of H_2NaPO_4, one leaf of each plant (n=10) was detached 8 weeks pmi and infested with two second instar *S. exigua* larvae (n=20 larvae). **(B)** Larvae feeding on leaves of plants (n=9) fertilized with 0.3 mM (light grey) or 0.7 mM (grey) of H_2NaPO_4. Two second instar larvae were added to one leaf of intact plants using clip-cages (n=18 per treatment) 6 weeks pmi, and infestation was maintained for three weeks more. Survival distribution comparisons were performed using the non-parametric Log-rank (Mantel-Cox) test.

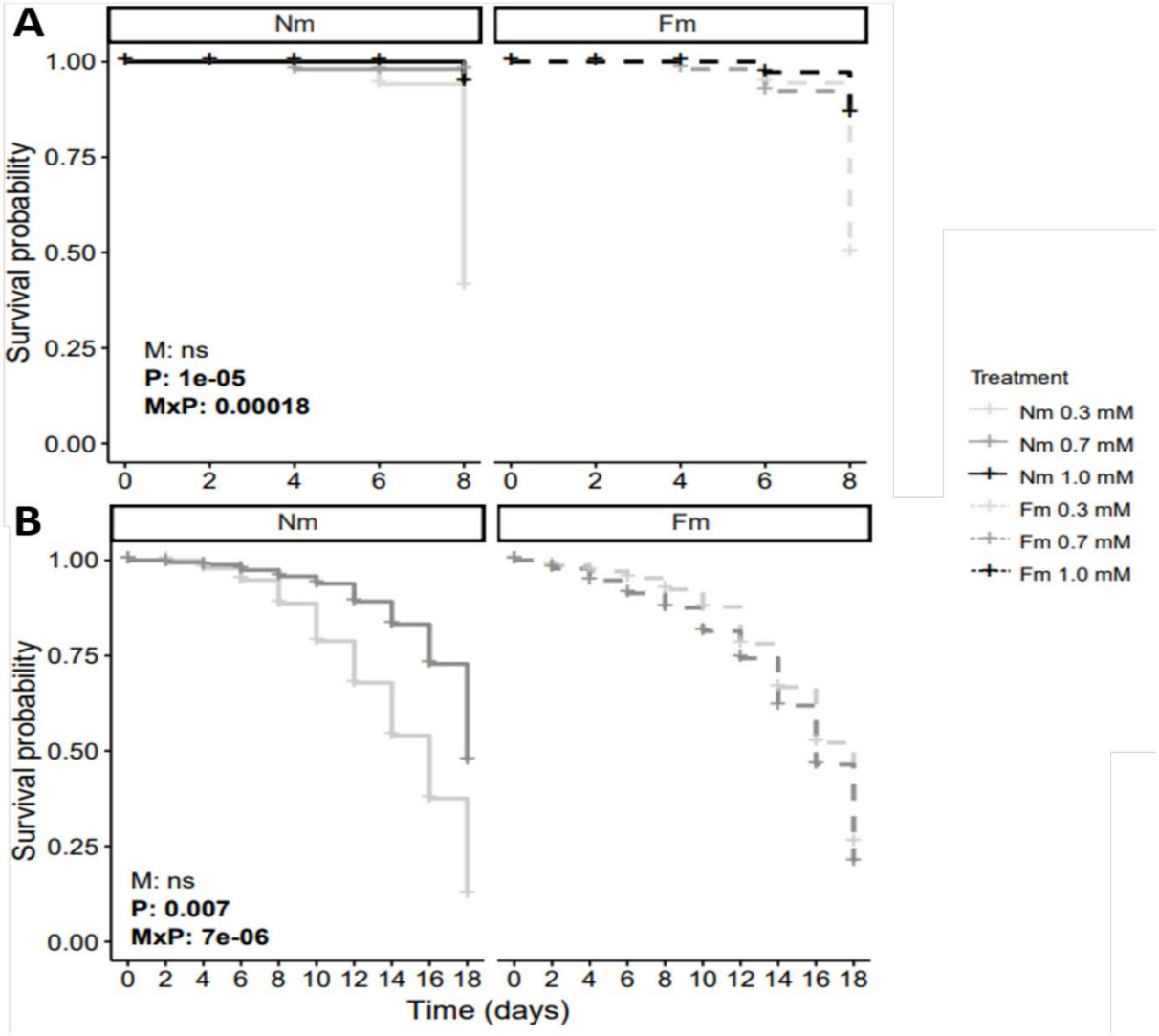

FIGURE S2. Salicylic acid content in non-mycorrhizal (Nm) and mycorrhizal tomato plants colonized by *F. mosseae* (Fm) grown under 0.3 mM or 0.7 mM P fertilization, and treated with 50 µg/ml OG solution (+OG; stripped bars) or water as control (-OG; filled bars), harvested 6h post elicitation. Multiway ANOVA using P fertilization (P) mycorrhizal (M) and OG treatment (OG) as factors was performed and significance is indicated in the upper right corner of the graph. Asterisks denote significant effect of a factor or their interaction. ns: no significant; *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001.

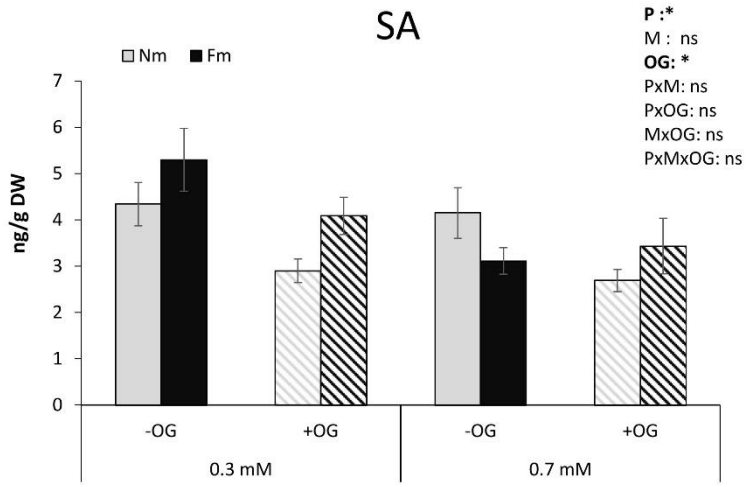

TABLE S1. Nutrient solution used for the experiments, modified from Hewitt (1966) in the total amount of H_2NaPO_4 to obtain the low P (0.3 mM), medium P (0.7 mM) and high P (1.0 mM) P fertilization regimes.

Hewitt solution						
Compound	**Mw**	**Stock solution (g/L)**	**mM**	**Low P 0.3 mM**	**Medium P 0.7 mM**	**High P 1.0 mM**
$MgSO_4$ * $7H_2O$	246.48	18.4	74.65	1.493	1.493	1.493
Fe-EDTA	367.05	2.45	6.67	0.067	0.067	0.067
$MnSO_4$ * H_2O	169.02	1.35	7.99	0.008	0.008	0.008
$CuSO_4$ * $5H_2O$	249.68	2.4	9.61	0.001	0.001	0.001
$ZnSO_4$* $7H_2O$	287.53	4.22	14.68	0.001	0.001	0.001
H_3BO_3	61.83	18.6	300.82	0.030	0.030	0.030
Na_2MoO_4*$2H_2O$	241.95	0.35	1.45	0.0001	0.0001	0.0001
KNO_3	101.1	30.3	299.7	2.997	2.997	2.997
$Ca(NO_3)_2$*$4H_2O$	236.15	101.54	429.98	8.600	8.600	8.600
H_2NaPO_4*H_2O	**137.99**	**18.4**	**133.34**	**0.333**	**0.667**	**1.000**

TABLE S2. List of primers used for qPCR analysis.

Primer name	PCR primers sequence (5' → 3')
SlEF	F- GATTGGTGGTATTGGAACTGTC
	R- AGCTTCGTGGTGCATCTC
Actin	F- TTGCTGACCGTATGAGCAAG
	R- GGACAATGGATGGACCAGAC
β-tubulin	F- TGGGCTGAAGATGGCATCCACG
	R- GCCTTGCGCCTGAACATGGC
TD	F- AGCTCAAACACACGCGCTGGA
	R- AACCCCCACCACCAACAGGT
LapA	F- ATCTCAGGTTTCCTGGTGGAAGGA
	R- AGTTGCTATGGCAGAGGCAGAG
MC	F- GAGAATTTCAAGGAAGTTCAA
	R- GGCTTTATTTCACACAGAGATA
LePGIP	F- CTCTCAGCTTCCGAATTTGC
	R- TTCCCGAACAAAAACGAAAC
PIN II	F- GAAAATCGTTAATTTATCCCAC
	R- ACATACAAACTTTCCATCTTTA
JAZ1	F- TTCCCTCAAGGTGGAATGAAGGCT
	R- TCCGAAACTCGGAACCACCAAATC
JAZ2	F- CGTCCGTTGAAACAAATCCT
	R- GGGGTTCTGTTTGTTGGCTA
SUS1	F- GGATTGAAAGCCACGGAAAGG
	R- ACCAGGCCTCAACGAATAGCA
LIN6	F- AGCACATTTATTCGCCTTCAAC
	R- TTTGTGACGTGGCATAATAAGA
SUT4	F- TCTCCGCTGATATTGGATGG
	R- GCAACATCGAGAAGCCAAAA

TABLE S3. Microelement concentration in leaf tissues. Data represent mean values (n=3). For each element (columns), treatments sharing a letter in common are not significantly different according to one way ANOVA followed by Fisher's Least Significant Difference (LSD) test ($p<0.05$). The global effects of the factors: AM symbiosis (M), P fertilization (P) and their interaction were analyzed by multiway ANOVA. Asterisks indicate significant differences: *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; $p<0.0001$.

ppm	Al	Ca	Cr	Cu	Fe	K	Li	Mg	Mn	Mo	Na	Ni	S	Si	Sr	Ti	Zn
Nm 0.3	50 a	21197 c	26 ab	9.4 c	517 b	17737 a	18 b	17717 b	401 c	2.2 b	288 a	18 b	3250 a	1029 a	64 b	2.3 a	27 b
Fm 0.3	50 a	19752 bc	30 b	9.5 c	404 a	18524 a	16 ab	15958 b	231 b	2.4 b	252 a	20 b	3011 a	1001 a	84 c	2.0 a	15 a
Nm 0.7	54 a	14824 a	21 ab	5.8 a	399 a	17515 a	19 b	10464 a	206 ab	1.4 a	515 b	14 ab	4114 b	1032 a	39 a	3.2 a	12 a
Fm 0.7	39 a	16927 ab	17 a	7.7 b	318 a	21484 b	13 a	10543 a	120 a	1.5 a	452 b	11 a	4523 b	945 a	68 b	2.0 a	9 a
M	1.74	0.09	0.01	4.62*	9.17**	11.43**	9.34**	0.7	16.01***	0.9	1.72	0.01	0.21	1.01	40.16***	2.35	7.31*
P	0.35	17.89***	6.04*	28.12***	10.13**	3.79	0.18	39.86***	22.95***	29.58***	31.57***	8.5**	41.76***	0.23	28.9****	1.01	13.44**
MxP	2.14	2.66	1.33	3.19	0.25	5.12*	1.17	0.84	1.78	0.15	0.13	1.36	3.1	0.27	1.62	0.99	2.47

TABLE S4. Gene expression data from real-time quantitative qRT-PCR normalized to housekeeping gene ß-tubulin. Gene expression levels in OG-treated or water-treated leaves of non-mycorrhizal (Nm) and mycorrhizal (Fm) plants grown under 0.3 mM and 0.7 mM P fertilization regimes. Values are the mean of 6 biological replicates.

Relative expression (average)

Gene	*LapA*	*PinII*	*TD*	*MC*	*LePGIP*	*JAZ1*	*JAZ2*	*SUS1*	*LIN6*	*SUT4*
Nm 0.3mM -OG	0.0036	0.0039	0.7661	0.0004	0.0245	1.1455	0.0681	0.0259	0.2323	0.0125
Fm 0.3mM -OG	0.0009	0.0009	0.1638	0.0004	0.0353	1.5034	0.1165	0.0496	0.4517	0.0269
Nm 0.3mM +OG	0.0016	0.0013	0.1834	0.0007	0.0313	1.6425	0.1286	0.0271	0.3561	0.0189
Fm 0.3mM +OG	0.0024	0.0016	0.2373	0.0003	0.0317	1.0439	0.0690	0.0308	0.1491	0.0236
Nm 0.7mM -OG	0.0005	0.0003	0.0785	0.0001	0.0203	0.9467	0.0511	0.0139	0.0916	0.0132
Fm 0.7mM -OG	0.0008	0.0003	0.2287	0.0002	0.0181	0.8868	0.0406	0.0135	0.0956	0.0127
Nm 0.7mM +OG	0.0003	0.0003	0.0734	0.0001	0.0211	1.1012	0.0599	0.0128	0.0980	0.0196
Fm 0.7mM +OG	0.0014	0.0008	0.1923	0.0003	0.0339	1.1425	0.0536	0.0152	0.1643	0.0177

Standard deviation (Relative xpression)

Gene	*LapA*	*PinII*	*TD*	*MC*	*LePGIP*	*JAZ1*	*JAZ2*	*SUS1*	*LIN6*	*SUT4*
Nm 0.3mM -OG	1.0E-03	1.4E-03	1.9E-01	2.1E-04	7.7E-03	1.0E-01	1.2E-02	6.2E-03	3.6E-02	2.0E-03
Fm 0.3mM -OG	1.0E-04	2.9E-04	1.7E-02	1.2E-04	5.5E-03	1.0E-01	1.2E-02	6.3E-03	2.7E-02	1.8E-03
Nm 0.3mM +OG	5.1E-04	2.9E-04	5.0E-02	2.7E-04	5.4E-03	2.0E-01	1.9E-02	4.9E-03	5.2E-02	2.1E-03
Fm 0.3mM +OG	8.3E-04	4.5E-04	2.8E-02	7.5E-05	3.3E-03	5.4E-02	9.9E-03	2.1E-03	3.8E-03	1.4E-03
Nm 0.7mM -OG	1.9E-04	1.3E-04	1.4E-02	4.2E-05	2.9E-03	4.8E-02	3.7E-03	2.6E-03	6.8E-03	1.9E-03
Fm 0.7mM -OG	1.6E-04	5.4E-05	6.6E-02	1.5E-04	3.8E-03	4.1E-02	7.7E-03	2.2E-03	1.4E-02	1.5E-03
Nm 0.7mM +OG	1.1E-04	1.4E-04	2.2E-02	2.0E-05	3.4E-03	1.6E-01	1.6E-02	3.2E-03	3.2E-02	2.4E-03
Fm 0.7mM +OG	4.0E-04	2.2E-04	5.3E-02	5.2E-05	1.4E-03	1.0E-01	1.6E-02	1.9E-03	3.4E-02	1.9E-03

TABLE S5. Fold changes in gene expression. Colors indicate up-regulation (red) and down-regulation (blue) and intensities are determined by the intensity of the changes: light color >1.5 or <0.75 and dark color >2 or <0.5 and significant effects are highlighted in bold. (A) Effect of P starvation on gene expression in Nm plants (fold change in Nm and Fm plants fertilized at 0.3 vs 0.7 mM). (B) Effect of OG elicitation on gene expression in Fm or Nm plants grown at 0.3 mM and 0.7 mM P (fold change +OG/-OG). (C) Effect of AM symbiosis on gene expression at 0.3 mM and 0.7 mM P (fold change Fm/Nm). Bold numbers and asterisks indicate significant differences (t-test, $p<0.05$) between (A) 0.3 vs 0.7 mM, (B) +OG vs -OG and (C) Fm vs Nm. +: p<0.1; *: p<0.05; **: p<0.01; ***: p<0.001

	Gene	Nm	Fm
FOLD CHANGE 0.3mM/0.7mM	***LapA***	**7.2***	1.08
	PinII	**11.96***	**3.09***
	TD	**9.76****	0.72
	MC	6.52+	1.61
	LePGIP	1.21	**1.95***
	JAZ1	1.21+	**1.7*****
	JAZ2	1.33	**2.87*****
	SUS1	1.87+	**3.68*****
	LIN6	**2.54****	**4.72*****
	SUT4	0.95	**2.12*****

	Gene	0.3 mM		0.7 mM	
		Nm	Fm	Nm	Fm
FOLD CHANGE +OG/-OG	***LapA***	0.43+	2.73+	0.63	1.74
	PinII	0.34+	1.77	1.02	**2.75***
	TD	**0.24***	**1.45***	0.94	0.84
	MC	1.74	0.75	1.04	1.37
	LePGIP	1.28	0.90	1.04	**1.87****
	JAZ1	**1.43***	**0.69****	1.16	**1.29***
	JAZ2	**1.89***	**0.59****	1.17	1.32
	SUS1	1.04	**0.62***	0.92	1.12
	LIN6	**1.53***	**0.33*****	1.07	1.72+
	SUT4	**1.5***	0.88+	**1.49***	**1.39***

	Gene	0.3 mM		0.7 mM	
		-OG	+OG	-OG	+OG
FOLD CHANGE Fm/Nm	***LapA***	**0.24***	1.53	1.60	**4.44***
	PinII	0.23+	1.22	0.89	2.39+
	TD	**0.21***	1.29	**2.91***	**2.62***
	MC	0.97	0.42+	3.93	**5.2****
	LePGIP	1.44	1.01	0.89	**1.6***
	JAZ1	**1.31***	**0.64***	0.94	1.04
	JAZ2	**1.71****	**0.54***	0.79	0.89
	SUS1	**1.91***	1.14	0.97	1.18
	LIN6	**1.94*****	**0.42****	1.04	1.68+
	SUT4	**2.15*****	1.25*	0.96	0.90

TABLE S6. Multiway ANOVA of gene expression data. Effects of the different factors: AM symbiosis (M), P fertilization (P) and elicitation (OG treatment, OG) and their interactions were analyzed. Data represent f values and significant effects are highlighted in bold. Asterisks indicate significant differences: +: p<0.1; *: p<0.05; **: p<0.01; ***: p<0.001

Gene	*LapA*	*PinII*	*TD*	*MC*	*LePGIP*	*JAZ1*	*JAZ2*	*SUS1*	*LIN6*	*SUT4*
M	0.13	1.9	1.6	0.01	2.75	0.65	0.58	**6.52***	0.96	**9.65****
P	**12.63*****	**13.13*****	**12.39****	**6.1****	**5.02***	**15.19*****	**23.38*****	**46.25*****	**76.68*****	**12.07****
OG	0.01	0.76	**6.21***	0.45	2.29	1.92	0.9	2.2	1.51	**7.14***
MxP	**4.79***	3.81+	**13.63*****	3.89+	0	0.47	0.02	**4.91***	0.47	**15.9*****
MxOG	**8.27****	**5.28***	**8****	0.49	0.12	**7.02***	**8.05****	2.24	**18.58*****	**4.23***
PxOG	0.42	2.12	**4.48***	0.06	1.03	1.33	0.06	2.51	**9.02****	2.43
MxPxOG	3.39+	3.00+	**9.68****	1.21	3.71+	**10.74****	**9.4****	3.89+	**33.46*****	2.31

Chapter 2

Tomato Cell Wall-Associated Kinase SlWAK1 impacts primary and secondary metabolism and plant resistance against herbivore attack

Tomato Cell Wall-Associated Kinase SlWAK1 impacts primary and secondary metabolism and plant resistance against herbivore attack

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ABSTRACT

A key step for the efficient activation of inducible defenses in plants is the perception of the attack. Cell wall-associated receptor kinases (WAKs) are specific plant receptors that play a crucial role in recognition of damage associated molecular patterns (DAMPs). In Arabidopsis, WAK1 is the best characterized WAK, shown to bind oligogalacturonides (OGs), derived from plant cell wall fragments by wounding or pathogen attack. The perception of these DAMPs triggers a cascade of reactions that lead to plant defense activation. How WAK1 influences the activation of plant defense responses against herbivores is still unknown. Here, we explore the role of the WAK1 orthologue in tomato, by using a tomato *Slwak1* mutant line, to test whether it is involved in the activation of plant responses to herbivore attack. Our study shows that the *Slwak1* genotype is more susceptible to the generalist chewing herbivore *Spodoptera exigua*, as caterpillar survival and development increased when feeding in these plants. *Slwak1* is not impaired in the activation of JA-regulated defenses, but it is deficient in the synthesis of certain defensive secondary metabolites upon herbivory. Transcriptomic and metabolomic analysis showed that *Slwak1* is compromised in phenylpropanoid, alkaloid and flavonoid biosynthesis. Profiling of enzymatic activities related to carbohydrate metabolism and sugars quantification revealed that *Slwak1* was strongly altered in primary metabolism. In the absence of herbivory, there are remarkable differences among the mutant and wildtype genotypes, with the mutant showing higher glycosidic activities, and lower sugars and tricarboxylic acid compounds content. Upon herbivory, wild-type tomatoes mobilized storage carbohydrates locally to obtain energy for defense, turning the wounded leaf into sink tissue. Local and systemic responses to herbivory were deficient in the mutant plants, with changes following opposite directions than in the wild type: *Slwak1* plants, actively reduced cell wall invertase activity, increased starch and sucrose synthesis and sucrose transporters activity in local injured leaves. *Slwak1* was unable to properly regulate the trade-offs between primary and secondary metabolism and carbon partitioning in the plant was altered, failing to activate the sink strength in challenged leaflets to maintain enough energy for an efficient defense response activation. In conclusion, SlWAK1 receptor is involved in the regulation of primary metabolism and required for the full activation of plant defense response against chewing herbivores.

INTRODUCTION

Plants have evolved sophisticated defense mechanisms to hinder the performance and fitness of potential aggressors. Some traits are constitutively present in plants as the first mechanical defense line like trichomes, waxy cuticles and spines that function as physical barriers to prevent attackers from reaching plant tissues (War et al., 2012; Ziv et al., 2018). Others, as toxic secondary metabolites and defense proteins, may constitutively be present in the plant or can be induced and accumulated only after damage perception (Chaudhary et al., 2018; Zaynab et al., 2018; Erb and Reymond, 2019).

Recently, it has been proposed that the position of receptors in the plant is crucial for the proper activation of immune responses (Kanyuka and Rudd, 2019). In particular, cell surface receptors are the first to recognize host damage-derived molecules and to activate plant defense responses due to their spatial location. These receptors are membrane-bound proteins with extracellular domains involved in apoplastic molecular recognition. Among them, cell wall-associated protein kinases (WAKs) have been recently discovered as receptors triggering plant immunity (Stephens et al., 2022). The WAK protein family are receptor-like kinases characterized by an intracellular serine/threonine kinase domain, a transmembrane helix, and an extracellular region that binds pectin fragments in the apoplastic space (He and Wu, 2016).

Different works have demonstrated the role of WAK receptors in plant development and immunity. Indeed, many immunity-related WAKs can modify the cell wall composition, supplying additional strength to the barrier (Wagner and Kohorn, 2001; Hou et al., 2005; Yin and Hou, 2017; Zhang et al., 2017; Zhang et al., 2019). Among WAK receptors, the Arabidopsis *WAK1* was the first WAK-encoding gene identified (He et al., 1999) and successively characterized in other plant species like rice and tomato (Li et al., 2009; Rosli et al., 2013). This receptor is considered a cell wall integrity sensor able to detect damage-associated molecular patterns (DAMPs) and trigger plant immunity (Kohorn, 2016). Arabidopsis *WAK1* expression is induced by wounding (Wagner and Kohorn, 2001), and transgenic plants overexpressing *WAK1* are more resistant to *Botrytis cinerea* (Brutus et al., 2010). Several studies have demonstrated the high affinity of Arabidopsis WAK1 to bind oligogalacturonides (OGs), the homogalacturonan fragmentation products, that are linear homopolymers of α-1,4-linked D-galacturonic acid (Decreux and Messiaen, 2005; Denoux et al., 2008; Ferrari et al., 2013; De Lorenzo et al., 2011; Hou, 2019). The orthologue in tomato, SlWAK1, has a high binding affinity to oligomeric pectin as shown by docking analysis (Kurt et al., 2020).

OGs are among the best-known plant DAMPs. They are released into the apoplast after cell wall hydrolysis by the cleavage activity of polygalacturonases (PG) (De Lorenzo et al., 2011; Ferrari et al., 2013; Benedetti et al., 2015; Pontiggia et al., 2020). These molecules trigger downstream signals that elicit multiple defense responses in different plant species as Arabidopsis and tomato (Ferrari et al., 2013; Gramegna et al., 2016; Zhang, 2020; Gamir et al., 2020).

Herbivory is one of the most prevailing threats to plant survival due to the diversity and abundance of feeders. Approximately two-thirds of known herbivorous insect species are leaf eaters such as caterpillars (Lepidoptera) that cause damage with mouthparts evolved for chewing (Howe and Jander, 2008). Chewing insects inflict mechanical damage by breaking plant cells, modifying the extracellular space by releasing intracellular components and cell wall fragments (Waterman et al., 2019). During insect herbivory, plants may perceive herbivore- and damage-associated molecular patterns (HAMPs and DAMPs) (Erb and Reymond, 2019). Their recognition triggers a complex and highly regulated cascade of reactions that activate plant immunity (Albert et al., 2020), leading to defensive strategies to reduce their suitability and digestibility (Howe and Jander, 2008). The activation of the plant immune system after herbivory is finely regulated by phytohormone-dependent signaling (Divekar et al., 2022). Hormonal networks connect perception and early signaling to broad transcriptional reorganization and defense induction (Erb and Reymond, 2019). It is well established that jasmonic acid (JA) is the main hormone regulating plant defense responses against chewing herbivores (Diezel et al., 2009; Inbar and Gerling, 2008; Wasternack and Hause, 2013). JA signaling coordinates plant defenses inducing transcriptional changes that lead to the accumulation of toxic, anti-feedant or anti-nutritional compounds, such as glucosinolates, benzoxazinoids and alkaloids (Divekar et al., 2022). JA also triggers the production of inducible proteins like polyphenol oxidase, arginase, threonine deaminase, leucine amino peptidase and proteinase inhibitors to reduce plant digestibility (Chen et al., 2006; Fowler et al., 2009; Wang et al., 2021). Other hormones, such as auxins may act synergistically with JA in response to wounding and herbivore attack (Hentrich et al., 2013; Pérez-Alonso et al., 2021). Hence, it was reported that indoleacetic acid (IAA) biosynthesis increases in leaves and roots under caterpillar attack (Machado et al., 2013; Machado et al., 2016) and its accumulation promotes the production of secondary metabolites as phenolics and flavonoids (Lulu et al., 2015; Mahdieh et al., 2015). The simultaneous application of IAA and methyl jasmonate induces the production of anthocyanins which may act as chemical repellents for the pest (Lev-Yadun et al., 2008), and jasmonate and auxin dependent

mechanisms regulate changes in carbohydrate dynamics and allocation upon herbivory (Machado et al., 2013).

Secondary metabolites considerably impact herbivore fitness via a diversity of modes of action (Agrawal and Weber, 2015; Mithöfer and Boland, 2012). In *Solanaceae*, like potato, tomato and eggplant, steroidal alkaloids and their glycosylated forms are toxic compounds involved in plant resistance against a wide range of pests, including insects (Milner et al., 2011; Cárdenas et al., 2016). Depending on the insect-plant specialization, plants can produce toxic compounds to contrast insect attacks. For example, in tomato, the generalist *Helicoverpa zea* strongly affects concentrations of defense-related metabolites (simple phenolics and precursor amino acids), while the specialized *Manduca sexta* alters compounds associated with carbon and nitrogen transport (Steinbrenner et al., 2011). While many studies have described the effect of herbivory on plant secondary metabolism, the impact on the primary metabolism is less characterized (Zhou et al., 2015).

Carbohydrates (CH), derived from photosynthesis, are used for the growth, development and maintenance of non-photosynthetic plant tissues, making their storage necessary for plants and herbivores. During herbivore attack, plants reallocate carbon photosynthetic products to provide substrates for defensive metabolites construction or to mobilize them toward sink tissues (Ferrieri et al., 2012; Schulz et al., 2013; Zhou et al., 2015). Generally, upon attack, plants reduce photosynthetic activity as a local response to limit energy availability for the herbivore. At the same time, plants increase carbon demand in attacked leaves promoting local catabolism of carbohydrates to produce defensive compounds (Zhou et al., 2015). Thus, plants increase the expression of genes encoding carbohydrate-related enzymes and invertases, which cleaves sucrose into glucose and fructose (Roitsch and Gonzales, 2004, Appel et al., 2014). These hexoses work as signal molecules inducing defense-related hormonal pathways and reinforcing cell walls (Veillet et al., 2016; Tauzin and Giardina, 2014). Hence, the photosynthetic leaves turn from source into sink tissues, and the CH are incorporated into defense-related compounds. For example, Ferrieri et al. (2012) demonstrated that wounded plants or methyl-jasmonate treated plants incorporate radioactively labeled CH into defensive compounds in sink leaves. This strategy is particularly effective against generalist herbivores that are less tolerant to specialized plant toxins (Steinbrenner et al., 2011).

This work aims to gain a deeper insight into the role of wall associated kinases and damage perception in plant-herbivore interactions. Among eleven *SlWAK* genes, *SlWAK1* shows the highest expression after damage, abiotic stress or pathogen attack (Sun et al., 2020; Zhang et al., 2020; Meco et al., 2020), but there is no information on their regulation or function during

herbivory. We explore the function of the tomato (*Solanum lycopersicum*) SlWAK1 in the plant responses to herbivory comparing the *Slwak1* mutant line (Meco et al., 2020) and its corresponding wild type by monitoring the performance of the generalist chewing herbivore *Spodoptera exigua*, and comparing the local and systemic responses of the plants to the larval attack. *S. exigua* survival was higher when feeding on *Slwak1*. Transcriptomic analysis showed that *Slwak1* was not impaired in the activation of the jasmonic acid dependent pathway, but showed deficient activation of auxin signaling and flavonoid and alkaloid biosynthetic gene expression. Furthermore, a non-targeted metabolic analysis confirmed differential metabolic profiles before and after herbivory in *Slwak1*, and a lower accumulation of some defensive metabolites. Finally, biochemical analyses revealed altered primary metabolism in the mutant, pointing to a reduced basal energy status in *Slwak1,* limiting the plant responses to herbivory. The results highlight the contribution of SlWAK1 to trigger efficient plant defense responses against herbivory.

MATERIAL & METHODS

Plant material and growing conditions

Tomato (*S. lycopersicum L.*) cv Moneymaker wild type (WT) and *Slwak1* mutant plants, with a single T-DNA insertion localized in the promoter region of SlWAK1, Solyc09g014720) were used (Meco et al., 2020).

Tomato seeds were surface disinfected by immersion in 4% NaClO (10 min), rinsed thoroughly with sterile water and incubated for 10 days in an open container with sterile vermiculite at 25°C. Two-week-old seedlings were transferred to 300 mL pots containing a sterile sand:vermiculite (1:1) mixture. Pots were randomly distributed in a controlled glasshouse at 24:18°C with a 16:8 h diurnal photoperiod and 70% humidity. Plants were fertilized with Hewitt nutrient solution (Hewitt, 1966) modified in the P content to 0.7 mM. Plants were watered twice a week with half strength of this modified nutrient solution, and water was supplied as needed.

Herbivore rearing and performance bioassays in tomato plants

S. exigua (Lepidoptera: Noctuidae) eggs were provided by Dr. S. Herrero's lab (ERI-BIOTECMED, Universitat de Valencia, Spain). Upon hutching, larvae were reared on artificial diet (Greene *et al.*, 1976) at 25°C with 16:8h light:dark regime and 70% relative humidity.

For the herbivore performance assay, seven-week-old WT and *Slwak1* tomato plants (n=15 per treatment) were infested with *Spodoptera exigua* larvae. Two L2-L3 stage larvae were placed in leaflets from the fifth true leaf of each tomato plant, using clip-cages to limit the feeding area and avoid their escape. Larvae were kept in individual cages to avoid cannibalism. Clip-cages were moved into new leaflets to ensure larvae always had food available. To monitor insect performance, larval weight was determined after 10 days, and mortality and pupation were monitored every two days for three weeks.

Leaflets were collected at different time points before and during herbivory (0, 2, 14 and 21 days post infection). For 2 and 14 dpi, wounded leaflets, where *S. exigua* was feeding, were harvested to analyze local responses (Local, L) and the non-damaged leaflets of the same leaf were sampled to study the systemic response (Systemic, S). Plants were harvested 21 days post larvae infestation, leaves and roots were immediately frozen in liquid N and stored at -80°C for further molecular analysis.

Determination of mineral nutrient content

Nutrient analyzes were performed at the Technical Services of the Estación Experimental del Zaidín (CSIC). Frozen leaves were grinded and lyophilized, and 100 mg aliquot of dry tissue was used per sample. Total P and micronutrient (Ca, Cu, K, Li, Mg, Mn, Na, Ni, P, S, Sr, Zn) concentrations were determined after acid digestion of samples, by inductively coupled plasma optical emission spectrometry (ICP-OES; Varian ICP 720-ES). Total C and N contents were analyzed using an Elemental Analyzer (LECO TruSpec CN), according to standard procedures. Five independent biological replicates were analyzed per treatment.

Analysis of Gene Expression by qPCR

Gene expression levels were determined in leaf tissue from the herbivory experiment described above and on leaves of tomato plants elicited by exogenous application of an 50 µg/ml aqueous OG solution as described in Dejana et al., (2022). Total RNA from tomato leaves was extracted and treated with DNase using the Direct-zol RNA MiniPrep kit (Zymo Research). Subsequently, the RNA was purified through a column using the RNA Clean and Concentrator-5 kit (Zymo Research), and stored at -80°C until use. The first-strand cDNA was synthesized with 1 µg of purified total RNA using the iScript cDNA Synthesis kit (Bio-Rad). All kits were used according to the manufacturer protocols. Four independent biological replicates were analyzed per treatment. The expression of marker genes for different metabolic pathways was analyzed by qPCR using gene specific primers (Table S1). Among them, genes related to JA pathway: multicystatin (*MC*), proteinase inhibitors II (*PinII*) and leucil aminopeptidase A (*LapA*). Genes related to auxin pathway: auxin efflux carrier protein (*PILS3*), auxin response factor (*PIN3*) and auxin-responsive gene (*GH3.8*).

Genes related to phenypropanoid, flavonoid and alkaloid pathways: phenylalanine ammonia-lyase (*PAL1-170*), methyl jasmonate-dependent regulator of phenylpropanoid-conjugate (*MybJS1*), chalcone synthases (*CHS1*), flavonoid-3'-hydroxylase (*F3'H*), flavonol synthase (*FLS*) and glycoalkaloid metabolism 9 (*GAME9*).

The expression of three different housekeeping genes, actin (Solyc03g078400), elongation factor 1-α (Solyc06g005060) and β-tubulin (Solyc04g081490) was analyzed to find the optimal normalization gene, using the Normfinder software (https://moma.dk/normfinder-software) (Andersen et al., 2004). According to the results, expression values were normalized using the housekeeping gene actin, and relative quantification of specific mRNA levels was performed using the comparative 2–Δ (ΔCt) method (Livak and Schmittgen, 2001).

Untargeted metabolomic profiling by liquid chromatography and electrospray ionization (LC-ESI) mass spectrometry (Q-TOF instrument)

Lyophilized freeze-dried leaves (30 mg) were homogenized in 1 ml of MeOH:H_2O (10:90) containing 0.01% of HCOOH. The homogenate was centrifuged at 15 000 *g* for 15 min at 4 °C, and the supernatant was recollected and filtered through 0.2 µm cellulose filters (regenerated cellulose filter, 0.20 µm, 13 mmD pk/100; Teknokroma, St Cugat, Spain). Subsequently, 20µl of the filtered supernatant was injected into an Acquity ultra-performance liquid chromatography system (UPLC; Waters, Mildford, MA, USA), which was interfaced with a hybrid quadrupole time-of-flight equipment (QTOF-MS Premier). An aqueous methanol gradient containing 0.01% HCOOH was used for elution. Solvent gradients and other chromatographic conditions were performed as previously described (Gamir et al., 2020). Six biological replicates were randomly injected for each treatment.

LC separation was performed using ultra-performance liquid chromatography (UPLC) Kinetex C18 analytical column with a 5 µm particle size, 2.1 x 100 mm (Phenomenex, Madrid, Spain). For a correct and accurate identification of the detected signals, a second fragmentation function was introduced into the TOF analyzer. This function was programmed in a t-wave ranging from 5 to 45 eV to obtain a fragmentation spectrum of each analyte (Gamir *et al.*, 2014).

Full scan data analysis

Data were acquired in centroid mode and subsequently transformed into .cdf files using the Databridge from MassLynx 4.1 software (MassLynx 4.1, Waters, USA). Chromatographic signals were processed using the software *R* for statistical purposes. Signals from positive and negative electrospray ionization (ESI+; ESI-) were processed separately. Peak peaking, grouping and signal corrections were performed using the XCMS algorithm (Smith *et al.*, 2006). Metabolites were analyzed based on normalized peak area units relative to the dry weight. Adduct and isotope corrections were performed by using the MarVis Suit 2.0 software tool (Kaever *et al.*, 2015). The same software was used for testing differential production across genotypes and herbivory via Kruskal–Wallis test (p<0.01). To determine the global behavior of the signals, principal component analyses (PCA), sparse partial least-squares discriminant analysis (sPLSDA) and heat map plots were generated using MetaboAnalyst 4.0 software (www.metaboanalyst.ca), a comprehensive web-based package for a range of metabolomics applications (Chong et al., 2019).

For compound identification, at least two of the following three criteria were required: matching the exact mass; matching the retention time between the internal library and experimental samples; and coincident fragmentation spectrum with those available in the Massbank and/or Metlin (www.massbank.jp; www.masspec.scripps.edu).

Quantification of soluble carbohydrates and starch

The extraction of sugars from leaves and roots was performed as described in De Rocchis et al. (2022). Ground frozen plant material was suspended in ultrapure water and 5 mM mannitol as internal standard was added, and centrifuged at 19,000 *g* for 10 min at 4 °C. The supernatant was transferred to a new tube, heated at 99 °C for 3 min to stop enzymatic activities, and centrifuged again at 19,000 *g* for 10 min at 4 °C. The supernatant was used for the analysis of glucose, fructose, and sucrose, while the pellets of the two centrifugation steps containing the starch were pooled, resuspended in a total volume of 1 mL containing 0.5 mM mannitol, and treated with 1.5 U of amyloglucosidase (Sigma-Aldrich, Saint Louis, USA) for 2 h at 37 °C. After two centrifugation steps at 19,000 *g* for 10 min at 4 °C with a 3 min heating step at 99 °C in between, the final supernatant was used for starch analysis. Starch and sugars were quantified by HPLC DIONEX ICS 3000 (Thermo Fisher Scientific, Waltham, USA) with a CarboPacTMPA200 AnalyticI (3 × 150 mm) column (Thermo Fisher Scientific, Waltham, USA).

Enzymatic activity assay

Semi-high throughput profiling was used for assessment of the activity of 12 key enzymes of carbohydrate metabolism (Jammer et al, 2022). Both roots and shoots frozen material (~300 mg fresh weight) were used for extractions, according to Jammer et al. (2015) with few adjustments, in order to analyze the activity of 12 key enzymes of primary carbohydrate metabolism: cell wall invertase (CwInv), vacuolar invertase (VacInv), cytoplasmic invertase (CytInv), fructokinase (FK), hexokinase (HXK), phosphoglucoisomerase (PGI), phosphofructokinase (PFK), aldolase (Ald), ADP-glucose pyrophosphorylase (AGPase), phosphoglucomutase (PGM), UDP-glucose pyrophosphorylase (UGPase) and glucose-6-phosphate dehydrogenase (G6PDH).

Briefly, ground material was homogenized with PVPP and was extracted with 1 ml extraction buffer (40 mM TRIS-HCl pH 7.6, 1 mM EDTA, 0.1 mM PMSF, 1 mM benzamidine, 14 mM β-mercaptoethanol, 24 µM NADP), cell wall-bound proteins were extracted from the

remaining pellet with a high-salt buffer (1 mM NaCl, 40 mM Tris–HCl, pH 7.6, 3 mM MgCl2, 1 mM EDTA).

Activities were determined photometrically by kinetic assays in a 96-well plate format and were normalized to the protein contents determined according to the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as a standard protein (Jammer *et al.,* 2015).

Statistical analyzes

All statistical analyzes (one- and two-way ANOVA, LSD test, PERMANOVA, log-rank test, exact binomial test and t-test applied when appropriate, as indicated in the corresponding figure legends) were conducted using STATGRAPHICS PLUS 3.1 (Rockville, MD, USA), *R* software v.3.5.2 (R Development Core Team) and the XCMS package. Survival distribution comparison between treatments was performed using the non-parametric log-rank test (Mantel-Cox). Survival analyzes were performed using *survival* and *survminer* R packages. Normality and homocedasticity assumptions were validated by using Shapiro-Wilk and Levene's tests, respectively. For analysis of the metabolomics data, data were normalized by sum, transformed by cube root, and scaled by Pareto method in order to obtain the sparse partial least squares discriminant analysis (sPLSDA) and heatmap plots, which were generated using MetaboAnalyst 4.0 software (www.metaboanalyst.ca), a comprehensive web-based package for a range of metabolomics applications (Chong *et al.*, 2019).

RESULTS

SlWAK1 is transcriptionally upregulated by OG elicitation and herbivory

Responses to herbivory have been proposed to be related to OG perception (see Dejana et al., 2022). To explore whether OG perception is involved in the plant response to herbivory, we aimed to address the regulation and function of the tomato Sl*WAK1* gene. WAK1 has been described as a major OG receptor in Arabidopsis, and in tomato, docking analysis points to a high affinity of the SlWAK1 for pectin oligomers. We first analyzed whether *WAK1* is transcriptionally regulated by OG application and in response to herbivory. *WAK1* expression was upregulated 3 fold in response to OG application (Figure 1A). It was similarly upregulated upon herbivory, with a 2,8 fold increase locally at the wounded leaflet, where the caterpillar fed. In the unwounded systemic leaves, transcript levels were not altered (Figure 1B).

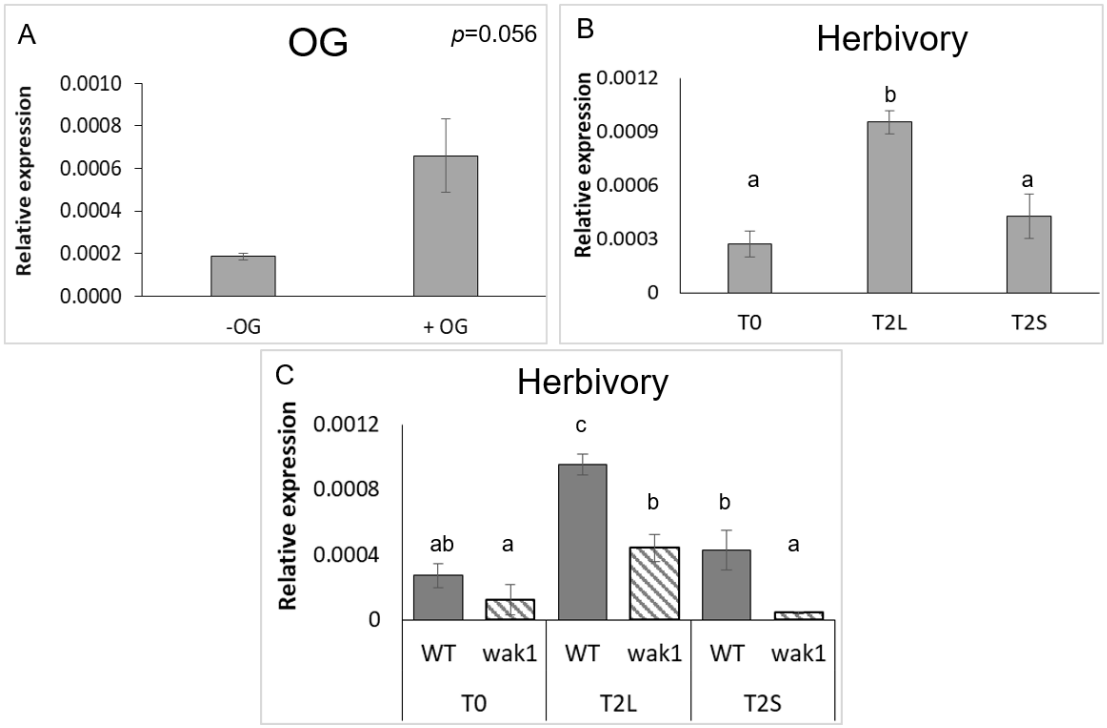

FIGURE 1. Transcriptional regulation of *SlWAK1* gene. Gene expression analysis by real-time qPCR of *SlWAK1* was determined in tomato leaves. **(A)** Expression analysis of *SlWAK1* in wild-type plant in water-treated (-OG) or oligogalacturonides-treated (+OG) leaves 6 h after local OG (50 mg/ml) application (t-test, p=0.056). Expression values are means of six biological replicates, normalized to the housekeeping gene tubulin. **(B)** Expression analysis of *SlWAK1* in tomato wild-type before (T0) and after 2 days of herbivory in local (T2L) and systemic leaves (T2S). **(C)** Expression analysis of *SlWAK1* in tomato wild-type (WT) (filled bars) and *Slwak1* (dashed bars) mutant plants before (T0) and after 2 days of herbivory in local (T2L) and systemic leaves (T2S). Expression values are means of four biological replicates, normalized to the housekeeping gene actin. Data not sharing a letter in common differ significantly according to the Fisher's LSD test ($p<0.05$).

To explore the function of the gene, we used the tomato mutant line *Slwak1* with deficient expression in *WAK1* described by Meco et al. (2020). This line was shown to have

reduced levels of expression and deficient response to salt stress (Meco et al., 2020). We explored the regulation of the gene in the mutant in response to herbivory. Expression levels were around 50% lower in the mutant than in the wildtype in the absence (T0) or in the local, wounded levels, and the reduction was even higher in systemic tissue (only a 10% compared to the levels in the wildtype) (Figure 1C).

Increased herbivore performance on the *Slwak1* mutant

To analyze the impact of SlWAK1 deficiency on *Spodoptera exigua* performance, we infested wild type (WT) and the *Slwak1* mutant tomato plants. *S. exigua* larvae feeding on *Slwak1* had a lower mortality rate than those feeding on WT (Log-rank test, $p<0.0001$) (Figure 2A). Indeed, after 21 days only 17% of the larvae died in *Slwak1* plants, while the mortality reached 41% for larvae on WT plants, even though the larval weight showed no statistical differences between genotypes (t-test, $p=0.2$) (Figure 2B). There was a higher percentage of individuals reaching pupation, with a 70% in *Slwak1* and only 50% in the WT (50%), although the differences were only marginally significant (Log-rank test, $p=0.098$) (Figure 2C). The percentage of larvae reaching the moth stage was also higher in the mutant, with more than two-fold moths than the WT (45% vs 41%, respectively), although differences were not significant (Exact binomial test, $p=0.5$) (Figure 2D).

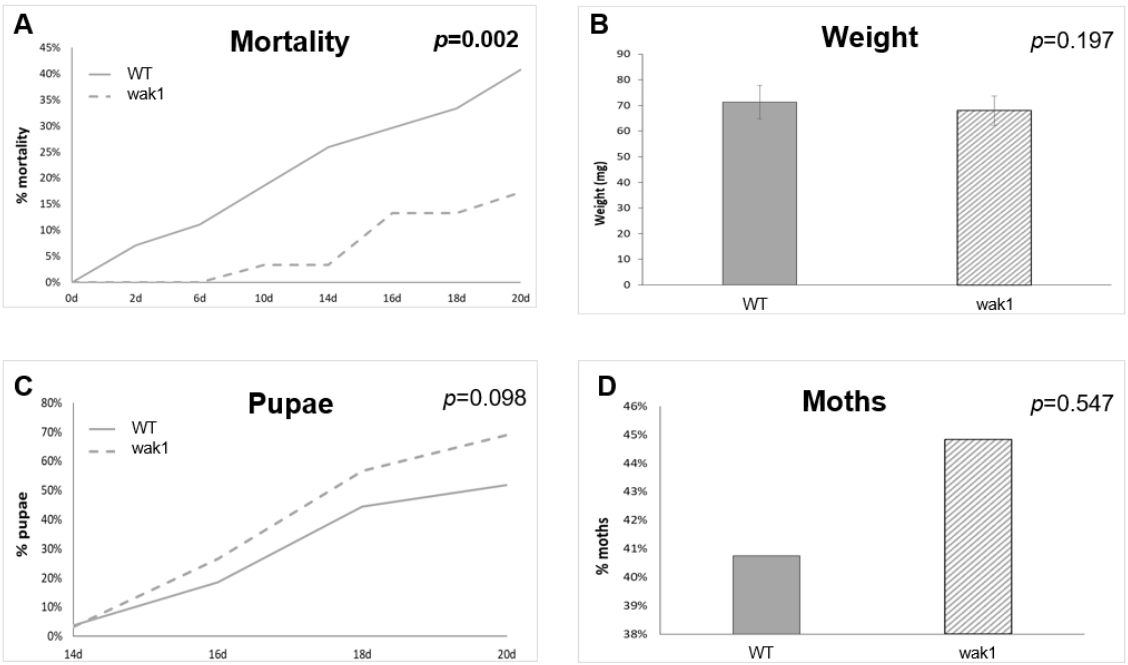

FIGURE 2. *Spodoptera exigua* performance is higher when feeding in *Slwak1*. Tomato wild type (WT) and *Slwak1* (wak1) plants (n=15 per treatment) were infested with second instar *S. exigua* larvae (two per plant, each one in a separate clip-cage). Mortality and pupation were recorded each two days for 3 weeks. **(A) *S. exigua* larvae mortality.** Percentage of mortality of *S. exigua* larvae fed on wild type plants (WT, continuous line) or *Slwak1* (wak1, dotted line); P value according to Log-rank (Mantel-Cox) test. **(B) *S. exigua* weight.** The weight of *S. exigua* larvae was recorded 10

days after plant infestation (t-test). **(C)** *S. exigua* larvae reaching pupae developmental stage. Percentage of caterpillars reaching pupae when fed on WT (continuous line) or wak1 (dotted line), P value according to Log-rank (Mantel-Cox) test. **(D)** *S. exigua* larvae reaching to moths. Percentage of caterpillar reaching moth state when fed on WT or wak1 (Exact binomial test). P values of the different analyses are shown in the graphs, and significant values highlighted in bold.

Nutrients content is altered in the *Slwak1* after herbivory

To explore whether the enhanced performance of *S. exigua* in the *Slwak1* mutant were related to differences in plant nutritional status, we evaluated the nutrient content in the plants. Before *S. exigua* infestation (T0), analysis of micro- and macronutrients in leaves showed no significant differences between *Slwak1* and WT (t-test, $p>0.05$, table 1). After 21 days of herbivory (T21), all nutrients, except carbon (C), reduced their content in leaves compared to T0 in both genotypes (t-test, $p<0.05$, Table 1). After herbivory, C and N values were also not different between the genotypes. However, micronutrient levels differed between both genotypes. In fact, the concentration of Cu, K, Mg, Mn, Na, Ni, P, S, Zn were significantly lower in *Slwak1* than WT (t-test, $p<0.05$). No significant differences in nutrient concentration were found in roots between both genotypes (t-test, $p>0.05$, Table 1).

Herbivory	T0		T21 Leaf		T21 Root	
Nutrients (ppm)	WT	wak1	WT	wak1	WT	wak1
C	398286	389552	420620	426264	328525	338576
N	43701	40534	25274	27522	17476	18967
Al	41	34	20	16	12101	9961
Ca	17088	19239	**14204**	**12555 +**	4619	4752
Cr	1.7	4.0	0	0	620	520
Cu	16	14	**7.0**	**5.25 ***	37	29
Fe	264	273	102	72	6793	5492
K	17022	15526	**12539**	**8753 ****	2809	1785
Li	8.0	8.9	**2.2**	**1.6 +**	3	3
Mg	11651	13242	**7236**	**5980 ****	27531	22719
Mn	163	180	**66**	**46 ****	131	132
Na	618	551	**641**	**456 ****	3973	4422
Ni	4.6	5.2	**2.9**	**1.8 ***	192	162
P	**2063**	**1562 +**	**1188**	**906 ****	949	1029
S	6369	5898	**5122**	**4092 ****	1566	1609
Si	472	412	352	287	104	96
Sr	47	55	**28**	**23 +**	19	20
Ti	1	0	0	0	1053	845
Zn	70	55	**36**	**23 *****	35	32

TABLE 1. Micro- and macro-nutrients content in leaves and roots of WT and *Slwak1* mutant. Macro and micronutrients in leaves of wild type (WT) and Slwak1 (wak1) were determined before *S. exigua* infestation (T0) in leaves, and after 21 days (T21) of herbivory in leaves and roots. Data represent the mean values of 5 biological replicates (ppm). Colored cells indicate changes in nutrient content in the mutant as compared to WT levels (blue, significant reduction; light blue, marginally significant reduction). Asterisks indicate statistically significant differences between genotypes within the same time point according to t-test. +:$p < 0.1$; *:$p < 0.05$; **:$p < 0.01$; ***:$p < 0.001$.

***Slwak1* is not impaired in JA-regulated defenses but it present deficient activation of auxin markers and biosynthesis genes of defensive secondary metabolites**

To understand how *Slwak1* modulates plant defense responses upon herbivory, we performed a transcriptional analysis on genes associated with the main herbivore-induced plant-defense signaling pathways (Figure 3). We collected chewed injured (local response, L) and uninjured leaflets (systemic response, S) from the same leaf 2 days after herbivory (T2). The analysis showed that **JA-** and **ethylene-related** marker genes were induced in both genotypes after *S. exigua* attack (two-way ANOVA, Table S1) and no differences were found across genotypes (Figures 3A, B) (two-way ANOVA, Table S1). Herbivory significantly induced locally the expression of the JA marker genes encoding multicystatin (*MC*), proteinase inhibitors II (*PinII*) and leucil aminopeptidase A (*LapA*) in both genotypes (Figure 3A; two-way ANOVA, Table S1). The ET marker genes encoding for the ET biosynthetic enzyme 1-aminocyclopropane-1-carboxylate oxidase 1 (*ACO1*), the ethylene receptor (*ETR3*) and an ethylene response factor (*ERF*) were significantly induced by herbivory also in both genotypes (Figure 3B; two-way ANOVA, Table S1).

Regarding **auxin**-related genes, the two-way ANOVA showed that the regulation of these genes was affected by both herbivory and genotype, and in the case of the auxin response factor (*PIN3*) by the interaction of both factors (Table S1). The transcriptomic levels of **auxin**-responsive gene (*GH3.8*), auxin efflux carrier protein (*PILS3*) and *PIN3* were similar in WT and *Slwak1* before the herbivory as for JA- and ET-related genes (Figure 3C). However, their expression profile changed upon herbivory (T2): they were upregulated in local (*GH3.8, PILS3*) and/or systemic leaves (*GH3.8, PIN3*,) in WT but not in *Slwak1* (Figure 3C), pointing to a deficient regulation of the auxin signaling pathway.

Regarding the **phenylpropanoid and alkaloid pathways,** the two-way ANOVA showed that the regulation of these genes was affected by both herbivory and genotype (except for the transcription factor (*MybJS1*), a key regulator of phenylpropanoid-related genes, that was affected only by herbivory) (Table S1). Similar expression levels of **phenylpropanoid**-related genes were found between WT and *Slwak1* plants before herbivory (T0), but their levels increased significantly more in WT than in *Slwak1* plants under *S. exigua* attack (T2). For instance, the gene coding for the phenylalanine ammonia-lyase (*PAL1-170*) and for *MybJS1* showed higher expression levels in local leaves of WT plants than *Slwak1* under herbivory (Figure 3D). The **glycoalkaloid** biosynthetic gene 9 (*GAME9*), key for the synthesis of steroidal alkaloids and isoprenoids, has also similar expression levels in both genotypes before herbivory.

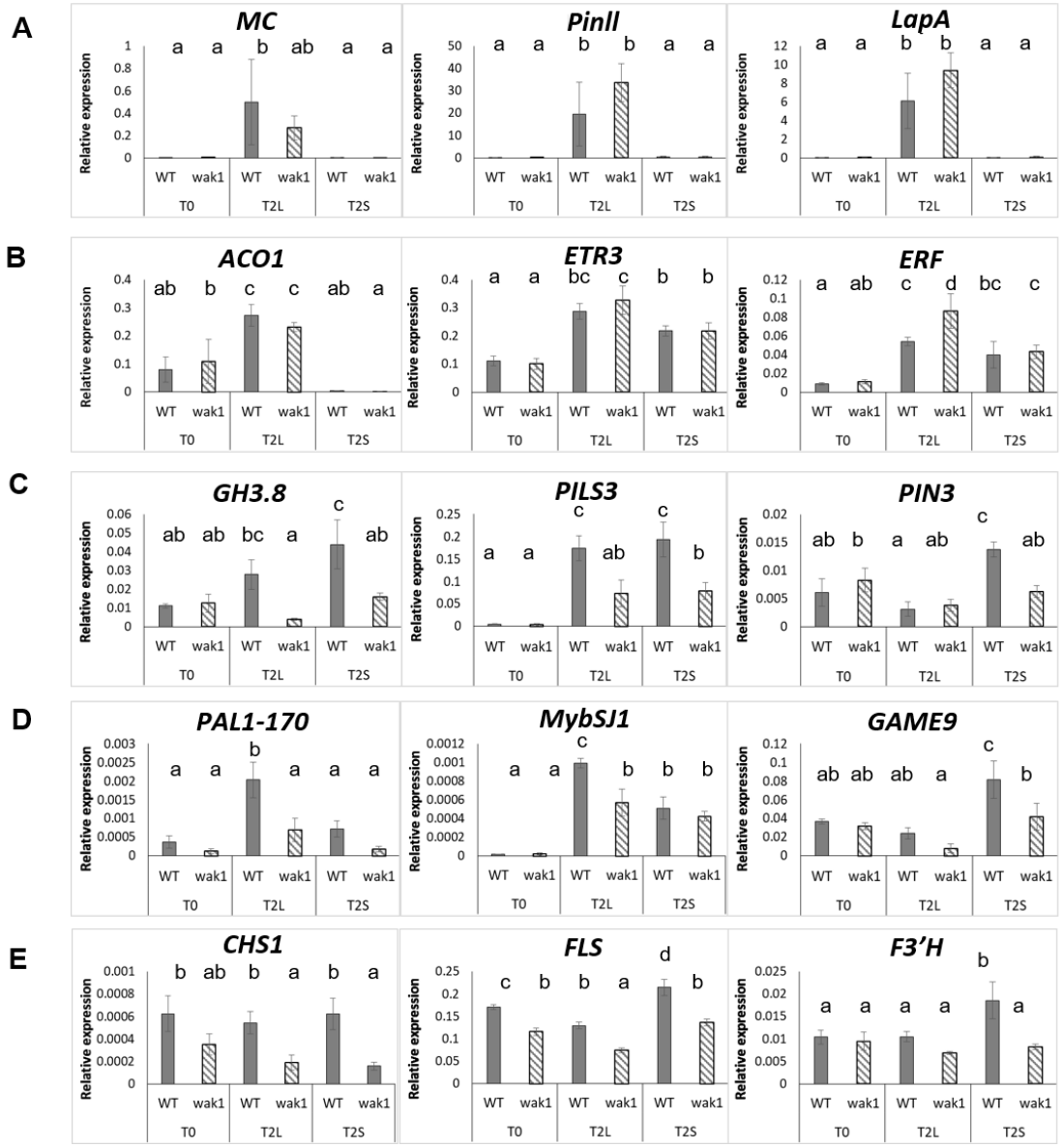

FIGURE 3. Transcriptional regulation of defense-related genes. Gene expression analysis by real-time qPCR was determined in wild type (grey, WT) and mutant *Slwak1* (grey lines, wak1) leaves before (T0) and after 2 days of herbivory in local (T2L) and systemic leaves (T2S). Expression values are means of four biological replicates, normalized to the housekeeping gene actin. **(A)** Jasmonate regulated defense-related genes coding for multicystatin (*MC*), proteinase inhibitor II (*PinII*) and Leucylaminopeptidase A (*LapA*). **(B)** Ethylene marker genes coding for 1-aminocyclopropane-1-carboxylate oxidase 1 (*ACO1*), ethylene receptor (*ETR3*) and ethylene response factor (*ERF*). **(C)** Auxin marker genes coding for auxin-responsive gene (*GH3.8*), auxin efflux carrier protein (*PILS3*) and auxin response factor (*PIN3*). **(D)** Phenylpropanoid and alkaloid-related marker genes coding for phenylalanine ammonia-lyase (*PAL1-170*), transcription factor (*MybJS1*), key regulator of phenylpropanoid-related genes and glycoalkaloid metabolism 9 (*GAME9*), key for the biosynthesis of steroidal alkaloids and isoprenoids. **(E)** Flavonoid-related marker genes coding for chalcone synthases (*CHS1*) and flavonol synthase (*FLS*) and flavonoid-3'-hydroxylase (*F3'H*). Data not sharing a letter in common differ significantly according to the Fisher's LSD test (p<0.05).

However, upon herbivory, its expression was significantly enhanced in systemic leaves compared to T0 in the WT, but not in Slwak1 (Figure 3D). Finally, the two-way ANOVA showed that **flavonoid** biosynthesis genes were mostly affected by the genotype (Table S1). Indeed, the transcription levels of chalcone synthases (*CHS1*) and flavonol synthase (*FLS*) was higher in WT than in *Slwak1* plants before (T0) and after herbivory in both local and systemic leaflets (T2L and

T2S, respectively). On the other hand, the expression of flavonoid-3'-hydroxylase (*F3'H*) was induced by herbivory in systemic leaflets (T2S) only in WT (Figure 3E). Thus, *Slwak1* is impaired in the upregulation of flavonoids related genes. Overall, these results reveal differential transcriptional regulation in WT and *Slwak1* both before and after herbivory, mostly associated with a lower response of *Slwak1* to herbivory in the auxin, alkaloids and flavonoid related genes (Table S1).

***Slwak1* is impaired in defensive metabolites production under herbivory**

Since the transcriptional analysis points to a deficient activation of the phenylpropanoid and flavonoid pathways, we hypothesized that *Slwak1* is compromised in the accumulation of defense-related secondary metabolites. To test this hypothesis, we performed an untargeted analysis to compare the metabolome leaves profile in WT and *Slwak1* plants before (T0) and after local herbivory (T2L).

A representation of sparse Partial Least Squares Discriminant Analysis (sPLSDA) revealed that plant genotype and herbivory had an impact on plant metabolism (Figure 4A). According to the two main components contributing to data variation, the differences were more pronounced in relation to genotype than to herbivory, since the groups were more distant between WT and *Slwak1* than between T0 and T2L (Figure 4A). Then, into the same genotype, the herbivory seems to have a stronger effect on the metabolome of *Slwak1* than in the WT.

Thus, we performed a heatmap analysis with the top 150 signals showing the more significant changes (Kruskal-Wallis test, $p<0.05$) to find metabolites that were deficiently accumulated in *Slwak1* upon herbivory by *S. exigua* (Figure 4B). For that, we selected clusters of metabolites that were more accumulated constitutively (T0) and/or under herbivory (T2L) in WT than in *Slwak1* (Figure 4B, black boxes).

Among the selected clusters, we identified compounds by exact m/z and/or individual spectrum matches with databases. Using *S. lycopersicum* KEGG and internal databases, we putatively identified 9 signals by m/z or/and fragmentation spectrum, corresponding to compounds with higher accumulation in WT than in *Slwak1*. WT plants accumulated more metabolites that putatively belong to alkaloid, phenol, flavonoid and terpene biosynthesis and signalling pathways than the *Slwak1*. Particularly: kaempherol-rhamnoside, Hesperetin 7-O-glucoside, the phenol feruloylquinic acid, the terpenes dihydroactinidiolide and heptylmalic acid, the alkaloid senecionine, the tripeptides Val-Ile-Ile and the lignan (-)-Secoisolariciresinol

(ANOVA one-way, LSD, p<0.05) (Figure 4C). Among oligopeptides, the tripeptide Phe-Ala-Lys, showed higher accumulation in WT than in mutant plants only upon herbivory (Figure 4C).

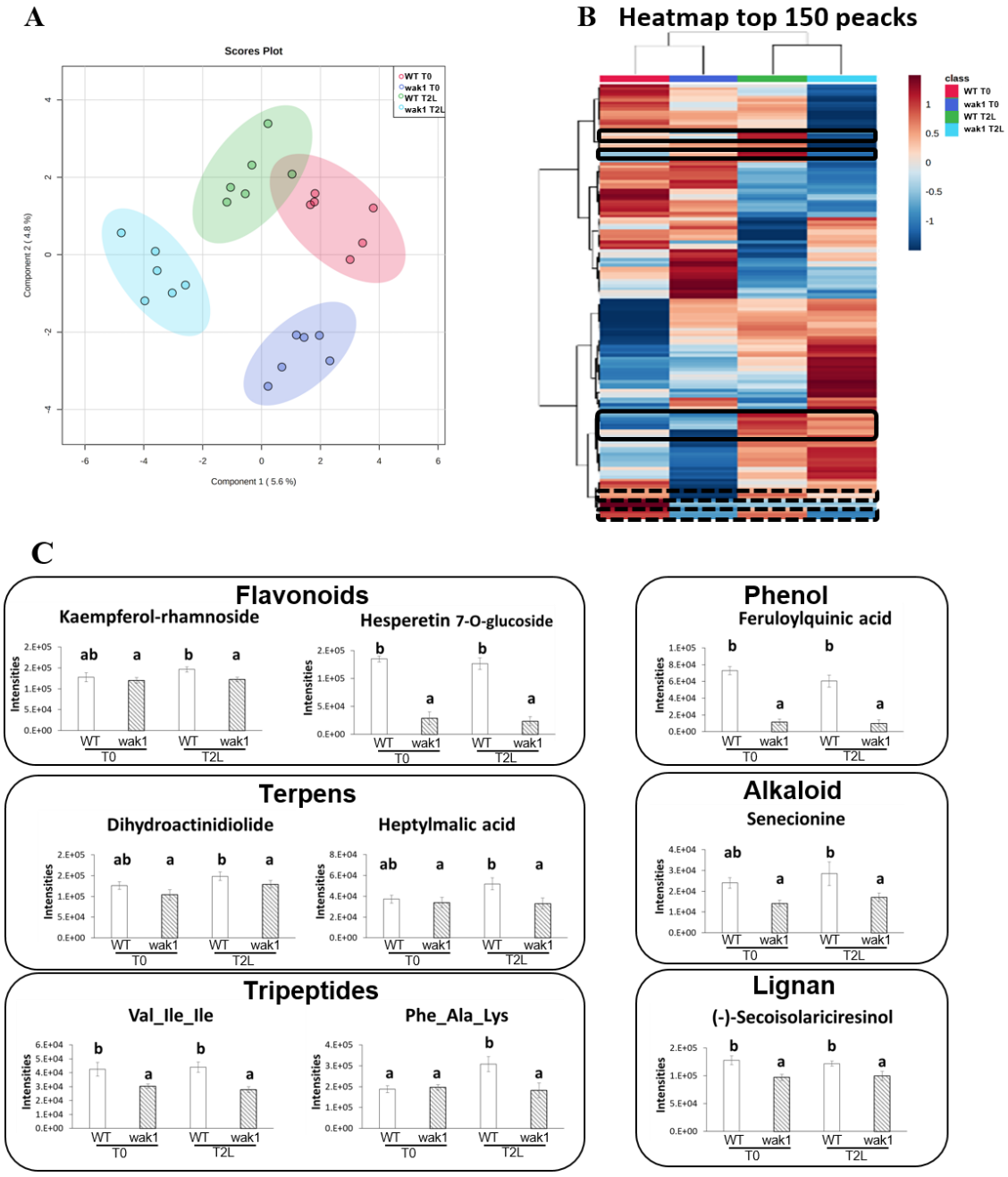

FIGURE 4. Untargeted metabolomic profiling of leaves before and after *S. exigua* attack in WT and *Slwak1* tomato plants. Wild type (WT) and *Slwak1* (wak1) tomato plants were infested with S. *exigua* larvae, and leaflets were harvested after two days of herbivory. Lyophilized leaflets of non-infested plants (T0) and leaflets where *S. exigua* fed (local response to herbivory, T2L) were analyzed with ultra-performance liquid chromatography (UPLC) (n=6). Signal intensity was determined in all samples after normalizing the chromatographic area for each compound to the dry weight of the sample. All registered signals from combination of both positive and negative electrospray ionization were compared using non-parametric Kruskal–Wallis test, and only data with significant differences between groups (p<0.05) were used for the supervised analysis. **(A)** Supervised three-dimensional sparse partial least squares discriminant analysis (sPLSDA) representation of the major sources of variability. **(B)** Heatmap and clustering representation of the 150 signals showing significant differences among treatments. Dotted squares represent metabolites accumulated more in WT than *Slwak1*; black squares represent over-accumulation of compounds in WT upon herbivory. **(C)** Putative differentially accumulated compounds. Signal intensities for the different compounds in

WT (white lines) and wak1 (gray lines) leaves before (T0) and after 2 days of herbivory (T2L). Data not sharing a letter in common differ significantly according to the Fisher's LSD test ($p<0.05$).

Slwak1 is altered in primary metabolism

Besides the activation of the secondary metabolism leading to the accumulation of defensive compounds, herbivory also triggered changes in the primary metabolism. Indeed, jasmonate and auxins are involved in carbohydrate reallocation in response to herbivory (Machado et al., 2013). Considering the deficiency in the activation of auxin related responses observed in the mutant (Figure 3), we hypothesized that the higher performance of the caterpillars in *Slwak1* plants could be also related to differential changes in the primary metabolism. We therefore explored the regulation of carbohydrate metabolism in both genotypes, comparing the sugar content (sucrose, glucose, fructose and starch) or the tricarboxylic acid (TCA) cycle intermediates (succinic, malic and citric acid) and the activity of several key enzymes related with glycolysis (HXK, PGI, PFK, Ald), sucrose biosynthesis (FK) or degradation (CwInv, VacInv, CytInv), cell wall biosynthesis (UGPase), oxidative pentose phosphate pathway (G6PDH) and starch biosynthesis (AGPase, PGM), both before infestation (T0) or in the local (L) and systemic (S) responses to herbivory after 2 or 14 days of herbivory (T2 and T14, respectively). Finally, we also analyzed sugar accumulation in roots and leaves at harvest, after 21 days of *S. exigua* herbivory (T21S and T21R, respectively). To explore possible changes at the transcriptional level, we also performed a transcriptomic analysis of genes involved in CH biosynthesis, hydrolysis and transport before (T0) or after herbivory (T2L).

Slwak1 displays a higher glycolysis activation than wild type in the absence of herbivory

Under basal conditions -before herbivory (T0)- the activity of the enzymes involved in the glycolysis and cell wall biosynthesis was higher in *Slwak1* than in WT: all, phosphoglucoisomerase (PGI), phosphofructokinase (PFK), aldolase (Ald), hexokinase (HXK) and UDP-glucose pyrophosphorylase (UGPase) displayed higher activity levels in the mutant (Figure 5A). On the other hand, phosphoglucomutase (PGM), related mainly to starch biosynthesis, showed reduced activity in *Slwak1* than WT, reflected in a lower starch concentration (Figure 5A-B). The activity of the cell wall (CwInv) and vacuolar (VacInv) invertases was also higher in *Slwak1* than in the WT (Figure 5C), correlating with a significant lower sucrose accumulation in the leaves of *Slwak1* (Figure 5B). The concentration of the compounds from the TCA cycle, as succinic and malic acid, was lower in the mutant, pointing to a possible reduced energetic status than in the WT (Figure 5B).

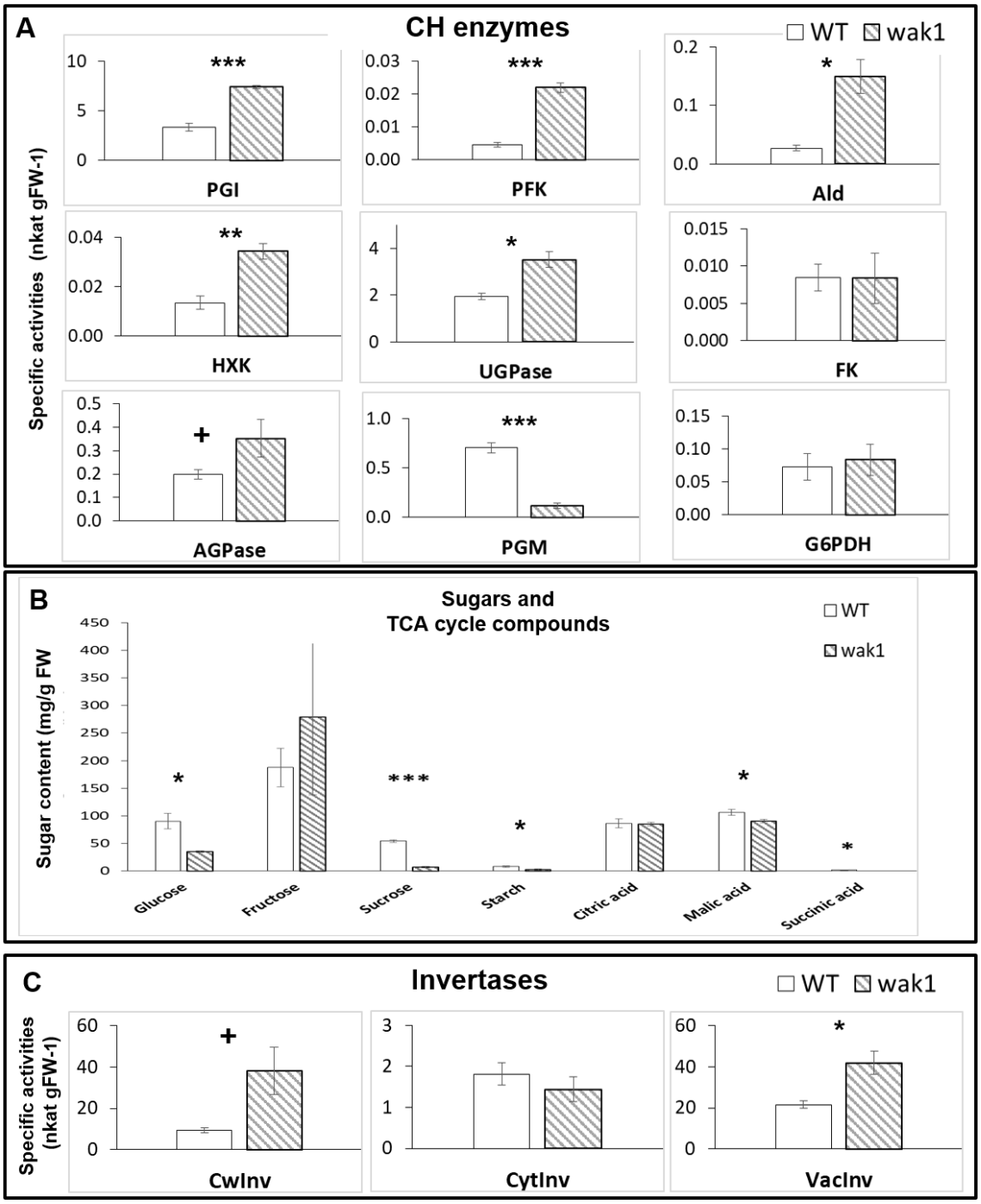

FIGURE 5. Enzymatic activities and compounds related to carbohydrate metabolism in the absence of herbivory (basal level). Lyophilized leaf material from control non-infested plants was determined in wild type (white, WT) and mutant (grey lines, wak1) plants. Data are means of 3 replicates ±SD. **(A)** Enzymatic activities (nkat gFW–1) of CH metabolism**.** Enzymes related with glycolysis (PGI, PFK, Ald, HXK), cell wall biosynthesis (UGPase), sucrose biosynthesis (FK), starch biosynthesis (AGPase, PGM) and oxidative pentose phosphate pathway (G6PDH). **(B)** Sugars (Glucose, Fructose, Sucrose, Starch) and TCA cycle intermediates (citric, malic and succinic acid) concentration (mg/g FW) and **(C)** invertases enzymes (nkat gFW–1) related with sucrose degradation (CwInv, VacInv, CytInv). Asterisks indicate statistically significant differences between genotypes according to t-test. +:$p < 0.1$; *:$p < 0.05$; **:$p < 0.01$; ***:$p < 0.001$.

Overall, these results revealed remarkable alterations in the primary metabolism of *Slwak1* tomato plants at the basal level, with higher glycolytic activity and reduced energy status in *Slwak1* than in WT, as summarized in the scheme (Figure 6).

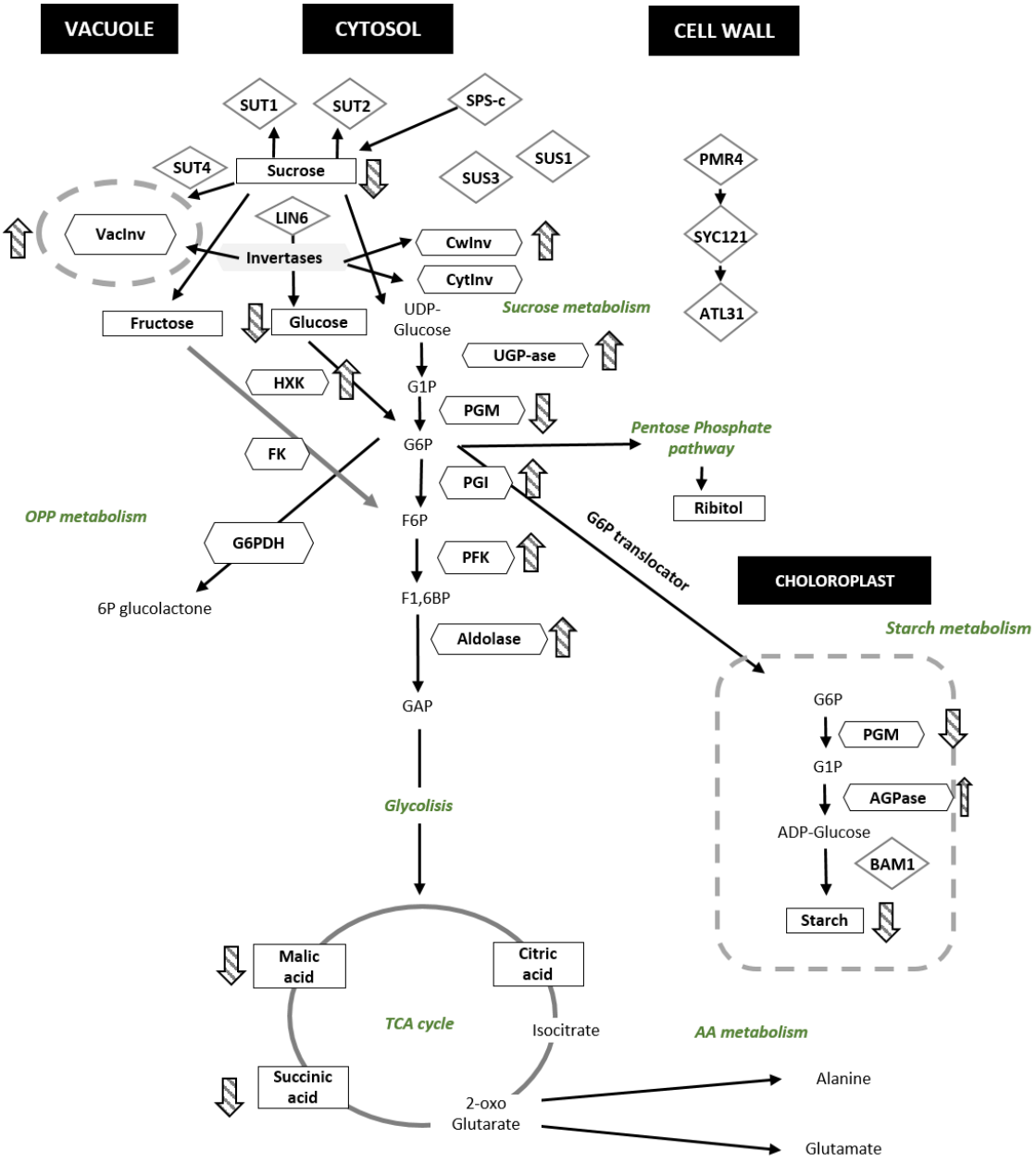

FIGURE 6. Schematic summary of the main differences in carbohydrate metabolism between *Slwak1* and WT in the absence of herbivory (basal level, T0) (*Slwak1* vs WT). Dashed grey arrows indicate changes between both genotypes. Thick ones indicate significant changes ($p<0.05$), thinner arrows indicate marginally significant changes ($p>0.05$ and <0.1). Arrows up and down indicate a higher accumulation/induction or reduction/repression of organic acids, sugars, enzymatic activity and gene expression in *Slwak1* vs WT. Rectangles represent metabolites -sugars and organic acids-; hexagons: enzymatic activity; rhombus: genes.

Contrasting response to herbivore attack in *Slwak1* and WT

A Permutational multivariate analysis of variance (PERMANOVA) of the enzymatic activities related to CH revealed a significant interaction at the local level between genotype and herbivory, while CH content showed only a significant effect of the genotype (Figure S1). . At systemic level, the enzymatic activities were significantly regulated only by herbivory - negative correlations of enzymatic activities with the herbivory in both genotype-, while CH content

showed significant effect of genotype and herbivory differences (Figure S1, S2). Indeed, only in WT, organic acids and soluble sugars displayed a significant negative correlation with the herbivory (Table S2). Hence, it seemed that primary metabolism of WT was more responsive to herbivory than *Slwak1*, mostly at the local level (Table S2).

Indeed, a higher number of significant correlations of enzymatic activities and sugar contents with time, i.e. herbivory, was seen in WT than in *Slwak1*. Locally, WT showed negative correlations of PGM and AGPase activities with the herbivory that leads to a starch reduction (starch correlated negatively with herbivory too). In WT, the enzymatic activity of Ald, PFK and HXK (enzymes related to glycolysis) showed a positive correlation with herbivory that was related with the significant negative correlation of glucose with herbivory. On the contrary, although the mutant showed positive correlations of the activities of PGI, PFK, and HXK (enzymes related to glycolysis), no significant correlation between soluble sugars and herbivory was detected at local level (Table S2).

In WT plants, the sucrose cleavage activity of CwInv increased at T2L and, consequently, reducing the sucrose content at T2L and T14L as compared to T0 (Figure 7A). In fact, WT plants displayed lower sucrose concentration and a significant fructose increase as free monosaccharide at T2L and T14L as compared to T0 (Figure 7A). On the contrary, the *Slwak1* reduced the sucrose cleavage activity of the CwInv enzyme upon herbivory and it did show an opposite trend than the WT in the sugars accumulation (Figure 7A).

Regarding transcriptional regulation of some CH related genes no significant changes were found between genotypes at T0, but opposite patterns were also observed in their response to herbivory (Figure S2). The expression of the sucrose synthase *SPS-C* gene (involved in sucrose biosynthesis) was marginally repressed in WT plants, while it was significantly enhanced in the mutant (Figure 7B). Moreover, only *Slwak1* lines showed elevated transcription levels of the sugar transporter *SUT2* upon herbivory (T2L vs T0).

With regards to starch biosynthesis, in WT plants PGM and AGPase activities were reduced upon herbivory (T2L and T14L compared to T0), correlating with a reduction in the starch content, suggesting a decreased energy storage in local leaves in the WT upon herbivory (Figure 7C). On the contrary, *Slwak1* plants increased the activity of starch biosynthesis enzyme ADP-glucose pyrophosphorylase (AGPase) at T2L, and the starch concentration was also higher upon herbivory (Figure 7C). Finally, the enzymatic activities related to glycolysis showed no significant changes after 2 days of herbivory (T2L) in both genotypes. In WT after 14 days of herbivory, the UGPase, PFK, HXK increased and G6PDH and Ald decreased their activity, while

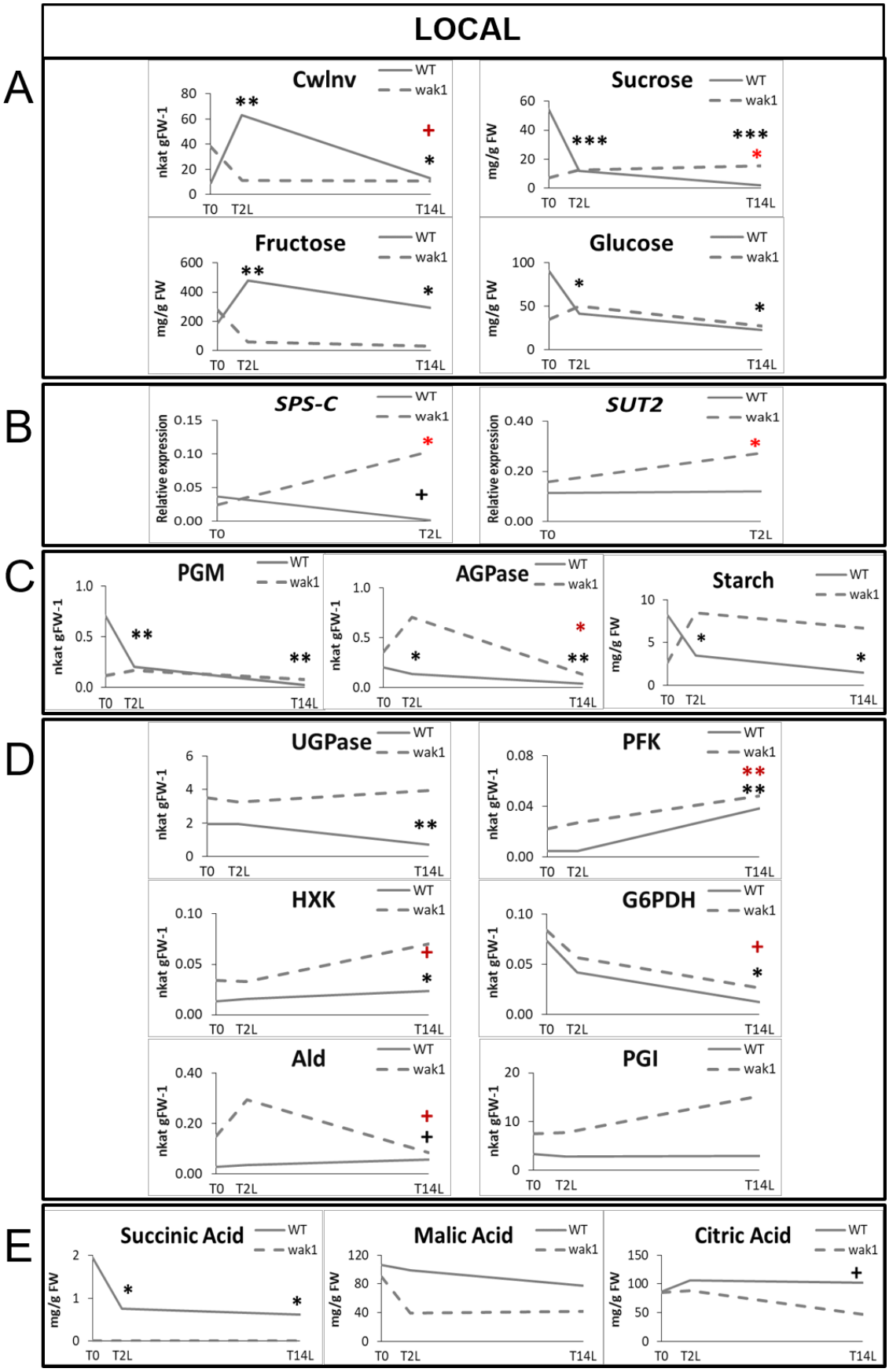

FIGURE 7. Changes in primary metabolism as a local response to herbivory in leaves of WT and *Slwak1* mutant tomato plants. Enzymatic activities (nkat gFW–1) and gene expression levels (relative expression) related to carbohydrate metabolism and sugar and organic acid content (mg/g FW) in wild type (WT; grey continuous line) and

mutant (wak1; dotted grey line) leaves before (0), and after 2 and 14 days of infestation with *S. exigua* (2L and 14L, respectively) in damaged leaflets of infested plants (Local response). **(A)** Cell wall invertase (CwInv) activity and sugars concentration of of Sucrose, Fructose, Glucose in WT and *Slwak1* (n=3). **(B)** Transcription level of *SPS-C* (sucrose synthase) and *SUT2* (sucrose transporter) before (T0) and at 2 days of *S. exigua* attack (T2L) in WT and *Slwak1* plants (n=6). **(C)** Activity of enzymes involved in starch biosynthesis (PGM and AGP-ase) and starch concentration (n=3). **(D)** Activity of enzymes involved in cell wall biosynthesis (UGPase), glycolysis and oxidative pentose phosphate pathway (PFK, HXK, G6PDH, Ald, PGI) in WT and *Slwak1* (n=3). (E) Organic acids concentration of Succinic, Malic and Citric acid in WT and *Slwak1* (n=3). Asterisks (black for WT and red for *Slwak1*) indicate significant effects of herbivory on each genotype between the indicated time point (T2L or T14L as compared to T0) within the same genotype according to t-test. +:p < 0.1; *:p < 0.05; **:p < 0.01; ***:p < 0.001.

the mutant showed only a significant increase of PFK at T14L compared to T0 (Figure 7D). Regarding TCA cycle compounds, only succinic acid was significantly lower accumulated in WT at T2L than T0 (Figure 7E).

In the unwounded leaflets, the systemic response to herbivory (T2S and T14S) also showed different patterns in both genotypes. In WT plants, the CwInv enzyme increased its activity after 2 days of herbivory in the wildtype (T2S vs T0). This increase led to a significant sucrose depletion after 2 and 14 days of herbivory (T2S and T14S) (Figure 8 A). However the expression levels of the *SPS-C* and *SUT2* genes significantly increased too (Figure 8B).

On the contrary, the CwInv activity decreased in *Slwak1* at 2 and 14 days upon herbivory (T2S and T14S) (Figure 8A), despite *SPS-C* and *SUT2* expression increased at T2S (Figure 8B). Additionally, the two enzymes involved in starch biosynthesis, PGM and AGPase, showed a significant decrease in their activity under herbivory in WT plants compared to uninjured leaves (T2S, T14S vs T0). However, *Slwak1* significantly increased the PGM activity at 2 days after herbivory (T2S) and increased starch accumulation upon herbivory (T14S) (Figure 8C). Noteworthy, WT increased the activity of UGPase and HXK systemically upon herbivory, while the mutant decreased the activity of all enzymes related to glycolysis, cell wall biosynthesis and oxidative pentose phosphate pathway (T2S, T14S vs T0) (Figure 8D). Finally, several compounds from the TCA cycle were less accumulated upon herbivory in systemic leaflets in both WT and *Slwak1* compared to T0 (Figure 8E). Overall, these results revealed remarkable alterations of primary metabolism of *Slwak1* tomato plants in response to herbivory, as summarized in the scheme (Figure 9).

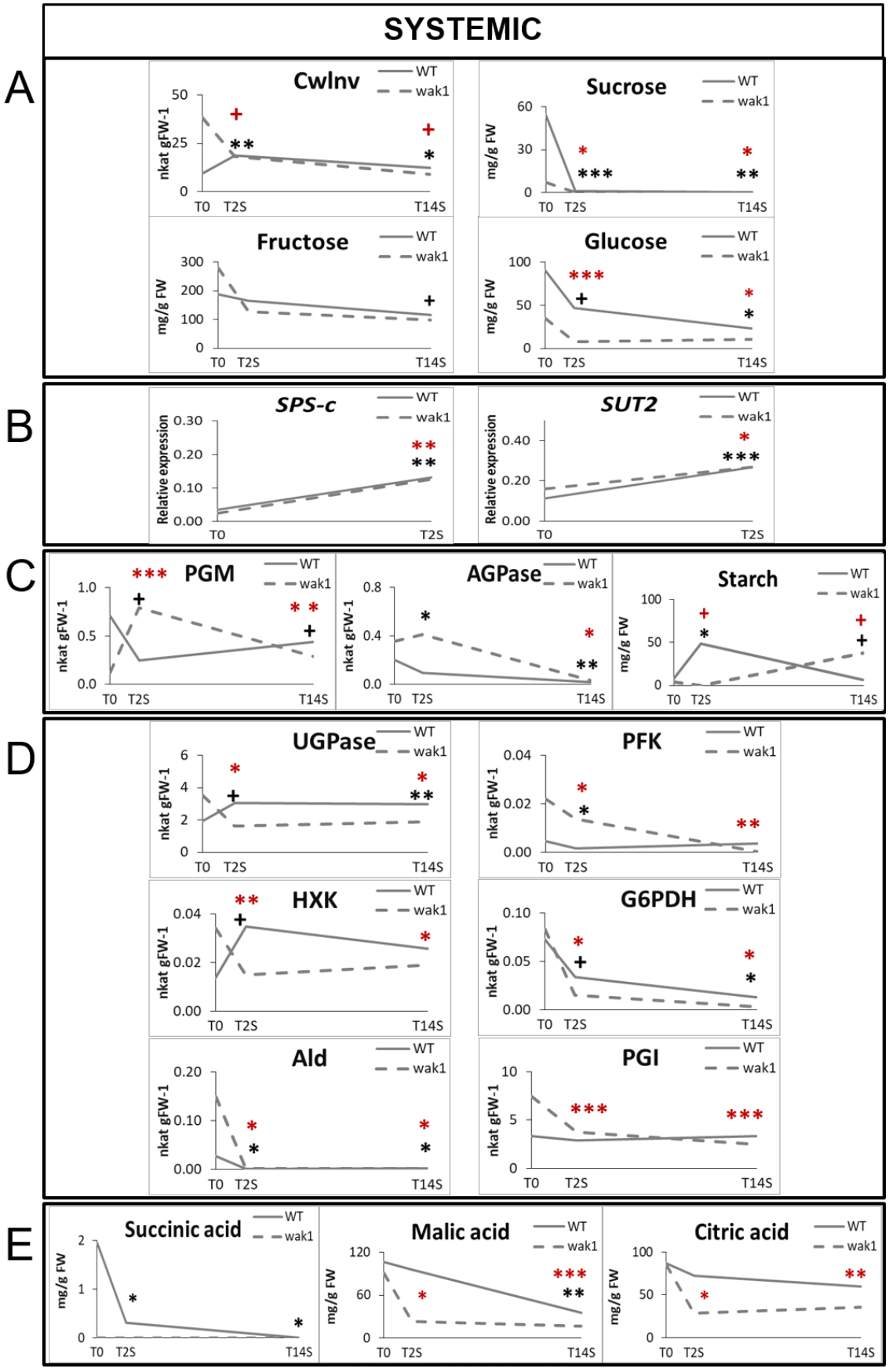

FIGURE 8. Changes in primary metabolism as a sytemic response to herbivory in leaves of WT and *Slwak1* mutant tomato plants. Enzymatic activities (nkat gFW–1) and gene expression levels (relative expression) related to the carbohydrate metabolism and sugar and organic acid content (mg/g FW) in wild type (WT; grey continuous line) and

mutant (wak1; dotted grey line) leaves before (0), and after 2 and 14 days of infestation with *S. exigua* (2S and 14S, respectively) in non-damaged leaflets from infested plants (Systemic response). **(A)** Cell wall invertase (CwInv) activity and sugars concentration of Sucrose, Fructose, Glucose in WT and *Slwak1* (n=3). **(B)** Transcription level of *SPS-C* (sucrose synthase) and *SUT2* (sucrose transporter) before (T0) and at 2 days of *S. exigua* attack (T2S) in WT and *Slwak1* plants (n=6). **(C)** Activity of enzymes involved in starch biosynthesis (PGM and AGP-ase) and starch concentration (n=3). **(D)** Activity of enzymes involved in cell wall biosynthesis (UGPase), glycolysis and oxidative pentose phosphate pathway (PFK, HXK, G6PDH, Ald, PGI) in WT and *Slwak1* (n=3). (E) Organic acids concentration of Succinic, Malic and Citric acid in WT and *Slwak1* (n=3). Asterisks (black for WT and red for *Slwak1*) indicate significant effects of herbivory on each genotype between the indicated time point (T2S or T14S as compared to T0) within the same genotype according to t-test. +:p < 0.1; *:p < 0.05; **:p < 0.01; ***:p < 0.001.

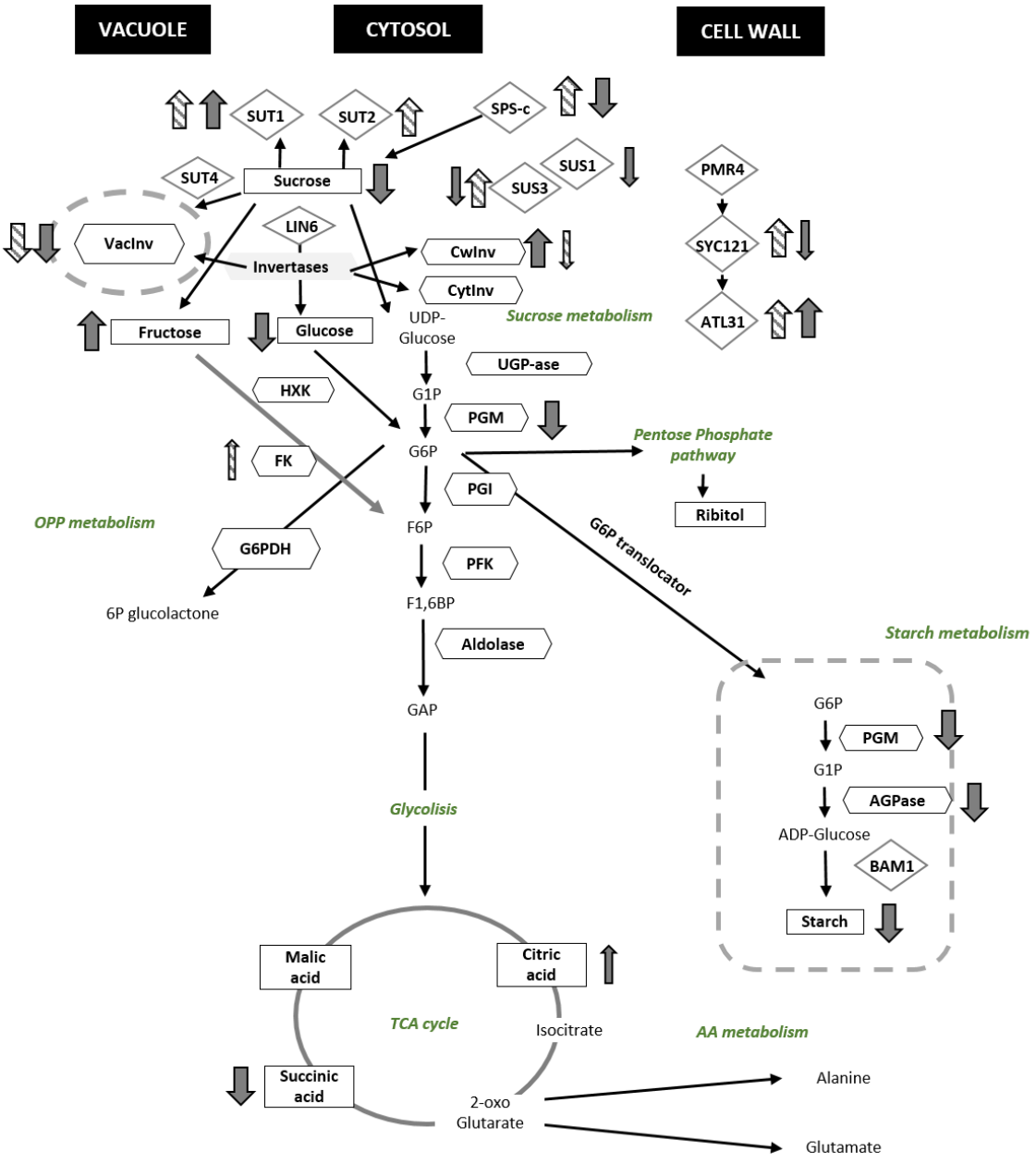

FIGURE 9. Schematic summary of the main differences in carbohydrate metabolism in response to local herbivory (T2L vs T0) in WT and *Slwak1*. Changes upon herbivory are indicated by dark grey-filled arrows in the WT and dashed grey arrows in *Slwak1*. Thick arrows indicate significant changes (p<0.05), and thinner arrows indicate marginally significant changes (p>0.05 and <0.1). Arrows up and down indicate a higher accumulation/induction or reduction/repression of organic acids, sugars, enzymatic activity and gene expression after herbivory (T2L vs T0) in WT and in *Slwak1.* Rectangles represent metabolites -sugars and organic acids-; hexagons: enzymatic activity; rhombus: genes.

***Slwak1* mutant show differential CH allocation under herbivory**

The analysis of soluble sugars and starch in shoots and roots at harvest, after 21 days of herbivory (T21) also revealed significant differences between the two genotypes (Figure 10). Levels of glucose, fructose and sucrose were higher in the leaves of WT plants than in the mutant, although the differences were significant only for sucrose (t-test, $p<0.05$). However, in roots, the levels of glucose and fructose were lower in WT than *Slwak1.* Thus, there is a strong difference in the root-to-shoot ratio, being 0.3 and 0.4 for glucose and fructose, respectively, in WT plants, but 0.8 and 1.3 in *Slwak1*. The levels of starch in leaves were 10 times bigger in the *Slwak1* than in the WT. Unfortunately, we did not detect starch in the roots.

Overall, the results show that WT plants reduce starch and increase soluble sugars in leaves, probably increasing energy sources to produce defensive metabolites, while *Slwak1* stores starch in leaves and increases hexoses in roots.

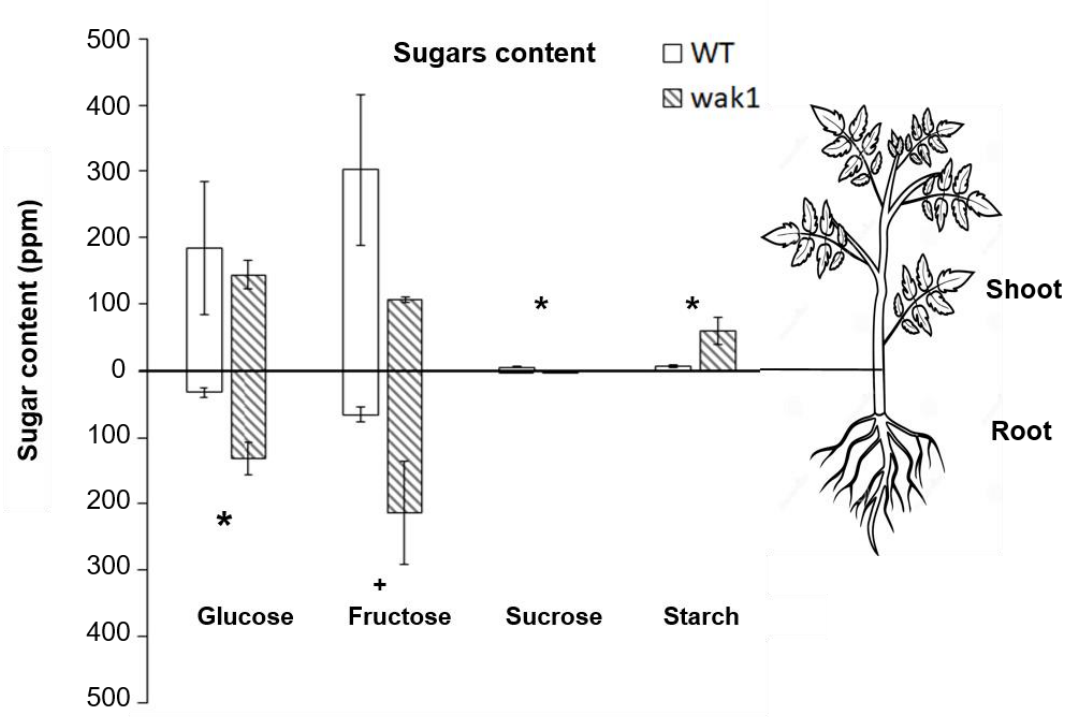

FIGURE 10. Sugar allocation within the plant after 21 day of herbivory in WT and *Slwak1* mutant. Sugars concentrations were determined after 21 days of herbivory in leaves (upper side of the graph) and roots (bottom side of graph) of wild type (white, WT) and mutant (grey lines, wak1). Data are the means of five biological replicates ±SD of Glucose, Fructose, Sucrose and Starch (mg/g FW). Asterisks within shoot or root indicate statistically significant differences between genotypes according to t-test. +:$p < 0.1$; *:$p < 0.05$.

DISCUSSION

The goal of this study was to analyze the role of the tomato receptor SlWAK1 in the regulation of plant responses to chewing herbivores. First, we showed that *SlWAK1* is transcriptionally upregulated locally in response to herbivory by S. *exigua*, and also by OG application, in agreement with its proposed role as OG receptor. *S. exigua* performed better on *Slwak1* mutant than in the WT plants, showing significantly higher survival and a higher percentage of individuals reaching the pupa stage, confirming that the lack of this receptor compromises the efficient activation of plant defenses. This improved performance does not seem to be related to major changes in nutritional value of the leaves: C and N content did not differ in the mutant, and other micronutrients were unaltered when herbivory started. Only the accumulation of several micronutrients decreased in *Slwak1* compared to WT under prolonged herbivory. Some of these elements, such as Cu and Zn, play an important role in secondary metabolite biosynthesis to maintain membrane structural integrity, and they are needed for protein synthesis, auxin regulation, phenol metabolism, and the biosynthesis of antioxidant defense enzymes (Bhat et al., 2020; Cabot et al., 2019). Additionally, P and Mn deficiencies in tomato plants alter biochemical and physiological processes, including energy transfer, protein metabolism, flavonoid content and the biosynthesis of aromatic amino acids and IAA (Bhat et al., 2020; Gupta et al., 2017). Thus, the micronutrients reduction that followed herbivory may correlate with the inability of the mutant to produce specific metabolites fundamental for inducible defense mechanisms.

Slwak1 is impaired in phytohormones and secondary metabolites pathways

To analyze the plant defense responses, we explored the main defense related to hormone signaling pathways. JA is a major hormonal signaling pathway regulating anti-herbivore defenses, leading to the accumulation of toxic, anti-feedant or anti-nutritional proteins and compounds (Divekar et al., 2022). JA biosynthesis and defensive JA responsive genes were indeed induced as a local response to herbivory, but the pattern was similar in both genotypes. Thus, the data do not support the role of SlWAK1 in the regulation of these responses. In plants as Arabidopsis, maize, rice and sweet orange, it was found that under attack *WAKs* induced the expression of the JA biosynthesis-related genes or JA accumulation (Moscatiello et al., 2006; Zuo et al., 2015; Yang et al., 2019; Li et al., 2020; Malukani et al., 2020). The lack of JA-related phenotype in the expression patterns analyzed may be explained by a

functional redundancy in the *WAK* genes. Indeed, the tomato has 11 SlWAKs, and *SlWAK1* and other 6 *SlWAK* genes in tomato have putative cis-acting regulatory elements that bind methyl jasmonate-related transcription factors (Sun et al., 2020). Thus, we can speculate that in tomato other *WAK* genes may have a role in JA-related responses.

However, auxin marker genes showed differential transcriptional regulation in the *Slwak1* mutant. Machado et al. (2016) described a strong, rapid and specific accumulation of IAA in herbivore-attacked *Nicotiana attenuata* leaves. Auxin accumulation promotes the production of secondary metabolites as phenolics and flavonoids (Lulu et al., 2015; Mahdieh et al., 2015). Moreover, auxin interacts with sugar pathways to fine-tune plant growth according to environmental conditions (Mishra et al., 2022) and carbohydrate reallocation in response to herbivory (Machado et al., 2013). Thus, the differential regulation of auxin signaling may contribute to the differential accumulation of some secondary metabolites and the alterations in the primary metabolism observed in the mutant.

Accordingly, we also found deficient induction of genes involved in the biosynthesis of secondary metabolites in *Slwak1* upon herbivory. Indeed, induced expression of phenylpropanoid, flavonoid and alkaloid-related genes as a local or systemic response to herbivory was only found in WT plants (except for MybSJ1 which was also induced in the mutant, although to a lower extent). Multiple experimental evidence supports the role of these genes in anti-herbivore defense: tomato plants accumulate increasing amounts of phenolic compounds and amino acid precursors in response to the generalist chewing herbivore *Helicoverpa zea* (Steinbrenner et al., 2011). The biosynthesis of flavonoids in wild tomato (chalcone, quercetin and kaempferol sugar conjugates) is associated with a reduction of leaf damage caused by phloem feeders and chewing herbivores (Yao et al., 2019; Kang et al., 2014; Ballester et al., 2016). The induction of the transcription factor MybSJ1 positively regulates the transcription of different phenylpropanoid-related genes, leading to the accumulation of methyl-jasmonate-derived molecules such as feruloylputrescine in *Nicotiana tabacum,* (Gális et al., 2006), and in tomato, the induction of GAME9 regulates the biosynthesis of steroidal alkaloids and isoprenoids, for example tomatine and tomatidine (Cárdenas et al., 2016).

Thus, we hypothesized that the failure of *Slwak1* plants to upregulate phenylpropanoid, flavonoids and alkaloid-related genes in response to herbivory lead to a deficient accumulation of defensive compounds that may explain the better performance of *S. exigua* in *Slwak1* plants than in WT plants. In fact, we identified, through untargeted metabolomic analysis different, flavonoid and alkaloid compounds with compromised accumulation in *Slwak1* in response to herbivory. For example, the tripeptide Phe-ala-lys, kaempferol -rhamnoside, feruloylputrescine,

dihydroactinolide and senescione are more accumulated in WT plants upon herbivory, but not in the mutant. These metabolites were previously shown to accumulate in tomato in response to herbivory, and after OGs application (Steinbrenner et al., 2011; da Silva Souza et al., 2020; Miklavčič Višnjevec et al., 2021; Rivero et al., 2021; Gamir et al., 2020), and reduced levels of kaempferol 3,7-dirhamnoside correlated with increased performance of the herbivore *Pieris brassicae* (Onkokesung et al., 2014). Together, these results support a role of SlWAK1 in the accumulation of these metabolites, likely through the perception of OGs. The reduction of these compounds that may directly repel herbivore attack (Ballester et al., 2016; Kang et al. 2014) may have contributed to the increase in *S. exigua* fitness in mutant plants.

***Slwak1* is altered in primary metabolism**

While both genotypes have comparable C levels, before and after herbivory, deep physiological phenotyping (Großkinsky et al., 2015) using enzyme activity profiling (Jammer et al., 2022) and sugars quantification in both genotypes revealed important alterations in CH metabolism. Under basal conditions, *Slwak1* has higher glycolytic activity, but lower starch biosynthesis, and lower content of soluble sugars and TCA cycle intermediates than the WT. Plants transform photoassimilates not required for leaf functions into sucrose or amino acids for translocation to sink organs (Osorio et al., 2014). This sugar transport is finely regulated, and sucrose-specific signaling is involved in transport activity (Julius et al., 2017). High sugar concentrations promote the storage of CH and plant growth, while low sugar levels increase the photosynthesis rate and the mobilization of storage compounds (Rolland et al., 2002; Amist and Singh, 2020). Thus, the high glycolytic activity and reduced sugar levels in *Slwak1* leaves suggest that the *Slwak1* mutant plants display a lower energy status.

Furthermore, the carbon skeletons of amino acids are converted into precursors or intermediates of the TCA cycle (Ramon et al., 2008). Thus, low concentrations in *Slwak1* of the components of TCA cycle, malic and succinic acid, are probably related to a lower amino acid concentration than in the WT. Indeed, some tripeptides were more accumulated in WT than in *Slwak1*. Several amino acids serve as signaling molecules themselves and/ or precursors for the synthesis of phytohormones or other secondary metabolites with signaling function (Halkier and Gershenzon, 2006; Hannah et al., 2010; Szabados and Savoure, 2010; Häusler et al., 2014). Thus, the reduction in amino acids may contribute to the reduction of secondary metabolites that we found in *Slwak1* mutants.

Amino acids, sugars and sugar alcohols are the most important primary metabolites whose concentrations are influenced by stress as a result of a complex regulatory network (Krasensky and Jonak, 2012; Arbona et al., 2013). Upon herbivory, plants face increasing energy and carbon demands to support the production of inducible defenses. Once the supply of CH provided by photosynthesis is reduced, the herbivore-attacked tissues have to find other CH and energy sources to produce defense-related metabolites, usually mobilizing resources from reservoir and promoting local catabolism to produce defense-related metabolites (Zhou et al., 2015). Under herbivore attack, CH are mobilized from injured to distal tissues, in a process known as CH sequestration (Orians et al., 2011; Osorio et al., 2014). This strategy reduces energy sources from the leaves, starving the herbivore feeder, and accumulating sugars in sink tissues for a possible future regrowth (Robert et al., 2014; Zhou et al., 2015). Here, we showed that the WT tomato plants reduced the starch content and biosynthesis (PGM and AGPase) and soluble sugars content (sucrose and glucose) under herbivore attack. On the contrary, *Slwak1* plants use the opposite strategy, increasing starch and sucrose content and biosynthesis (PGM, AGPase and *SPS-C*) and sucrose transport (*SUT2*). The starch degradation, induced by stress, increases carbon flux into the hexose phosphate pool (Dong et al., 2019). Therefore, fructose and glucose provide an additional source of energy for wounded tissues and also work as signaling molecules (Rolland et al., 2002; Arnold et al., 2004; Zeeman 2007; Dong et al., 2019). In our study, the *Slwak1* mutant seems to be compromised in the carbohydrate mobilization strategy under *S. exigua* attack. Moreover, WT plants increase the activity of cell wall invertases upon herbivory, while *Slwak1* plants are unable to do it. CwInv plays a crucial role in CH reallocation and sink strength of the tissues (Sturm and Tang, 1999; Schultz et al., 2013). These enzymes, involved in sucrose partitioning, are critical elements for the “sink strength”, influencing the capacity to import assimilates (Roitsch and Gonzales, 2004; Dong et al., 2019). Upon herbivory attack, CwInv activity increases, metabolizing sucrose to hexose and leading to a reduction of long distance sucrose export (Veillet et al., 2016). Thus the increasing activity of this enzyme in WT, but not in the mutant, impairs mutant ability to import CH to fuel the induction of plant defenses.

Conclusion

Overall, we demonstrated that deficiencies in SlWAK1, probably through deficient perception of damage associated signals as OGs, compromises the activation of efficient defense responses against *S. exigua*. Our results show an important alteration of the primary metabolism in the *Slwak1* mutant related to carbohydrate metabolism and partitioning. Plants usually turn local-wounded leaves into sink tissue to reduce the level of energetic compounds and synthetize

secondary metabolites as chemical defenses (Arnold and Schultz, 2002, Steinbrenner 2011; Ferrieri et al., 2012). However, *Slwak1* seems unable to regulate the trade-offs between primary and secondary metabolism, thus showing reduced resistance/tolerance against herbivores. Hence, the results support a role for SlWAK1 in the coordination of tomato defense responses against chewing insects by regulating primary metabolism and the biosynthesis of defensive secondary metabolites. This study reveals that the coordination of defense responses with primary carbohydrate metabolism and assimilate partitioning, with an induction of sink and a repression of source activities, originally observed for infection by microbial pathogens (Ehneß et al., 1997, Berger et al., 2007) is also operational in response to herbivores.

Our research highlights the contribution of SlWAK1, likely through damage perception, to the regulation of the primary metabolism and to activation of a correct defense response against herbivory. Thus, future studies are necessary to elucidate how SlWAK1 can modulate the metabolic source-sink flux to explore the complex network between primary and secondary metabolism in plant defense. This study provides, to the best of our knowledge, the first experimental evidence on the role of WAK1 in the regulation of responses to herbivory.

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SUPPLEMENTARY DATA

TABLE S1. Two-way ANOVA of expression levels of JA, ET, auxin, gibberellin, ABA, phenylpropanoid, alkaloid and flavonoid marker genes. Asterisks show statistically significant differences: +:p < 0.1; *:p < 0.05; **:p < 0.01; ***:p < 0.001.

JA pathway	*LoxD*	*Mc*	*PinII*	*PS*	*LapA*	*Jar*
WT T0	0.009 a	0.0001 a	0.006 a	0.08 a	0.008 a	0.21 a
wak1 T0	0.009 a	0.0002 a	0.005 a	0.11 a	0.02 a	0.29 a
WT T2L	0.12 b	0.5 b	33.5 b	0.27 b	6.12 b	0.65 b
wak1 T2L	0.15 ab	0.27 ab	33.6 b	0.23 b	9.4 b	0.75 b
WT T2S	0.0007 a	0.003 a	0.33 a	0.01 a	0.02 a	0.37 a
wak1 T2S	0.002 a	0.0008 a	0.37 a	0.02 a	0.09 a	0.74 b
Herbivory	**70.5*** **	3.42+	**16.34*** **	**21.7*** **	**19.39*** **	**23.04 *** **
Genotype	0.85	0.31	0	0	0.92	**11.15** **
HxG	0.75	0.31	0	0.5	0.85	2.9+

ET pathway	*ACS1*	*ACS6*	*ACO1*	*ETR3*	*ERF*	*ER21*
WT T0	0.0003 a	0.0001 a	0.08 ab	0.11 a	0.01 a	0.13 bc
wak1 T0	0.0008 a	0.0001 ab	0.27 b	0.10 a	0.01 ab	0.22 bc
WT T2L	0.005 ab	0.0003 ab	0.11 c	0.29 bc	0.05 c	0.13 c
wak1 T2L	0.005 ab	0.0004 ab	0.23 c	0.33 c	0.09 d	0.072 ab
WT T2S	0.009 b	0.0003 ab	0.004 ab	0.22 b	0.04 bc	0.02 a
wak1 T2S	0.005 ab	0.0005 b	0.001 a	0.22 b	0.043 c	0.005 a
Herbivory	3,01+	2.63	**24,39 *** **	**23.68*** **	**17,92*** **	**9,33** **
Genotype	0.41	0.74	0.03	0.17	2.35	3,43+
HxG	0.5	0.16	0.47	0.41	1.45	2,96+

Growth hormones	Auxin			Gibberellin	ABA
	GH3.8	*PILS3*	*PIN3*	*DELLA*	*NCED*
WT T0	0,01 ab	0,005 a	0.006 ab	0,09 ab	0.06 a
wak1 T0	0,01 ab	0,004 a	0.008 b	0,1 ab	0.09 ab
WT T2L	0,03 bc	0,17 c	0.003 a	0,1 ab	0.3 d
wak1 T2L	0,004 a	0,07 ab	0.004 ab	0,05 a	0.23 cd
WT T2S	0.04 c	0.19 c	0.014 c	0.14 b	0.15 abc
wak1 T2S	0.02 ab	0.08 b	0.006 ab	0.08 ab	0.2 bc
Herbivory	**4.18 ***	**17.77*** **	**7.91** **	1.57	**15.4*** **
Genotype	**9.73** **	**13.03** **	1.28	3.08+	0
HxG	2.93+	3.22+	**5***	1.79	2.04

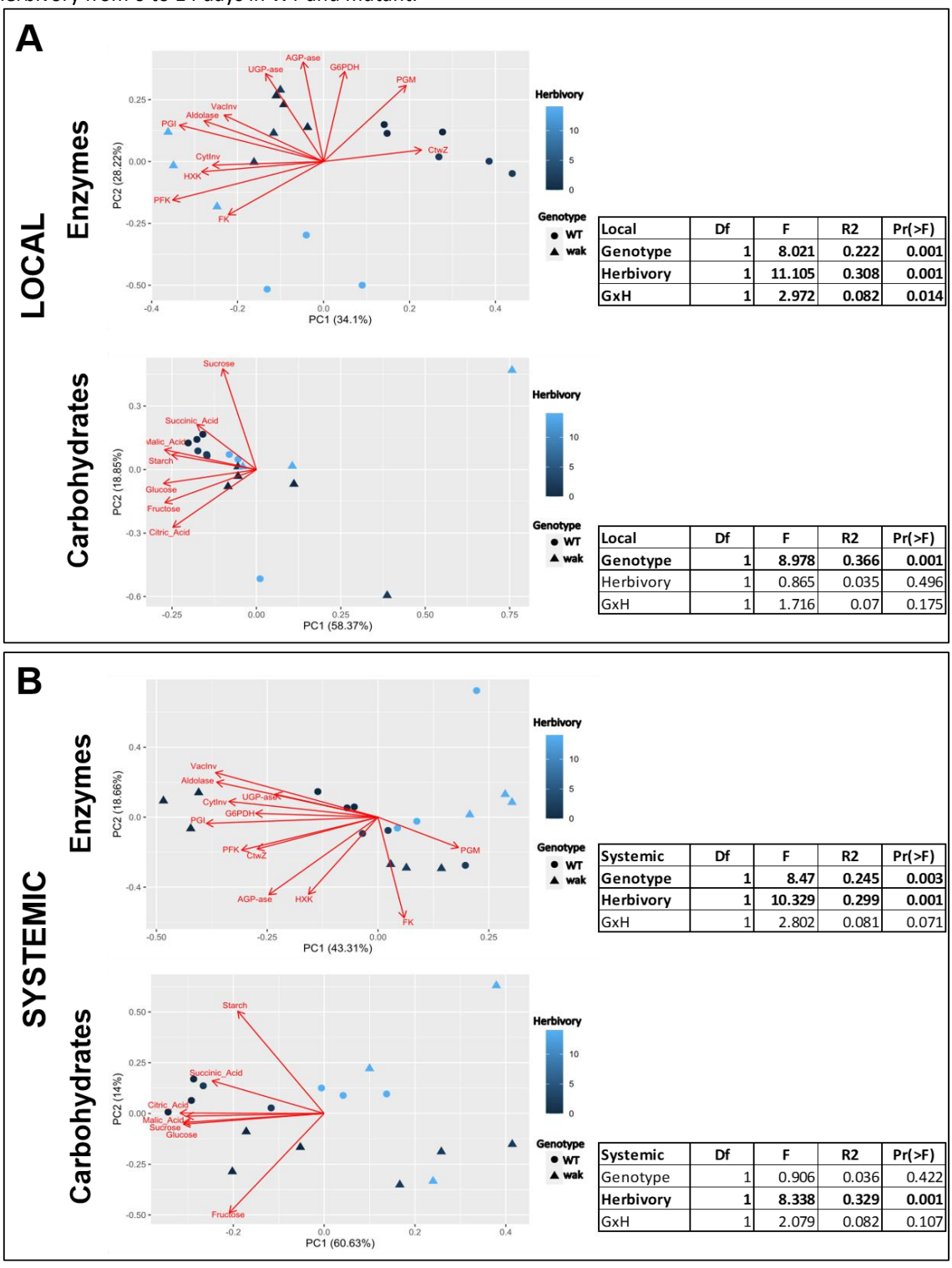
Phenylpropanoid & alkaloid	PAL1-170	MybSJ1	GAME 9
WT T0	3.70E-04 a	1.78E-05 a	3.70E-02 ab
wak1 T0	1.22E-04 a	2.38E-05 a	3.17E-02 ab
WT T2L	2.03E-03 b	9.93E-04 c	2.42E-02 ab
wak1 T2L	6.94E-04 a	5.71E-04 b	7.87E-03 a
WT T2S	7.15E-04 a	5.13E-04 b	8.15E-02 c
wak1 T2S	1.77E-04 a	8.06E-04 b	4.18E-02 b
Herbivory	**10.39** **	**10.92*** **	**8.36** **
Genotype	**11.00** **	0.07	**4.91***
HxG	2.33	2.11	1.1

Flavonoids	CHS1	F3'H	FLS
WT T0	6.26E-04 b	1.04E-02 a	1.71E-01 c
wak1 T0	3.52E-04 ab	9.49E-03 a	1.16E-01 b
WT T2L	5.45E-04 b	1.05E-02 a	1.30E-01 b
wak1 T2L	1.90E-04 a	6.90E-03 a	7.41E-02 a
WT T2S	6.24E-04 b	1.85E-02 b	2.15E-01 d
wak1 T2S	1.59E-04 a	8.21E-03 a	1.37E-01 b
Herbivory	0.71	2.79	**28.74*** **
Genotype	**17.15 *** **	**8.63** **	**59.16*** **
HxG	0.4	2.79 +	0.89

TABLE S2. Pearson correlation of CH enzymatic activity, organic acids and sugar content with the herbivory. Correlation of with enzymatic activity (intensity), organic acids and sugars concentration (mg/g FW) with the time of herbivory (T0, 2, 14 days) in wild type (WT) and mutant (wak1) in local and systemic leaflets. Lyophilized leaf material from non-infested plants (T0), infested leaflets (L, Local response to herbivory) and non-damaged leaflets from infested plants (S, Systemic response) was determined at two and fourteen days upon herbivory (n=3). Data show in the table are r values of correlation. Colors indicate up (red) or down (blue) regulation of enzymatic activities or accumulation of organic acids or sugars. Bold numbers indicate significant differences ($p<0.05$) and the intensity of the color indicate a significant (red or blue) or marginally significant (light red or light blue) correlation.

		LOCAL		SYSTEMIC	
	p value	WT	wak1	WT	wak1
Enzymes	CwInv	-0.237	-0.618	0.116	**-0.818**
	CytInv	0.577	0.541	**-0.795**	-0.511
	VacInv	-0.091	-0.217	-0.69	-0.479
	PGI	-0.175	**0.712**	0.121	**-0.842**
	PGM	**-0.933**	-0.518	0.06	0.106
	FK	**0.704**	0.678	-0.203	-0.075
	UGP-ase	**-0.953**	0.147	0.442	-0.426
	AGP-ase	**-0.958**	-0.693	**-0.804**	**-0.95**
	Aldolase	0.61	-0.604	-0.433	-0.648
	PFK	**0.898**	**0.837**	-0.17	**-0.984**
	G6PDH	**-0.762**	-0.675	-0.624	**-0.899**
	HXK	0.583	0.642	-0.301	-0.363
Organic acids	Citric Acid	0.239	-0.492	-0.599	-0.325
	Malic Acid	-0.575	-0.349	**-0.878**	-0.352
	Succinic Acid	-0.509		**-0.818**	
Sugars	Glucose	**-0.852**	-0.407	**-0.845**	-0.575
	Fructose	0.105	-0.682	**-0.749**	-0.475
	Sucrose	-0.616	0.258	**-0.999**	-0.61
	Starch	**-0.792**	0.065	-0.452	0.302

FIGURE S1. Permanova analysis of carbohydrate enzymatic activity, sugars and organic acids content in leaves of WT or *Slwak1* plants before (T0) or after herbivory (T2 and T14) for 2 or 14 days upon herbivory. A) Analysis of local responses in *S. exigua* attacked leaves, and B) analysis of systemic changes in unwounded leaves. Enzymatic activity (intensity) related to carbohydrate metabolism, sugar and organic acid content (mg/g FW) were determined in wild type (WT) and mutant (wak1) plants. Lyophilized leaf material from non-infested plants (T0), infested leaflets **(A) (Local response to herbivory)** and non-damaged leaflets from infested plants **(B) (Systemic response to herbivory**) was determined at two and fourteen days upon herbivory (T2 and T14). In the figure data represented are the means of 3 replicates. Permutational multivariate analysis of variance (Permanova) biplot built in R software libraries with all CH enzymatic activity and CH content during herbivory attack (blue). Color intensity indicates herbivory from 0 to 14 days in WT and mutant.



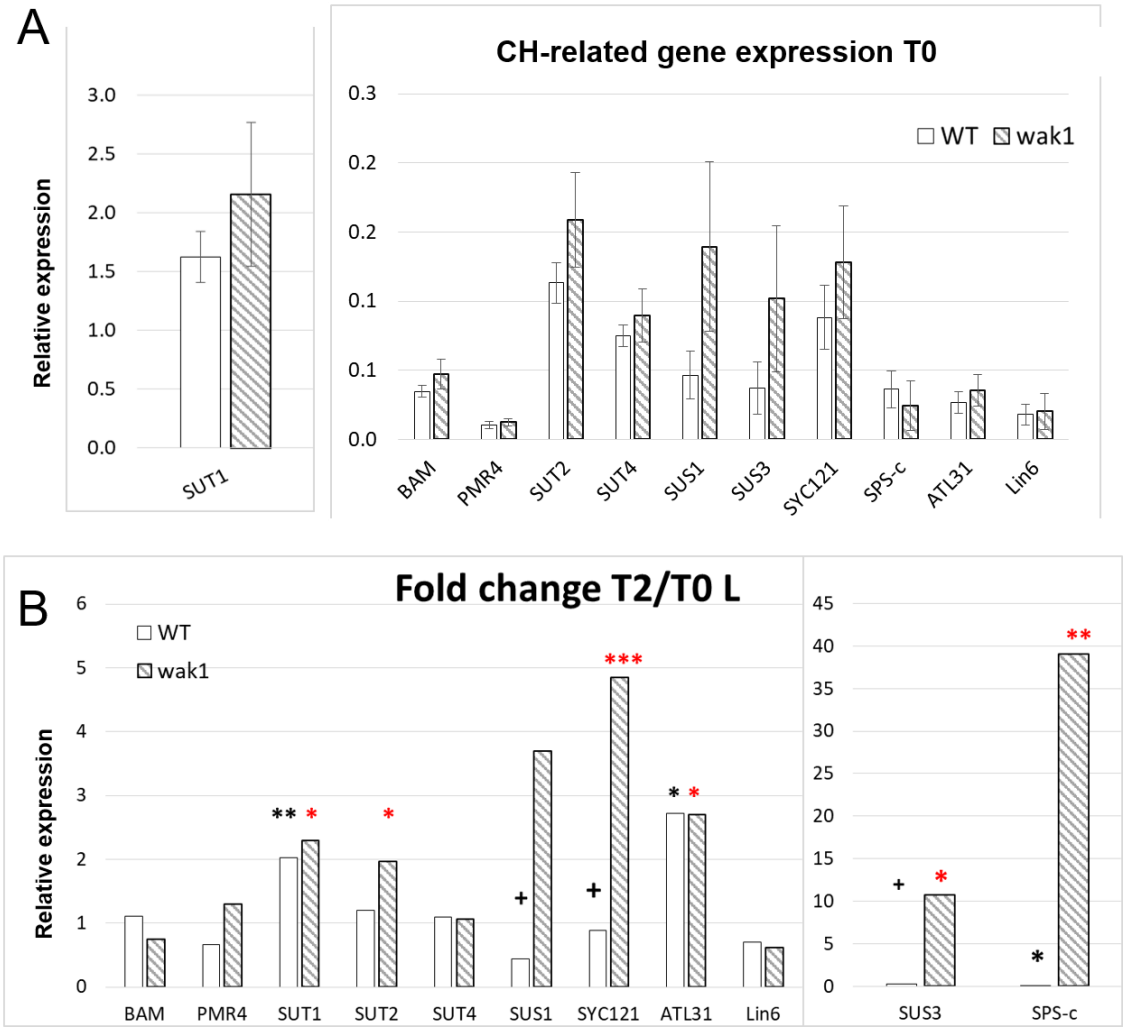
Local	Df	F	R2	Pr(>F)
Genotype	**1**	**8.021**	**0.222**	**0.001**
Herbivory	**1**	**11.105**	**0.308**	**0.001**
GxH	**1**	**2.972**	**0.082**	**0.014**

Local	Df	F	R2	Pr(>F)
Genotype	**1**	**8.978**	**0.366**	**0.001**
Herbivory	1	0.865	0.035	0.496
GxH	1	1.716	0.07	0.175

Systemic	Df	F	R2	Pr(>F)
Genotype	**1**	**8.47**	**0.245**	**0.003**
Herbivory	**1**	**10.329**	**0.299**	**0.001**
GxH	1	2.802	0.081	0.071

Systemic	Df	F	R2	Pr(>F)
Genotype	1	0.906	0.036	0.422
Herbivory	**1**	**8.338**	**0.329**	**0.001**
GxH	1	2.079	0.082	0.107

FIGURE S2. Transcriptional regulation of CH-related genes in WT (white) and wak1 mutant (dashed) before **(A)** and after herbivory **(B). (A)** Expression levels in leaves in absence of herbivory (T0). **(B)** Fold change in transcription levels upon local herbivory in wounded leaves compared to their basal levels (T2L vs T0). Asterisks show statistically significant differences between T0-T2 days of the same genotype (black WT and red wak1) according to t-test. +:p < 0.1; *:p < 0.05; **:p < 0.01; ***:p < 0.001.



Chapter 3

Mycorrhizal colonization finetunes primary metabolism and primes plant defenses upon herbivory in wild type and SlWAK1 deficient tomato

Mycorrhizal colonization finetunes primary metabolism and primes plant defenses upon herbivory in wild type and WAK1 deficient tomato

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) are soil microorganisms that establish mutualistic symbioses with plant roots, usually enhancing plant resilience. The symbiosis increases plant tolerance against different stresses, including enhanced resistance to biotic aggressors. In tomato, Mycorrhiza Induced Resistance (MIR) against the chewing herbivore *Spodoptera exigua* is achieved by priming of plant defenses, including primed accumulation of anti-herbivory proteins and defensive secondary metabolites. Previously, we demonstrated that mutant tomato plants deficient in the Wall Associated Kinase 1 (SlWAK1) are more susceptible to *S. exigua* attack, mostly as a consequence of deficient regulation of carbohydrate metabolism. The role of WAK1 in plant immunity has been shown in different plant species, associated with their role as receptors of oligogalacturonides (OGs). OGs are peptidoglycans fragments and act as damage-associated molecular patterns leading to the activation of defense signaling. We hypothesized that SlWAK1, through a role in damage perception and early signaling of defense responses, is required for MIR. To test this, we used *Slwak1* mutant lines inoculated with two different AMFs - *Funneliformis mosseae* or *Clareidoglomus etunicatum*- and infested with *S. exigua.* Mycorrhizal colonization was not impaired in the mutant. The symbiosis negatively impacted *S. exigua* development and survival in wild-type (WT) but also in *Slwak1* plants, although the reduction in larval biomass associated with AM observed in the WT was lost in the mutant line. Thus, the mutation influences only partially the efficiency of MIR. To address the mechanisms underlying the observed phenotypes, transcriptomic, metabolomics and enzymatic activities analyses were performed. WT and *Slwak1* mycorrhizal plants increased the expression of jasmonic acid marker genes upon herbivory, thus, both genotypes displayed primed JA-dependent defenses upon mycorrhization. Furthermore, the untargeted metabolomic analysis confirmed that *C. etunicatum* modifies the metabolites profile of both tomato genotypes, displaying a primed accumulation of defensive compounds related to alkaloids, phenylpropanoids and lignans upon herbivory. Finally, sugars quantification and profiling of enzymes related to carbohydrate metabolism revealed that mycorrhizal plants increased carbohydrate (CH) reallocation after *S. exigua* attack, and this effect was stronger in the *Slwak1* mutant than in WT plants. Overall, our results provide evidence that mycorrhizal colonization primes plant defense responses against herbivores in WT and *Slwak1* plants, and compensates for the deficient regulation of the primary metabolism in the mutant. The results support that the mycorrhizal symbiosis can buffer the increased susceptibility observed in the *Slwak1* line.

INTRODUCTION

The soil-borne arbuscular mycorrhizal fungi (AMF) are obligate symbionts that colonize the roots of 80% of terrestrial plant species in natural and agricultural systems, improving plant development and plant resistance against biotic and abiotic stresses (Mitra et al., 2021). The nutrient exchange between the plant and AMF is tightly regulated for the correct function of the symbiosis (Smith and Smith, 2011): the fungi found in the host physical support and supply of organic metabolites such as carbohydrates and lipids (Bago et al., 2000; Salmeron-Santiago et al., 2021) and in return, the symbiosis improves plant nutrient uptake, growth, and tolerance to multiple stresses (Chen et al., 2018; Mitra et al., 2021). Thus, the symbiosis requires additional plant energy and alters carbon (C) partitioning. Indeed, the symbiosis acts as an additional carbon sink in the root, using up to 20% of the total carbon photoassimilates (Valentine et al., 2013). Thus, AMF colonization modifies C distribution in the plant altering the biosynthesis, transport, and catabolism of sucrose (Salmeron-Santiago et al., 2021).

Balancing the costs and benefits of AMF-plant symbiosis is essential for cooperation. Multiple reports have demonstrated that mycorrhizal plants activate faster and stronger defense responses under herbivore or pathogen attacks than non-mycorrhizal plants (Jung et al., 2012; Rivero et al., 2018; Sánchez-Bel et al., 2016; Shafiei et al., 2022). This phenomenon, known as Mycorrhizal-Induced Resistance (MIR), is associated with defense priming, a preconditioning plant tissues for more efficient immunity responses through various physiological, transcriptional and metabolic changes (Pozo & Azcón-Aguilar, 2007; Jung et al., 2012; Pieterse et al., 2014). MIR is functional against below- and above-ground attackers, reducing the spread of pathogens and herbivore performance on plants. The lifestyle of the insect influences MIR, and the reduced performance of chewing insects in AM plants has been demonstrated in several systems defenses (Song et al., 2013; Pozo et al., 2020; Rivero et al., 2021; Selvaraj and Thangavel, 2020). For example, the caterpillar *Helicoverpa armigera* showed reduced fitness when feeding on mycorrhizal tomato plants due to a stronger induction of jasmonic acid (JA) signaling priming plant systemic defenses (Song et al., 2013). This hormone is a crucial regulator of plant defenses against chewing herbivores (Erb and Reymond, 2019; Gruden et al., 2020), and primed activation of the JA signaling pathway is a primary molecular mechanism in MIR (Pozo et al., 2010). Upon herbivory, the induction of several toxic metabolites, like alkaloids or antifeedant proteins, occurs in a JA-dependent manner (Wasternack and Hause, 2013; Erb and Reymond, 2019), and the dependence of MIR on JA

signaling has been confirmed (Song et al., 2013; Mora-Romero et al., 2014). Still, the molecular mechanisms shaping the final plant response to the herbivore are to be elucidated.

Only few studies have addressed the changes in the complete metabolomics profile in mycorrhizal plants, and rarely under herbivory. Schweiger et al. (2014) found basal changes in the metabolic profile of leaves related to mycorrhizal colonization in different plant species, in some cases potentially involved in direct plant defense against generalist herbivores as a strong accumulation of the iridoid glucoside catapol (Bowers, 1988; Gange et al., 1994). Rivero et al. (2021) demonstrated that tomato plants colonized by *F. mosseae* showed a primed accumulation of alkaloids, fatty acid derivatives and phenylpropanoid-polyamine conjugates in response to the herbivore *S. exigua,* reducing its performance. They confirmed the toxic effect of specific compounds such as the alkaloid physostigmine, the fatty acid derivatives 4-oxododecanedioic acid and azelaic acid on larval performance highlighting that AMF primed the reprogramming of leaf metabolomic profile under herbivory.

Plants need to reprogram their metabolism in function of the extensive damage inflicted by chewing insects. These herbivores cause mechanical damage, break plant cells and modify the apoplast releasing cell wall fragments (Erb and Reymond, 2019). Intercellular molecules and cell wall fragments, collectively called Damage Associated Molecular Patterns (DAMPs), are released into the surrounding apoplastic space and perceived by adjacent intact cells, triggering an alarm state in the plant (Tanaka and Heil, 2021). The small molecules oligogalacturonides (OGs) are among the best-characterized plant DAMPs. OGs are linear homopolymers of α-1,4-linked D-galacturonic acid that derive from homogalacturonan fragmentation, the major polysaccharide component of cell-wall pectins (Ferrari et al., 2013; Hou et al., 2019). OGs recognition triggers plant defense responses, in particular the activation of the JA signaling pathway as shown in Arabidopsis (Howlander et al., 2020) and tomato (Gamir et al., 2020). Plant cells sense OGs through the extracellular membrane-bound receptor, and the Wall-Associated Kinase 1 (WAK1) receptor was confirmed as the main OG receptor in Arabidopsis (Brutus et al., 2010; De Lorenzo et al., 2011). In tomato, WAK is a multigenic family with 29 members, 11 WAK and 18 WAK-like proteins (Sun et al., 2020). Kurt et al. (2020) reported by a docking analysis, that SlWAK1 has a high affinity to bind oligomeric pectin. *WAK1* expression levels increase during biotic and abiotic stresses in tomato (Zhang et al., 2020; Meco et al., 2020, Chapter 2), and its contribution to the regulation of plant defense responses to herbivory has been recently shown (Dejana et al., 2022, Chapter 2).

During an herbivore attack, plants actively reduce photosynthetic rates to optimize their defenses (Reymond et al., 2004; Lawrence et al., 2008). It is demonstrated that the JA signaling pathway is required for photosynthesis repression (Wasternack and Hause, 2013; Attaran et al., 2014). Thus, the photosynthesis reduction is an active plant response that results in a direct decrease of carbohydrate supply and consequently energy for the herbivores. However, plants increase the energetic and carbon demands to activate the plant immune system, requiring the reallocation of primary metabolites to support the production of inducible defenses (Zhou et al., 2015). Thus, upon herbivore attack, several plants promote local catabolism of energy storage compounds as sucrose or starch at the site of insect feeding and/or a systemic carbon reallocation at the site of undamaged tissues (Schwachtje et al., 2006; Zhou et al., 2015). Invertase enzymes play a crucial role in carbohydrate partitioning. They include cytosolic invertases, localized in the cytosol where the glycolytic pathway takes place; the acidic or vacuolar invertases, located in the vacuole, where sucrose is stored; the cell wall-bound invertases, located in the apoplast of sink tissues, where sucrose is unloaded, contributing to transport from source to sink (Roitsch and Gonzalez, 2004) and, thus, controlling the differential sink strength of plant tissues (Schultz et al., 2013). These enzymes break down sucrose into the hexoses glucose and fructose that, in turn, act as signaling molecules (Schwachtje et al., 2008; Cho and Yoo, 2011) along with the jasmonates during induction of plant defenses (Tauzin and Giardina, 2014). Glucose acts as a signaling molecule in the hexokinase regulatory pathway, and fructose seems to be involved in ABA signaling and ethylene response (Tauzin and Giardina, 2014; Horacio and Martinez-Noel, 2013; Cho et al., 2009). The invertases activity increase in injured leaves (Appel et al., 2014; Castrillòn-Arbelàez et al., 2012) leading to a decrease of sugars in local leaves to re-allocate carbohydrates in systemic tissues (Kundu et al., 2018) and incorporating them into defensive compounds (Ferrieri et al., 2012). Thus, the complex plant-microbe-insect interaction depends on the efficient perception among the parties to fine-tune the plant response to the different interactions. In general, the plant responses in three-way interactions (plant-microbe-arthropod) are more complex than the sum of independent responses in the two-way (plant-microbe or plant-arthropod) ones (Gruden et al., 2020). Understanding the molecular mechanisms linking the perception of the aggressor and the mounting of MIR is a hot spot in research that may contribute to improving the application of mycorrhizal inoculants for crop protection.

In the previous Chapter, we showed that the tomato gene *SlWAK1* contributes to the proper activation of plant defense responses against the chewing herbivore *Spodoptera exigua*.

Here, we hypothesize that the *SlWAK1* gene is required for full MIR. To test this, we evaluated *S. exigua* performance and tomato defense responses in wild-type (WT) tomato (*S. Lycopersicum* cv *Moneymaker*) and the *Slwak1* mutant inoculated or not with the AMFs *Funneliformis mosseae* or *Clareidoglomus etunicatum*. *S. exigua* biomass was lower when feeding in mycorrhizal WT plants, but not in the *Slwak1* mutant. However, larval fitness survival and the percentage of individuals reaching pupa and moth stages were reduced when feeding on mycorrhizal plants of both WT and *Slwak1*; thus, SlWAK1 was only partially required for MIR. Transcriptomic analysis showed that MIR is associated with primed jasmonic acid-dependent defenses in both genotypes, and the metabolic profiling confirmed a higher accumulation of some specific defensive secondary metabolites in AM plants upon attack. Finally, we found that the profound impairment in the primary metabolism of *Slwak1* mutant plants, shown in the previous Chapter, is partially reversed by mycorrhizal colonization. The results highlight that the defense priming associated with MIR is mostly independent of SlWAK1, and that the mycorrhizal colonization is able to buffer the deregulation of the primary metabolism in the mutant lines, partially contrasting its negative effects on herbivore resistance.

MATERIAL AND METHODS

Fungal material, plant and growing conditions

Arbuscular mycorrhizal fungi (AMF) isolates of *Funneliformis mosseae* (BEG12) obtained from the International Bank of Glomeromycota (http://www.i-beg.eu) and *Claroideoglomus etunicatum* (EEZ163) isolated from a highly salinized soil from Cabo de Gata Natural Park (Almeria, Spain) (Estrada et al., 2013), were maintained in open-pot cultures of *Trifolium repens* mixed with *Sorghum vulgare* plants in the greenhouse. The AMF inoculum consists of the soil of those cultures including root fragments, mycelia and spores.

Tomato (*S. lycopersicum L.*) cv Moneymaker wild type (WT) and *Slwak1* mutant plants, with a single T-DNA insertion localized in the promoter region of *SlWAK1* (Solyc09g014720) were used (Meco et al., 2020). Tomato seeds were surface disinfected by immersion in 4% NaClO (10 min), rinsed thoroughly with sterile water and germinated for 10 days in an open container with sterile vermiculite at 25°C. Plantlets were then transferred to 300 mL pots containing sterile sand:vermiculite (1:1) mixture. Pots for mycorrhizal treatments were inoculated by adding 10% (v/v) *F. mosseae* or *C. etunicatum* inoculum. All plants received a 3 ml aliquot of a filtrate (<20 μm) of inoculum, in order to provide the general microbial population but free of AMF propagules also to non-inoculated plants.

Thus, the experiment consisted of a fully randomized design with two genotypes (WT, *Slwak1*) and 3 mycorrhizal treatments: (Non mycorrhiza -Nm-, mycorrhizal plants colonized by *F. mosseae* -Fm- and mycorrhizal plants colonized by *C. etunicatum* -Ce-. Plants were randomly distributed in a climatic controlled greenhouse and grown at 24/16°C with a 16/8 h photoperiod and 70% humidity. They were fertilized twice a week with half-strength Hewitt nutrient solution (Hewitt, 1966) modified to a 0.7 mM P content. Water was supplied as needed.

Herbivore rearing and herbivory bioassays

Spodoptera exigua (Lepidoptera: Noctuidae) eggs were provided by Dr. S. Herrero's lab (ERI-BIOTECMED, Universitat de Valencia, Spain). Once hutching, larvae were reared on artificial diet (Greene et al., 1976) at 25°C with 16:8h light:dark regime and 70% relative humidity.

5 weeks after mycorhizal inoculation, *Slwak1* and WT tomato plants (n=15 per treatment) were infested with the generalist herbivore *Spodoptera exigua*. For each plant, two L2-L3 stage larvae per plant were placed in a leaflet from the fourth true leaf using clip-cages

(one for each larva to avoid cannibalism) to limit the feeding area and avoid their escape. Clip-cages were moved into new leaflets to make sure they always had food available. To monitor insect performance, larval weight, survival, pupation and moths were monitored for 3 weeks. Survival and pupation were counted every two days, larval weight was determined after 10 days and larvae reaching to moth stage were counted at the final time point.

Leaflet samples were collected before and during herbivory (T0, 2, 14 and 21 dpi). During the infestation (T2 and 14 dpi), leaflets where *S. exigua* directly fed (Local, L) and non-damaged leaflets of the same leaf (Systemic, S) were separately harvested. Two leaflets per plant and timepoint were collected, frozen in liquid nitrogen, and stored at -80°C for further molecular analysis. Three weeks post infestation, both shoots and roots were harvested, shoot and root fresh weight determined, and samples stored at -80 for nutrient content determination and molecular analyses (Figure S1).

Determination of Mycorrhizal Colonization

After the final harvest, eight weeks post-inoculation, tomato roots were washed and an aliquot of the roots was sampled to determine mycorrhizal colonization. Roots were cleared in KOH (10%) and fungal structures were stained with 5% ink in 2% acetic acid (Garcia et al., 2020). The extent of mycorrhizal colonization (expressed as a percentage of total root length colonized by the AMF) was estimated according to the gridline intersection method (Giovannetti & Mosse, 1980) using a BOECO zoom stereo microscope (Model BST-606).

Determination of mineral nutrient

Nutrient analyses were performed at the Technical Services of the Estación Experimental del Zaidín (CSIC). Frozen leaves or roots were ground and lyophilized, and 100 mg aliquot of dry tissue was used per sample. Total P and micronutrient (Al, Ca, Cr, Cu, Fe, K, Li, Mg, Mn, Na, S, Si, Sr, Ti, Zn) concentrations were determined after acid digestion of samples, by inductively coupled plasma optical emission spectrometry (ICP-OES; Varian ICP 720-ES). Total C and N contents were analyzed using an Elemental Analyzer (LECO TruSpec CN), according to standard procedures. Five independent biological replicates were analyzed per treatment.

Analysis of Gene Expression by qPCR

The expression of marker genes for different metabolic pathways was analyzed by qPCR. Total RNA from tomato leaves was extracted and treated with DNase using the Direct-

zol RNA MiniPrep kit (Zymo Research). Subsequently, the RNA was purified through a column using the RNA Clean and Concentrator-5 kit (Zymo Research), checked for integrity and quantified using a NanoDrop 2000 (ThermoFisher). The first-strand cDNA was synthesized with 1 µg of purified total RNA using the iScript cDNA Synthesis kit (Bio-Rad). All kits were used according to the manufacturer's suggested protocols. Four independent biological replicates were analyzed per treatment.

The expression levels of three different housekeeping genes, actin (Solyc03g078400), elongation factor 1-α (Solyc06g005060) and β-tubulin (Solyc04g081490) were determined, and the Normfinder software (https://moma.dk/normfinder-software) was used to determine the best normalization gene for the set of samples (Andersen et al., 2004). According to the results, expression values of the target genes were normalized using the housekeeping gene actin, and relative quantification of specific mRNA levels was performed using the comparative 2–Δ (Δ*Ct*) method (Livak & Schmittgen, 2001).

Untargeted metabolomic profiling by liquid chromatography and electrospray ionization (LC-ESI) mass spectrometry (Q-TOF instrument)

Lyophilized freeze-dried leaves (30 mg) were homogenized in 1 ml of MeOH:H2O (10:90) containing 0.01% of HCOOH. The homogenate was centrifuged at 15 000 *g* for 15 min at 4 °C, and the supernatant was recollected and filtered through 0.2 µm cellulose filters (regenerated cellulose filter, 0.20 µm, 13 mmD pk/100; Teknokroma, St Cugat, Spain). Subsequently, 20µl of the filtered supernatant was injected into an Acquity ultra-performance liquid chromatography system (UPLC; Waters, Mildford, MA, USA), which was interfaced with a hybrid quadrupole time-of-flight equipment (Q-TOF-MS Premier, Waters, Mildford, MA, USA). An aqueous methanol gradient containing 0.01% HCOOH was used for elution. Solvent gradients and other chromatographic conditions were performed as previously described (Gamir et al., 2020). Six biological replicates were randomly injected for each treatment.

LC separation was performed using ultra-performance liquid chromatography (UPLC) Kinetex C18 analytical column with a 5 µm particle size, 2.1 x 100 mm (Phenomenex, Madrid, Spain). For the correct and accurate identification of the detected signals, a second fragmentation function was introduced into the TOF analyser. This function was programmed in a t-wave ranging from 5 to 45 eV to obtain a fragmentation spectrum of each analyte (Gamir et al., 2014).

To identify metabolites, we used the *S. lycopersicum* KEGG and internal databases and we follow at least two of these three criteria: matching the exact mass; matching the retention time between the internal library and experimental samples; and contrasting the obtained fragmentation spectrum with those available in the Massbank and/or Metlin (www.massbank.jp; www.masspec.scripps.edu).

Full scan data analysis

Data were acquired in centroid mode and subsequently transformed into .cdf files using the Databridge from MassLynx 4.1 software (MassLynx 4.1, Waters, USA). Chromatographic signals were processed using the software *R* for statistical purposes. Signals from positive and negative electrospray ionization (ESI+; ESI-) were processed separately. Peak peaking, grouping and signal corrections were performed using the XCMS algorithm (Smith et al., 2006). Metabolite were analysed based on normalized peak area units relative to the dry weight. Adduct and isotope correction were performed by using the MarVis Suit 2.0 software tool (Kaever et al., 2015). The same software was used for testing differential production of across genotypes and herbivory via Kruskal–Wallis test (p<0.01). To determine a global behavior of the signals, principal component analyzes (PCA), sparse partial least-squares discriminant analysis (sPLSDA) and heat map plots were generated using MetaboAnalyst 4.0 software (www.metaboanalyst.ca), a comprehensive web-based package for a range of metabolomics applications (Chong et al., 2019).

For compound identification, at least two of the following three criteria were required: matching the exact mass; matching the retention time between the internal library and experimental samples; and coincident fragmentation spectrum with those available in the Massbank and/or Metlin (www.massbank.jp; www.masspec.scripps.edu).

Quantification of soluble carbohydrates and starch

The extraction of sugars from leaves and roots was performed as described in De Rocchis et al. (2022). Ground frozen plant material was suspended in ultrapure water and 5 mM mannitol as internal standard was added, and centrifuged at 19,000 g for 10 min at 4 °C. The supernatant was transferred to a new tube, heated at 99 °C for 3 min to stop enzymatic activities, and centrifuged again at 19,000 g for 10 min at 4 °C. The supernatant was used for the analyses of glucose, fructose, and sucrose, while the pellets of the two centrifugation steps containing the starch were pooled, resuspended in a total volume of 1 mL containing 0.5 mM

mannitol, and treated with 1.5 U of amyloglucosidase (Sigma-Aldrich, Saint Louis, USA) for 2 h at 37 °C. After two centrifugation steps at 19,000 g for 10 min at 4 °C with a 3 min heating step at 99 °C in between, the final supernatant was used for starch analysis. Starch and sugars were quantified by HPLC DIONEX ICS 3000 (Thermo Fisher Scientific, Waltham, USA) with a CarboPacTMPA200 AnalyticI (3 × 150 mm) column (Thermo Fisher Scientific, Waltham, USA).

Enzymatic activity assay

Semi-high throughput profiling was used for the assessment of the activity of 12 key enzymes of carbohydrate metabolism (Jammer et al, 2022). Both roots and shoots frozen material (~300 mg fresh weight) were used for extractions, according to Jammer et al. (2015) with few adjustments, in order to analyze the activity of 12 key enzymes of primary carbohydrate metabolism: cell wall invertase (CwInv), vacuolar invertase (VacInv), cytoplasmic invertase (CytInv), fructokinase (FK), hexokinase (HXK), phosphoglucoisomerase (PGI), phosphofructokinase (PFK), aldolase (Ald), ADP-glucose pyrophosphorylase (AGPase), phosphoglucomutase (PGM), UDP-glucose pyrophosphorylase (UGPase) and glucose-6-phosphate dehydrogenase (G6PDH).

Briefly, ground material was homogenated with PVPP and was extracted with 1 ml extraction buffer (40 mM TRIS-HCl pH 7.6, 1 mM EDTA, 0.1 mM PMSF, 1 mM benzamidine, 14 mM β-mercaptoethanol, 24 µM NADP) and cell wall-bound proteins were extracted from the remaining pellet with a high-salt buffer (1 mM NaCl, 40 mM Tris–HCl, pH 7.6, 3 mM MgCl2, 1 mM EDTA).

Activities were determined photometrically by kinetic assays in a 96-well plate format as described in Jammer et al., (2015). The results were normalized to the protein contents determined according to the Bradford method using BSA as a standard protein (Bradford, 1976).

Statistical analyses

All statistical analyses (one- and two-way ANOVA, LSD test, PERMANOVA, exact binomial test and t-test applied when appropriate, as indicated in the corresponding figure legends) were conducted using STATGRAPHICS PLUS 3.1 (Rockville, MD, USA), *R* software v.3.5.2 (R Development Core Team) and the XCMS package. For analysis of the metabolomics data, data were normalized by sum, transformed by cube root, and scaled by Pareto method in order to obtain the sparse partial least squares discriminant analysis (sPLSDA) and heatmap

plots, which were generated using MetaboAnalyst 4.0 software (www.metaboanalyst.ca), a comprehensive web-based package for a range of metabolomics applications (Chong et al., 2019).

RESULTS

Arbuscular mycorrhizal symbiosis reduces the fitness of *Spodoptera exigua*

To determine whether SlWAK1 contributes to MIR in tomato, we compared *S. exigua* performance when feeding in non-mycorrhizal (Nm) plants or plants colonized by *Funneliformis mosseae* or *Claroideoglomus etunicatum* (Fm or Ce, respectively) both in the wild type (WT) and the *Slwak1* mutant. Two larvae per plant were allowed to feed for 3 weeks in fully developed leaves, and their performance was evaluated by determining larval weight, mortality, and percentage of individuals reaching the pupae or moth stage.

In WT plants, larvae fed in AM showed significantly lower biomass than those fed in Nm (LSD test, $p<0.05$). In contrast, larval biomass was not different in AM and Nm plants when feeding in the *Slwak1* mutant (Figure 1A). Thus, the negative effect of mycorrhization on larval biomass was lost in the mutant. However, AMF establishment had a negative effect on *S. exigua* in both genotypes in terms of mortality and development, with Ce showing stronger effects (Figure 1B, C, D).

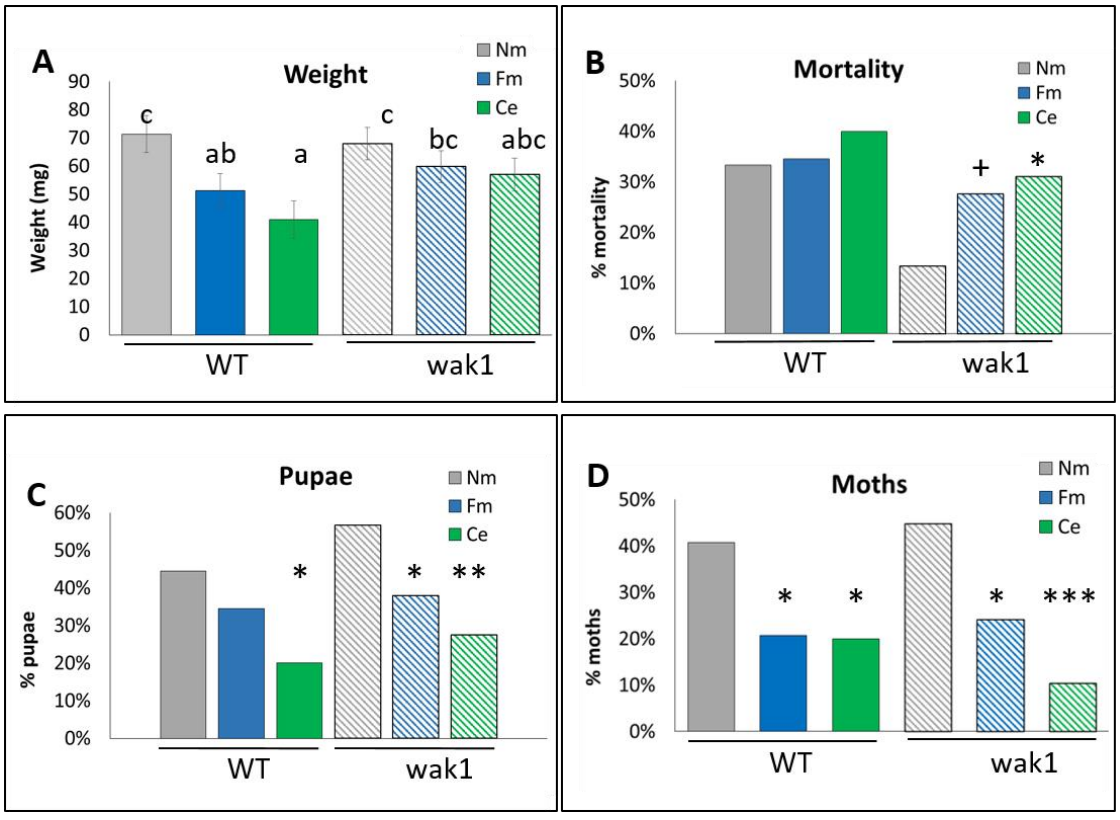

FIGURE 1. Arbuscular mycorrhizal symbiosis reduces the fitness of *Spodoptera exigua* in WT and *Slwak1.* Tomato wild type (WT; filled bars) and *Slwak1* (wak1; stripped bars) plants (n=15 per treatment) non-colonized (Nm; grey) or colonized by *Funneliformis mosseae* (Fm; blue) or *Clareidoglomus etunicatum* (Ce; green) were infested with second instar *S. exigua* larvae (two per plant, each one in a separate clip-cage). **(A)** Weight of *S. exigua* 10 days after plant infestation. **(B)** Percentage of *S. exigua* mortality 18 days after plant infestation. **(C)** Percentage of larvae reaching pupae stage 18 days after plant infestation. **(D)** Percentage of larvae reaching moth state 8 weeks after

infestation. For **(A)**, data not sharing a letter in common differ significantly according to Fisher's Least Significant Difference (LSD) test ($p<0.05$). For **(B)**, **(C)** and **(D)** statistical analyses were done separately for WT and *Slwak1* and asterisks indicate significant differences according to the exact binomial test; +:p < 0.1; *:p < 0.05; **:p < 0.01; ***:p < 0.001.

Herbivore mortality was only slightly higher in Ce plants than in the Nm WT, but the mortality in mycorrhizal plants was up to 2-fold higher in AM than in Nm plants in the *Slwak1* mutant (Figure 1B). Regarding larval development, the percentage of individuals reaching the pupae stage was lower in mycorrhizal plants in both genotypes, with a significant reduction in Ce of both genotypes and in Fm of *Slwak1* plants (Figure 1C). Finally, mycorrhizal colonization significantly reduced the number of larvae reaching the moth stage as compared to those fed on Nm in the WT. The reduction was higher in the mutant, down to 10% for those feeding in Ce-inoculated *Slwak1* plants (Fig 1D). Thus, mycorrhizal colonization reduced the survival and development of *S. exigua* in both genotypes.

Mycorrhizal colonization promotes plant growth and changes nutrients content in both genotypes

We check whether the worst *S. exigua* performance in mycorrhizal plants could be related to changes in the nutritional value of the plants, evaluating plant biomass at the final harvest (T21) and nutrient content before (T0) and after herbivory (T21). First, we analyzed the mycorrhizal status of the plants (Table S1). The absence of fungal structures was confirmed in the roots of the Nm controls. The staining of fungal structures confirmed a well-established mycorrhizal symbiosis in both AMF treatments with well-developed arbuscules. The plants inoculated with Ce showed higher colonization levels than Fm in both genotypes, although differences were higher in the mutant since *Slwak1* roots displayed a reduction in mycorrhizal levels for Fm (LSD, $p<0.05$). The symbiosis had a significant growth promotion effect on shoots of both WT and *Slwak1* plants, with a higher growth promotion achieved by Ce than by Fm (Table S1). Root biomass was not affected by mycorrhization in the WT, but it increased in mycorrhizal mutant plants. As a result, the shoot-to-root ratio significantly increased in mycorrhizal plants in the WT, but not in the mutant (Table S1).

Regarding nutrient content, C and N levels were not affected by mycorrhization in WT nor *Slwak1* leaves or roots (except for an increase of C in Ce roots). In contrast, mycorrhizal colonization significantly impacted the concentration of several micronutrients as Ca, Cu, K, Li, Mg, Mn, Sr, and Zn in leaves and roots, but the changes followed similar trends in both genotypes, especially after herbivory (Table S2). In the absence of herbivory, basal levels of K,

Mn, Str were elevated by the AM treatment only in the WT, while Ca and Ni were reduced by mycorrhiza in the mutant. Thus, mycorrhizal colonization promotes plant growth (increasing plant biomass and modifying nutrient levels), regardless of the genotypes.

Mycorrhizal-induced priming of plant defenses in both WT and *Slwak1*

To find potential differences in plant defenses, we analyzed transcriptional regulation of plant defense responses in tomato leaves before (T0) and after two days of herbivory (T2L, local response). In the previous study, we found that the highest susceptibility of *Slwak1* to *S. exigua* was independent of **JA signaling**. Here, we showed that the expression of the two defense-related JA-responsive genes coding for the Proteinase Inhibitor II (*PinII*) and Multicystatin (*MC*) and the JA-biosynthesis gene Lipoxygenase D (*LoxD*) was induced by herbivory and showed moderately increased induction in AM than in Nm plants in both genotypes, WT and *Slwak1* (significant for *PinII*, LSD test, $p<0.05$) (Figure 2A).

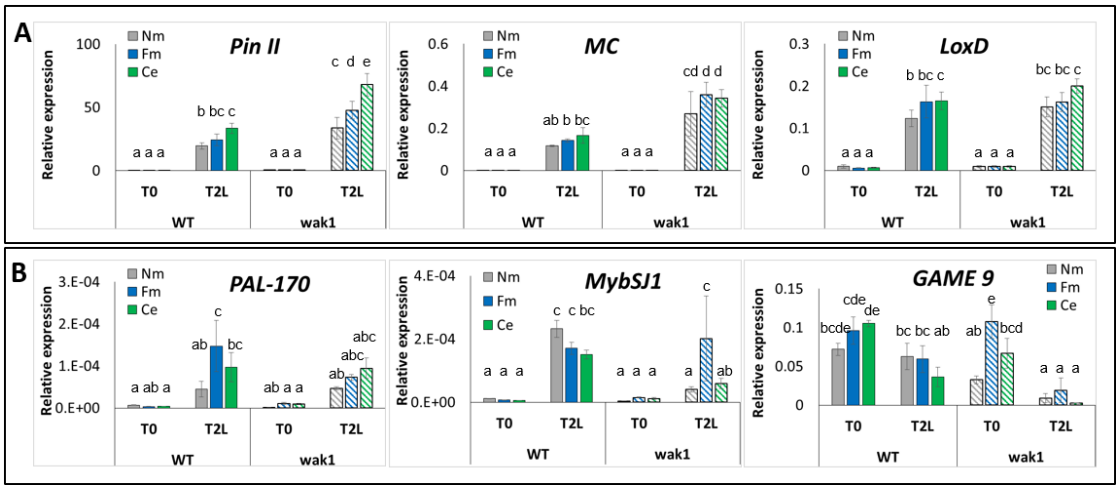

FIGURE 2. AM induces priming of plant defenses in both *Slwak1* and WT. Transcriptional regulation of defense-related genes. Gene expression analysis by real-time qPCR was determined in wild type (filled bars, WT) and mutant *Slwak1* (stripped bars, wak1) leaves before (T0) and after 2 days of local herbivory (T2L) in non-mycorrhizal (Nm, grey) and mycorrhizal (Fm, blue or Ce, green) plants. Expression values are means of four biological replicates, normalized to the housekeeping gene actin. **(A)** Jasmonate-regulated defense-related genes coding for proteinase inhibitor II (*PinII*), multicystatin (*MC*), and Lipoxygenase (*LoxD*). **(B)** Phenylpropanoid and alkaloid-related marker genes coding for phenylalanine ammonia-lyase (*PAL1-170*), transcription factor (*MybJS1*), key regulator of phenylpropanoid-related genes and glycoalkaloid metabolism 9 (*GAME 9*), key for the biosynthesis of steroidal alkaloids and isoprenoids. Data not sharing a letter in common differ significantly according to ANOVA, Fisher's Least Significant Difference (LSD) test ($p<0.05$).

In the previous Chapter, we found that the regulation of phenylpropanoid and alkaloid pathways was higher in WT than in *Slwak1* plants. Here, we found that the gene coding for the phenylalanine ammonia-lyase (*PAL1-170*) increased in both genotypes under herbivory, and the induction was higher in AM plants than in Nm (significant for Fm WT, LSD, $p<0.05$; Figure

2B). The expression of the transcription factor MybJS1 (*MybJS1*) -a key regulator of phenylpropanoid-related genes- was also induced upon herbivory in WT regardless of AM symbiosis, while in S*lwak1* plants the increase was only significant in Fm under herbivory (LSD, $p<0.05$; Figure 2B). Surprisingly, the glycoalkaloid biosynthetic gene 9 (*GAME9*) -key for the synthesis of steroidal alkaloids and isoprenoids- tends to be upregulated in AM compared to Nm plants at basal level (T0) (significant for Fm *Slwak1*; LSD, $p<0.05$). On the contrary, *GAME9* was downregulated under herbivory (T2L) in both genotypes, although the reduction was only significant in AM plants (significant for Fm and Ce in *Slwak1*; LSD, $p<0.05$ and only Ce in WT, Figure 2B).

Regarding flavonoid biosynthetic genes, chalcone isomerase 1 (*CHI1*), flavonoid-3'-hydroxylase (*F3'H*) and flavonol synthase (*FLS*) were downregulated by mycorrhiza regardless of herbivory, but intriguingly they were not regulated by mycorrhiza in the mutant, while the effect of herbivory -induction of *CHI1*, reduction of *FLS*- was maintained in both genotypes (Figure S1). Although there is not a clear pattern in the regulation of phenylpropanoid, alkaloid and flavonoid pathways, the AM symbiosis induces priming of plant defenses JA-related in both WT and *Slwak1.*

Herbivory changes metabolic profile in both genotypes. Mycorrhizal colonization, mainly *C. etunicatum,* induces more defensive metabolites than non-mycorrhizal plants

Since *S. exigua* fitness was negatively impacted by mycorrhization in both genotypes, WT and *Slwak1*, we hypothesized that mycorrhizal plants had higher levels of secondary metabolites related to defense. To test this, we performed an untargeted metabolomic analysis to compare leaves of non-mycorrhizal (Nm) and mycorrhizal plants colonized by any of the AMF (Fm and Ce plants) before herbivory (T0) or after two days of *S. exigua* feeding (T2L, local response). The sparse Partial Least Square-Discriminant analysis (sPLSDA) showed that the metabolic profile of WT plants was similar between Nm and AM plants in the absence of stress.

However, herbivory had a differential impact on the different treatments: while the metabolic profile of herbivore-attacked Nm and Fm plants overlapped, the profile differed in herbivore-attacked Ce-colonized plants. On the contrary, mycorrhizal colonization impacted the metabolic profile of *Slwak1* also in the absence of herbivory, since the loading plots of mycorrhizal *Slwak1* plants differ from Nm plants before and after herbivory (Figure 3A). The clustering in the heatmap analysis pointed that the greatest difference between treatments was due to herbivory, clearly separating the metabolome into two main clusters: before (T0) and after herbivory (T2L) (blue branch, Figure 3B). Notably, regardless of the herbivory, there

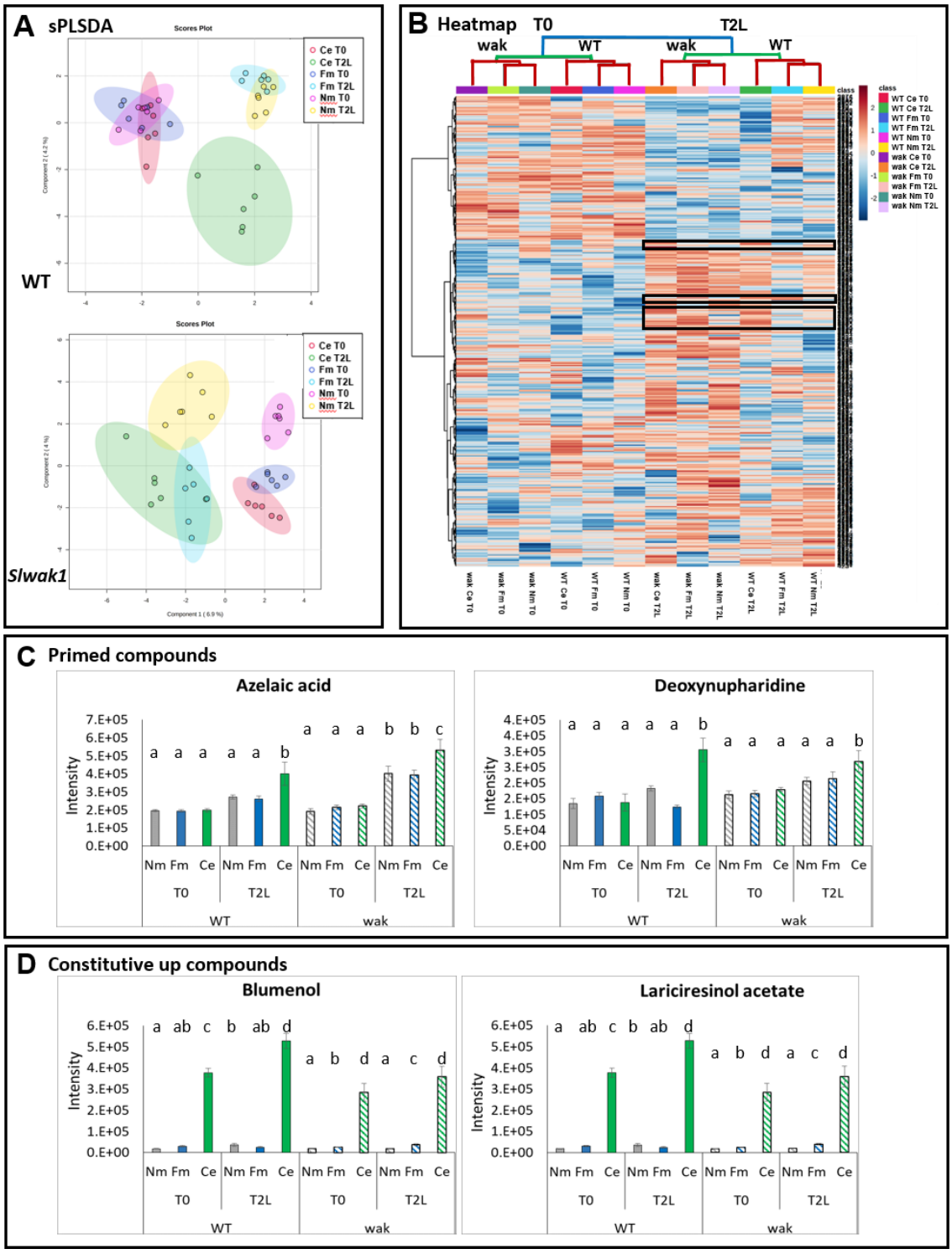

FIGURE 3. Herbivory changes the metabolic profile in both genotypes. Wild type (WT) and *Slwak1* (wak) tomato plants non-colonized (Nm) or colonized by *F. mosseae* (Fm) or *C. etunicatum* (Ce) were infested with *S. exigua* larvae, and leaflets were harvested after two days of herbivory. Lyophilized leaflets of non-infested plants (T0) and leaflets where *S. exigua* fed (local response to herbivory, T2L) were analyzed with ultra-performance liquid chromatography (UPLC) (n=6). Signal intensity was determined in all samples after normalizing the chromatographic area for each compound to the dry weight of the sample. All registered signals from the combination of both positive and negative electrospray ionization were compared using the non-parametric Kruskal–Wallis test, and only data with significant differences between groups (p<0.05) were used for the supervised analysis. **(A)** Supervised three-dimensional sparse partial least squares discriminant analysis (sPLSDA) representation of the major sources of variability. **(B)** Heatmap and clustering representation of signals showing significant differences among treatments. Black squares represent 3 clusters of over-accumulated compounds in Ce or both AM plants of both genotypes upon herbivory. **(C)** Putative metabolites primed upon herbivory in Ce-colonized plants of both genotypes. **(D)** Putative metabolites constitutively

up in Ce-colonized plants of both genotypes. For (C) and (D), data not sharing a letter in common differ significantly according to the Fisher's LSD test (p<0.05)

is a clear distinction between *Slwak1* (wak) and the WT, revealing a clear impact of the genotype on the metabolome (green branch, Figure 3B). Mycorrhization influenced the metabolomic profile differentially depending on the colonizing fungus, with Ce-colonized plants showing a greater divergence from the metabolomic profiles in Nm and Fm plants regardless of the genotype or the herbivory (red branch, Figure 3B).

Since larval performance was more severely impaired in Ce-colonized plants, and the heatmap analysis confirmed a more divergent metabolic profile in plants, we aimed to identify defense metabolites differentially accumulated in Ce as potentially involved in the higher larval mortality in Ce plants. For that, we focused the analysis on clusters of signals differentially accumulated in Ce as compared to Nm plants. The combined heatmap analysis of ESI- and ESI+ signals showed a set of compounds -divided into 3 clusters- with a primed accumulation in Ce T2L of both genotypes (highlighted by a black square, Figure 3B). To establish a tentative identity of the metabolites with differential accumulation in Ce plants, we annotated several secondary metabolites based on exact masses, fragmentation spectra with online databases or internal library matching. Hence, we give a putative identification of 30 signals, and among the most representative, we identified alkaloids, terpenoids, phenylpropanoids and lignans (Table S3). We highlight the accumulation of two herbivore-induced, mycorrhiza-primed compounds: the fatty acid derivative azelaic acid and the alkaloid deoxynuphardine (Figure 3C). Interestingly, we also found five compounds (MarVis Suit 2.0 software, Kruskall-Wallis test, p<0.01) constitutively over-accumulated in Ce plants, of which we putatively identified two: blumenol and lariciresinol acetate (Figure 3D).

In summary, still from basal conditions (no herbivory), the metabolome analysis revealed distinct profiles between Ce-colonized and Nm plants. Further identification of putative defensive compounds confirmed that Ce plants exhibited both constitutive (before herbivory) and induced accumulation (after herbivory) of secondary metabolites, then Ce-induced priming of plant defenses.

Mycorrhizal colonization changes primary metabolism

Slwak1 plants display alterations in the primary metabolism, with higher glycolysis-related activities and a lower basal energy status than WT plants without herbivory (see Chapter

2). As mycorrhizal colonization can alter plant primary metabolism too, here we analyzed the impact of mycorrhizal colonization on the primary metabolism of plants from both genotypes.

In the absence of herbivory, the activity levels of carbohydrate-related enzymes were significantly impacted by the mycorrhizal treatment AMF ($p< 0.05$), the plant genotype ($p< 0.001$) and the interaction between both, AMF and genotype ($p< 0.01$) according to the PERMANOVA analysis. A principal component analysis showed that 63.53% of variance (PC1) was related to Genotype (Figure 4A). PC1 correlated with most of the enzyme activities, increasing towards *Slwak1*. PC2, explaining ca. 20% of variance, clearly differentiated between mycorrhizal treatments, being driven by PGM and CytInv (Figure 4A). In this case, major differences were found for the *Slwak1* mutant and between the mycorrhizal and non-mycorrhizal treatments (Figure 4A). In contrast, only the genotype significantly led to differences in carbohydrate concentration (PERMANOVA, p<0.001). Accordingly, PC1 explained 41.54% of the variance, clearly differentiated between genotypes showing a higher concentration of carbohydrates in WT (Figure 4B).

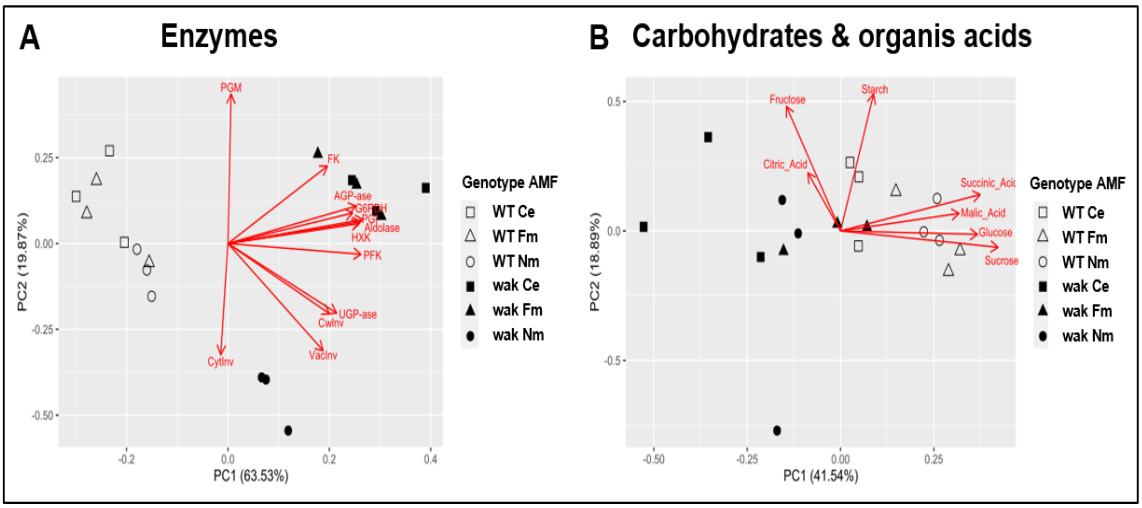

FIGURE 4. Mycorrhizal colonization affected primary metabolism at the basal level. Principal component analysis (PCA) of CH enzymatic activity, CH and organic acids content in leaves of WT and *Slwak1* (wak) plants non-colonized (Nm) or colonized by *F. mosseae* (Fm) or *C. etunicatum* (Ce) before herbivory (T0). **(A)** Enzymatic activity (nkat gFW–1) related to CH metabolism and **(B)** CH and organic acids content (mg/g FW). In the figure data represented are the means of 3 replicates. The shape of the symbols indicates the mycorrhizal treatment and the color indicates the genotype.

When looking at the carbohydrate-related enzymatic activities in detail, most of them (phosphoglucoisomerase (PGI), phosphofructokinase (PFK), Aldolase (Ald) and hexokinase (HXK) glycolysis-related; glucose-6-phosphate dehydrogenase (G6PDH), oxidative pentose phosphate pathway-related and fructokinase (FK), sucrose biosynthesis-related) showed higher levels in *Slwak1* plants, as previously discussed (see Chapter 2), but this increase was further

promoted by mycorrhization, showing maximum levels in mycorrhizal *Slwak1* plants (Figure 5A).

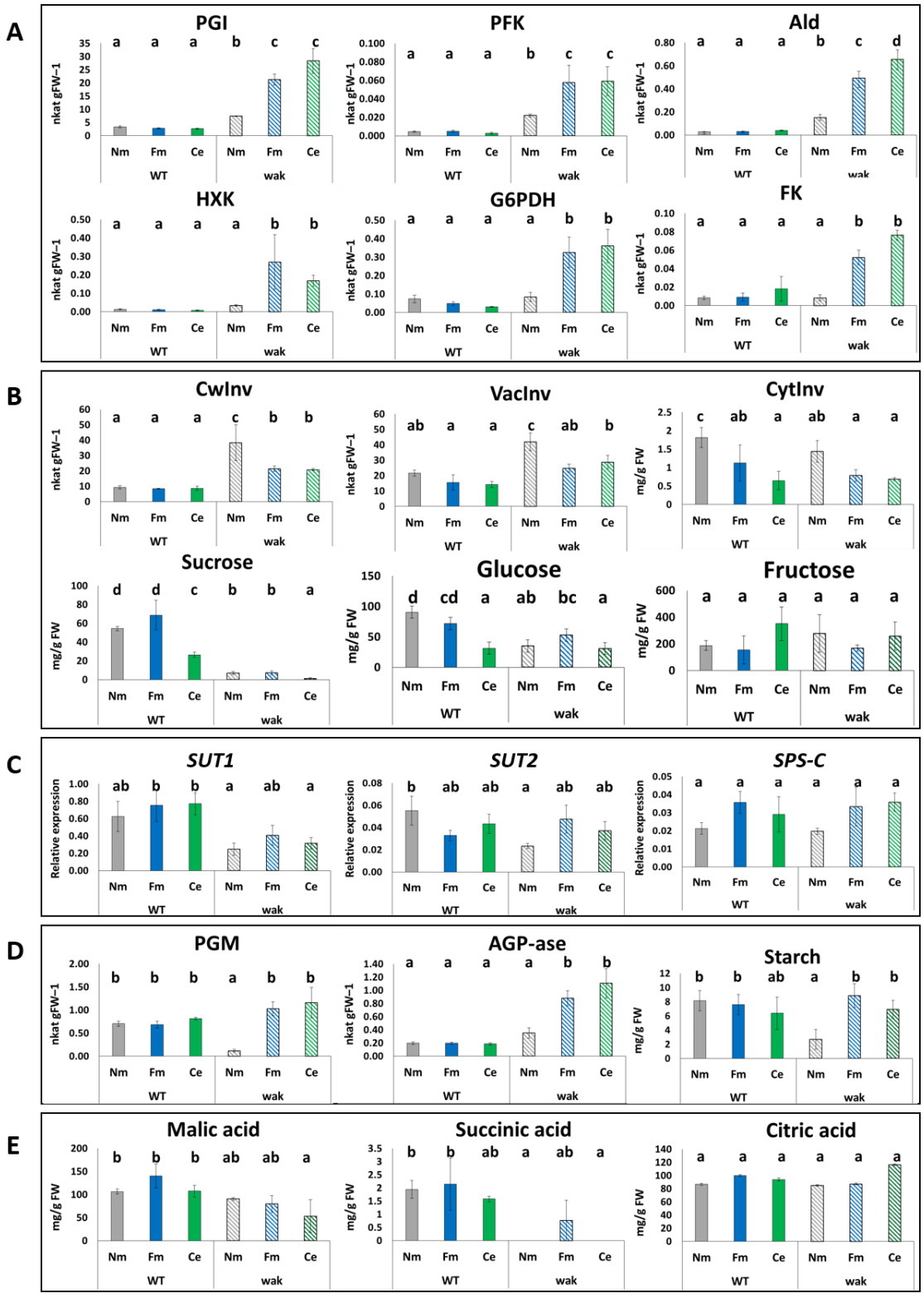

FIGURE 5. Enzymatic activities, compounds and gene expression related to carbohydrate metabolism in the absence of herbivory (T0, basal level) varies between genotypes. Leaf material from control non-infested plants was determined in wild type (WT, filled) and mutant (wak, dashed) plants non-colonized (Nm, grey) or colonized by *F. mosseae* (Fm, blue) or *C. etunicatum* (Ce, green). Enzymatic activities (nkat gFW–1) of CH metabolism, **(A)** enzymes related with glycolysis (PGI, PFK, Ald, HXK), cell wall biosynthesis (UGPase), sucrose biosynthesis (FK), and oxidative

pentose phosphate pathway (G6PDH) (n=3). **(B)** Invertases enzymes related to sucrose degradation (CwInv, VacInv, CytInv) (nkat gFW–1) and sugars (Sucrose, Glucose, Fructose) content (mg/g FW) (n=3). **(C)** Gene expression analysis by real-time qPCR of sucrose transporter 1 and 2 and sucrose phosphate synthase C (*SUT1, SUT2* and *SPS-C,* respectively) (n=4). **(D)** Starch content (mg/g FW) and enzymes related to starch biosynthesis (AGPase, PGM) (nkat gFW–1) (n=3). **(E)** TCA cycle intermediates (succinic, malic and citric acid) content (mg/g FW) (n=3). Data not sharing a letter in common differ significantly according to ANOVA, Fisher's Least Significant Difference (LSD) test ($p<0.05$).

Invertase activities were also higher in the *Slwak1* plants, but in this case, their activities were reduced by mycorrhization in the mutant background, getting the levels closer to the ones in the WT lines (Figure 5B). In fact, the cleavage activity of cell wall invertase (CwInv) decreased in mycorrhizal *Slwak1* plants, while the vacuolar invertase (VacInv) and cytosolic invertase (CytInv) decreased in the mycorrhizal plant of both genotypes (Figure 5B). In *Slwak1*, these higher invertase levels correlate with reduced soluble sugar levels. Sucrose and glucose were strongly reduced in the leaves of the *Slwak1* genotype, and remarkably, the levels of sucrose and glucose were significantly reduced in both genotypes by Ce colonization.

Transcript levels of the sucrose transporters 1 and 2 (*SUT1* and *SUT2)* were reduced in the *Slwak1* mutant in Nm plants, with a moderate buffering effect of mycorrhiza, as the levels in mycorrhizal plants in both genotypes were similar. The expression of the sucrose phosphate synthase (*SPS-C*) was similar in both genotypes (Figure 5C).

Finally, the starch biosynthesis activity and concentration showed no differences between AM and Nm in WT plants. However, the *Slwak1* mutant mycorrhizal plants showed higher activity of phosphoglucomutase (PGM) and ADP-glucose pyrophosphorylase (AGP-ase), leading to a higher starch concentration in mycorrhizal than in Nm plants in the mutant (Figure 5D). Regarding the tricarboxylic acid (TCA) cycle compounds, we did not observe mycorrhizal-related differences in their accumulation in any of the genotypes. However, the levels of malic and succinic acid in WT plants were slightly higher than in *Slwak1* (Figure 5E).

Without herbivory, the findings revealed that AM symbiosis, in mutant plants, modified the carbohydrate flux compensating for the deficient energy status of Nm *Slwak1*.

AM symbiosis in *Slwak1* plants changed the source-sink relations under herbivore attack

We monitored the levels of CH-related enzymatic activities and CH during herbivory, by evaluating their changes in leaves at time 0, 2 and 14 days post infestation both at the local and systemic level. PERMANOVA analysis of the data showed that in the local response, the herbivory, the genotype, and their interaction significantly impacted carbohydrate-related enzymatic activities ($p<0.001$), but there was no effect of AMF inoculation nor its interaction. Accordingly, a principal component analysis (PCA) showed that 41.22% of variance (PC1) was

related to plant genotype (Figure 6A). This axis (PC1) correlated with most of the enzymatic activities, increasing towards WT. PC2, explaining 21.6% of variance, clearly differentiated for herbivory, being mainly driven by CytInv, but this effect was more evident for WT than for *Slwak1* (Figure 6A).

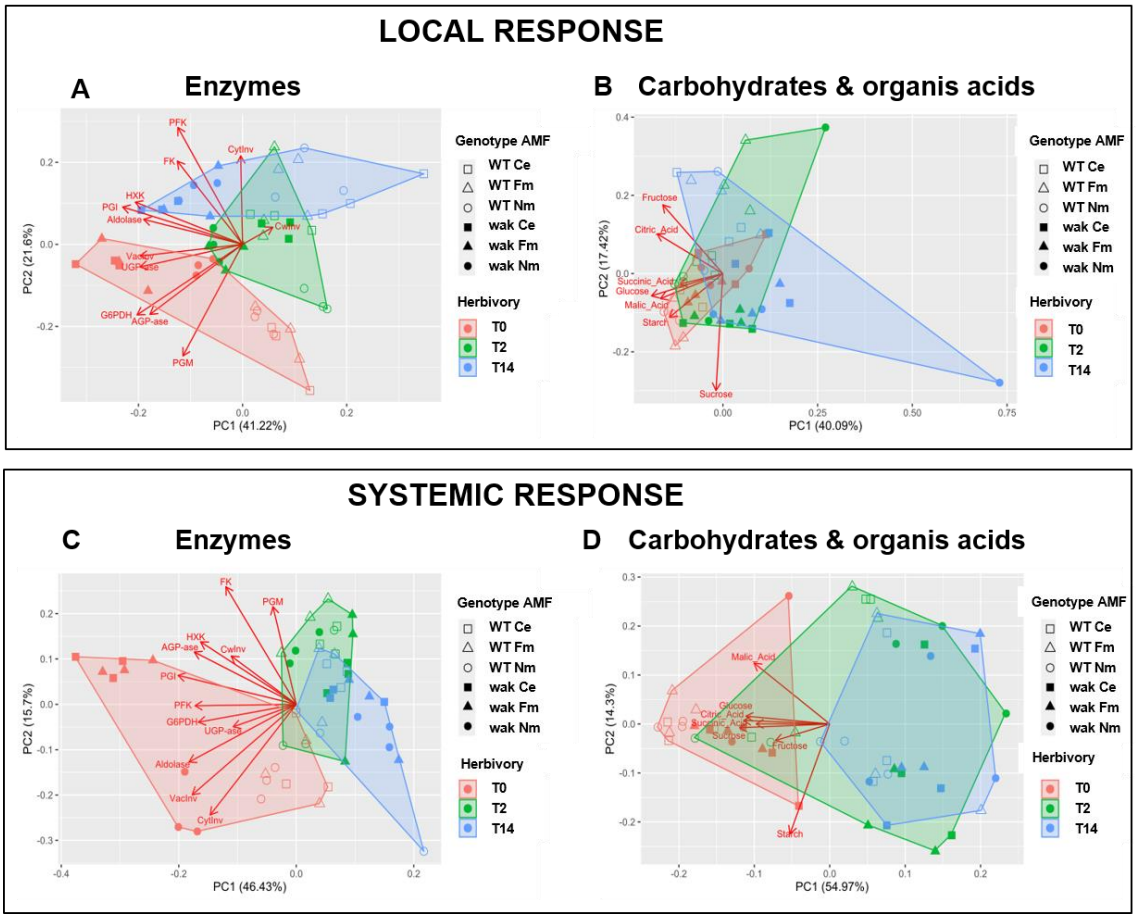

FIGURE 6. Primary metabolism is regulated by herbivory. Principal component analysis (PCA) of CH enzymatic activity, CH and organic acids content in leaves of WT or *Slwak1* (wak) plants non-colonized (Nm) or colonized by *F. mosseae* (Fm) or *C. etunicatum* (Ce) upon herbivory (T0: no herbivory; T2: 2 days of herbivory; T14: 14 days of herbivory). **(A, C)** Enzymatic activity (nkat gFW–1) related to CH metabolism and (**B, D**) CH and organic acids content (mg/g FW) were determined. **(A, B)** Analysis of local responses in *S. exigua* attacked leaves, and **(C, D)** analysis of systemic changes in unwounded leaves. In the figure data represented are the means of 3 replicates. Colors indicate the herbivory; the shape of the symbols indicates the treatment (genotype and AMF).

Regarding CH content, the PERMANOVA showed that genotype (p<0.001) and herbivory (p<0.01) significantly impacted CH accumulation under herbivory and significant interactions of herbivory*genotype, herbivory*AMF and genotype*AMF (p<0.05) were found. This result of PERMANOVA could be driven by differences in multivariate dispersion across analyzed groups, as was revealed by the PCA ordination. The PCA showed that PC1, explaining 40% of variance, was correlated with most of CH and organic acids, while PC2 was driven mainly by sucrose (Figure 6B).

In systemic tissues, the PERMANOVA showed that genotype (p<0.05), herbivory (p<0.001) and their interaction (p<0.05) significantly led to differences in CH-related enzymatic activities. Again, we found no effect of AMF nor its interaction. Accordingly, the PCA showed that 46.43% of the variance (PC1) was clearly related to herbivory (Figure 6C), but this trend was more pronounced in *Slwak1* than in WT. PC1 correlated with most enzymatic activities, increasing towards T0 in *Slwak1*. Regarding CH content, PERMANOVA analysis showed that genotype (p<0.05) and herbivory (p<0.001) but not AMF significantly impacted CH accumulation under herbivory. Accordingly, the PCA showed that ca. 55% of variance (PC1) was mainly related to herbivory and, at any time, WT tended to have higher concentrations of CH than *Slwak1*. PC1 was correlated with almost all CH and organic acids, while PC2 was mainly driven by starch (Figure 6C).

As shown in Figure 6, most changes occur early, in the first two days and then the levels are generally maintained. Comparing local and systemic responses, the herbivory and the genotype impacted the enzymatic activities, CH and organic acids content at both local and systemic levels. However, the analysis showed that the interaction between AMF*herbivory and AMF*genotype influences (p<0.05) the carbohydrate content only at the local level.

Then, since most changes occur within the first two days at the local level, we focused the analyses on local leaves 2 days upon herbivory. Consistent with our previous results (Chapter 2), the behavior of *Slwak1* under herbivory differs from WT, with enzymatic activities related to CH and sugars accumulation, changing generally in an opposite direction in *Slwak1* than in WT. Indeed, the enzymatic activities related to the glycolysis, oxidative pentose phosphate pathway and sucrose biosynthesis (PGI, PFK, Ald, HXK, G6PDH, FK), did not significantly change upon herbivory in Nm plants, regardless of the genotype (WT or *Slwak1*). On the contrary, the effect of mycorrhization on the enzymatic activities upon herbivory followed opposite patterns: they increased in AM WT, while they decreased in AM *Slwak1* plants (Figure 7A).

Regarding invertase activities in the WT, the sucrose cleavage activity of VacInv decreased in both Nm and AM plants after herbivory. However, CwInv and CytInv activity increased, mostly in AM plants (significant for Ce). The sucrose content strongly decreased upon herbivory and fructose levels increased (Figure 7B). In *Slwak1,* VacInv and CytInv followed a similar trend to WT, decreasing and increasing, respectively, after herbivory. In contrast, the CwInv showed the opposite trend in the mutant than in WT in both Nm and AM, reducing its activity upon herbivory. This reduction leads to an increase in sucrose content in *Slwak1* plants

(with exception of Fm) (Figure 7B). Thus, *Slwak1* mutant plants applied partially the opposite strategy than WT in response to herbivory, leading to a higher sucrose content than WT.

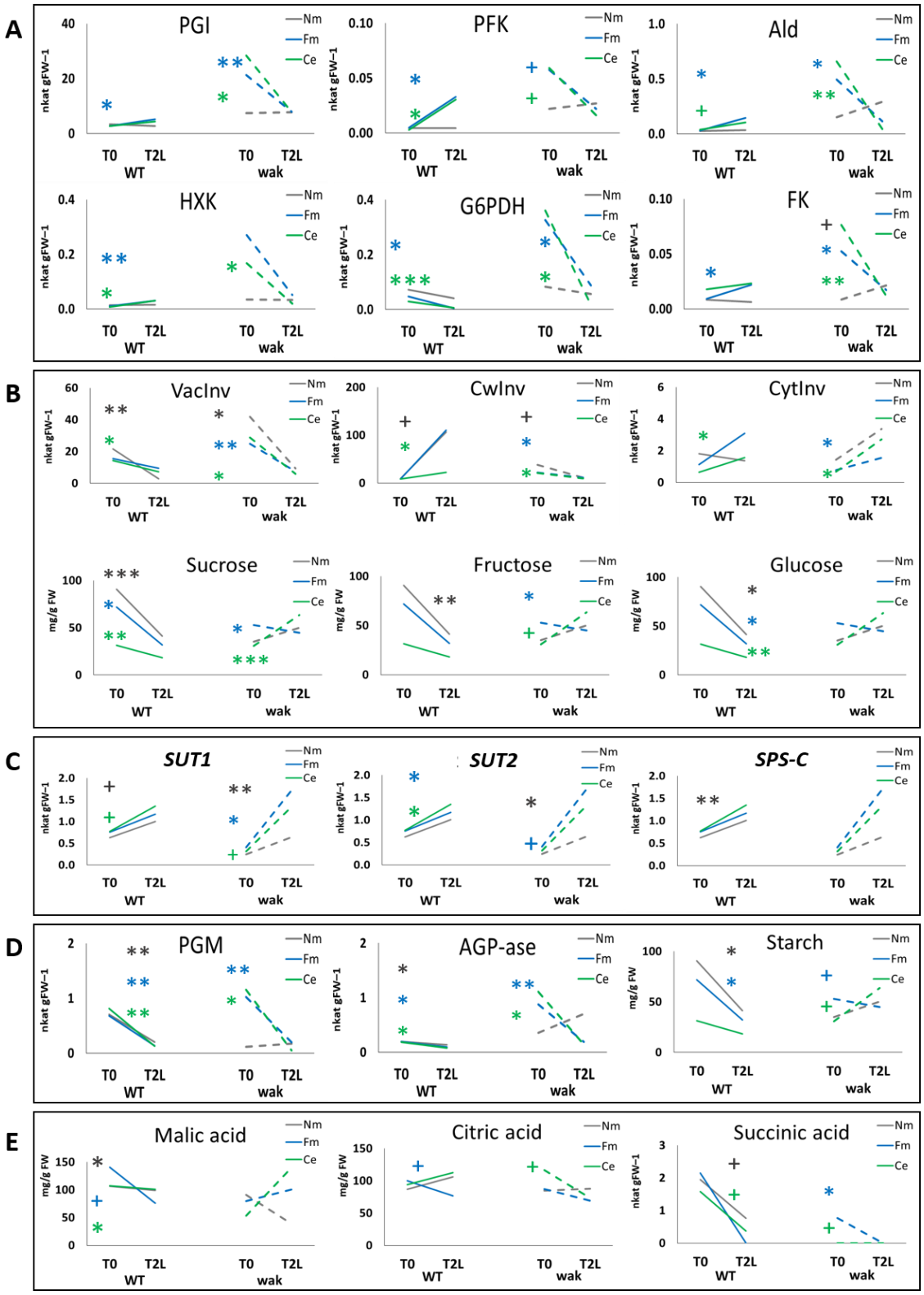

FIGURE 7. Enzymatic activities, compounds and gene expression related to carbohydrate metabolism upon herbivory varies between genotypes. Leaf material from control non-infested plants was determined in wild type (WT, continuous line) and mutant (wak, dotted line) plants non-colonized (Nm, grey) or colonized by *F. mosseae* (Fm, blue) or *C. etunicatum* (Ce, green) before (T0) and after (T2L) 2 days of local herbivory. Enzymatic activities (nkat

gFW–1) of CH metabolism, **(A)** enzymes related with glycolysis (PGI, PFK, Ald, HXK), cell wall biosynthesis (UGPase), sucrose biosynthesis (FK), and oxidative pentose phosphate pathway (G6PDH) (n=3). **(B)** Invertases enzymes related with sucrose degradation (CwInv, VacInv, CytInv) (nkat gFW–1) and sugars (Sucrose, Glucose, Fructose) content (mg/g FW) (n=3). **(C)** Gene expression analysis by real-time qPCR of sucrose trasporter 1 and 2 and sucrose phosphate synthase C (*SUT1, SUT2* and *SPS-C,* respectively) (n=4). **(D)** Starch content (mg/g FW) and enzymes related to starch biosynthesis (AGPase, PGM) (nkat gFW–1) (n=3). **(E)** TCA cycle intermediates (succinic, malic and citric acid) content (mg/g FW) (n=3). Asterisks indicate significant effects of herbivory (T2L vs T0) on each mycorrhizal treatment (grey WT; blue Fm; green Ce) within the same genotype according to t-test. +:$p < 0.1$; *:$p < 0.05$; **:$p < 0.01$; ***:$p < 0.001$.

In addition, the sucrose transporters 1 and 2 (*SUT1* and *SUT2*, respectively) increased significantly their transcription levels in AM plants of both genotypes in injured leaves, while Nm plants maintain the same transcriptional levels as before herbivory (Figure 7C). The sucrose phosphate synthase (*SPS-C*) maintains similar expression levels (with exception of Nm WT) before and after herbivory (Figure 7C).

Noteworthy, both AM and Nm WT plants decreased PGM and AGP-ase activity, leading to a reduction of starch upon herbivory (T2L vs T0). However, despite no significance, mutant Nm *Slwak1* plants showed a trend to increase the PGM and AGP-ase activity upon herbivory, leading to an increase in the starch concentration. Surprisingly, the activities in AM *Slwak1* plants followed a similar trend to the WT, significantly reducing PGM, AGP-ase activity and starch content upon herbivory. Thus, mycorrhizal colonization reverted the altered phenotype of the mutant regarding starch metabolism (Figure 7C). Finally, the changes upon herbivory on the levels of TCA cycle compounds showed a similar trend in AM and Nm plants in the WT, while in the *Slwak1* mutant the concentrations of malic and citric acid followed opposite trends in AM and Nm plants (Figure 7D).

We also analyzed the long-term effects of herbivory (21 days of herbivory, T21) on the CH content and distribution in the plants. Starch was only detected in leaves, and the concentration was about 10 times higher in the *Slwak1* leaves than in the WT, independently of AM colonization (Table 1A). On the contrary, sucrose and fructose were lower in the mutant leaves regardless of mycorrhizal colonization (Table 1A).

In roots, hexoses (glucose and fructose) and citric acid showed higher levels in the *Slwak1* mutant than in the WT (Table 1B), confirming a differential carbon partitioning in the mutant. Regarding the effect of mycorrhiza, the levels of glucose and sucrose were lower in mycorrhizal roots as compared to non-mycorrhizal plants (Table 1B). Regarding the root/shoot ratio the mutant showed a lower ratio for sucrose and higher for glucose and fructose and TCA intermediates compared to the WT. In mycorrhizal plants, there was a trend for a higher root-to-shoot ratio for glucose and fructose and citric and malic acids (Table 1C).

		A T21 Shoot			B T21 Root			C Root/Shoot		
Sugars and organic acids	Genotype	Nm	Fm	Ce	Nm	Fm	Ce	Nm	Fm	Ce
Starch	WT	6 a	8 ab	6 a	ND	ND	ND	-	-	-
	wak	59 bc	39 abc	61 c	ND	ND	ND	-	-	-
Sucrose	WT	4.2 b	0.0 **a**	2.9 ab	0.8 a	0.0 a	1.2 a	0.56	-	0.54
	wak	0.0 a	0.5 a	0.7 a	0.4 a	0.7 a	0.4 a	-	-	0.04
Glucose	WT	184 b	44 ab	53 ab	32 a	18 a	48 ab	0.17	0.41	0.89
	wak	144 b	65 ab	39 **a**	132 c	96 bc	78 abc	0.92	1.48	2.01
Fructose	WT	303 ab	435 b	290 ab	65 a	222 **b**	138 ab	0.22	0.51	0.47
	wak	107 a	140 a	190 ab	214 ab	241 b	197 b	2.01	1.72	1.04
Citric Acid	WT	81 ab	32 a	110 b	1 a	0 a	17 a	0.01	0.00	0.15
	wak	86 ab	73 ab	124 b	6 a	18 a	21 a	0.07	0.24	0.17
Malic Acid	WT	172 b	57 **a**	149 ab	13 a	22 a	34 a	0.08	0.39	0.23
	wak	192 b	138 ab	146 ab	27 a	29 a	40 a	0.14	0.21	0.28
Succinic Acid	WT	2.1 a	0.3 a	1.2 a	0	0	0	0	0	0
	wak	1.2 a	0.5 a	0.6 a	0	0	0	0	0	0

TABLE 1. AM symbiosis in *Slwak1* plants (partially) changes the direction of sugars movement after 21 day of herbivory in WT and *Slwak1* mutant. Sugars and organic acid concentrations (mg/g FW) were determined in WT and mutant (wak) in non-myccorhizal (Nm) and mycorrhizal (Fm or Ce) plants after 21 days of herbivory in **(A)** leaves (T21 Shoot) and **(B)** roots (T21 Root) (n=3). **(C)** The root/shoot ratio (Root/Shoot). For **(A, B)** one-way ANOVA was done for each compound in specific plant tissue (Shoot or Root) with Nm and AM plants of both genotypes. Data not sharing a letter in common differ significantly according to ANOVA, Fisher's Least Significant Difference (LSD) test (p<0.05).

Overall these results showed that the most and highest changes in CH metabolism occurred early, in the first two days, in the directly challenged leaves (local response). Although the direction of enzymatic activities and the corresponding sugar flux were generally opposite between WT and *Slwak1,* in both genotypes the mycorrhizal colonization had a stronger impact on CH metabolism than Nm plants upon herbivory.

DISCUSSION

The present study shows how primary and secondary metabolism are differentially regulated during mycorrhiza induced resistance to herbivore attack, and explores if the SlWAK1 receptor kinase is involved to any extent in this process. We demonstrate that AMF colonization by *F. mosseae* and *C. etunicatum* significantly impacts the interaction of the generalist herbivore *S. exigua* both in WT tomato and *Slwak1* deficient plants. The larvae feeding on mycorrhizal plants performed worst in both genotypes, with reduced survival and development. Previous works demonstrated the positive effect of AMF on plant resistance against generalist chewing herbivores -including *Helicoverpa arimigera*, *H. punctigera, Spodoptera exigua*, *S. littoralis, S. litura, Trichoplusia ni* Hübner-, with larval fitness reduced when feeding in mycorrhizal plants (Song et al., 2013; Rivero et al., 2021; Formenti and Rasmann, 2019; Frew and Wilson, 2021; Selvaraj and Thangavel, 2020; Schoenherr et al., 2019).Indeed, mycorrhizal tomato plants colonized by *F. mosseae* were more resistant against *S. exigua,* increasing larval mortality through primed induction of toxic metabolites in leaves (Rivero et al., 2021). Experiments with different AMF species demonstrated that they could differentially affect insect herbivore performance (Barber et al., 2013; Vannette et al., 2013; Malik et al., 2018), but the molecular mechanisms responsible for such differential responses are yet to be understood. Here, both AMFs induced tomato resistance in WT and *Slwak1* against *S.* e*xigua,* being the protective effect stronger in *C. etunicatum* than in *F. mosseae* colonized plants. Although colonization levels were higher in Ce-inoculated plants, protection has been shown not to be directly dependent on the colonization level. Thus, higher resistance showed in Ce-colonized tomato plants may be related to the differential impact of the fungus on the plant than to the differences in the colonization levels.

We hypothesized that the SlWAK1 receptor has a relevant role in developing MIR, and thus in the mutant mycorrhiza could not protect against the pest attack. However, mycorrhizal *Slwak1* tomato plants display enhanced protection against the herbivore as compared to the non-mycorrhizal ones; the increase in mortality and the reduction in the percentage of individuals reaching to pupae and moths by mycorrhiza was more pronounced in the mutant, and only the weight reduction was lost. In view of the results, SlWAK1 is not essential for MIR, although it may mediate some of the effects (related to the reduced effect on larval biomass).

Mycorrhizal-induced priming of plant defenses in both WT and *Slwak1*

In the previous Chapter, we demonstrated that the JA signaling pathway is not impaired in the *Slwak1* mutant, and so is not responsible for its enhanced susceptibility to *S. exigua*. Several studies demonstrated that the priming of mycorrhizal plants is JA-dependent, being one of the main MIR "strategies" (Song et al., 2013; Mora-Romero et al., 2014). As expected, mycorrhizal plants showed primed responses to herbivory regarding JA-dependent genes, and we found that mycorrhizal-associated priming in response to herbivory was stronger in *Slwak1* plants, with mycorrhizal mutant plants showing the strongest JA response to the herbivory, especially in the case of *C. etunicatum*. Mycorrhizal colonization was shown to induce leaf resistance to insect herbivores through a higher accumulation of metabolites with toxic effects against herbivores, either constitutively (Bennett et al., 2009) or upon attack (Rivero et al., 2021). Here, we found that genes related to the biosynthetic pathways of these compounds, such as MybSJ1 (positive regulator of the synthesis of polyamine conjugates), PAL 170 (phenypropanoids) and GAME9 (alkaloids), with reduced expression in the *Slwak1* mutant than the WT, showed higher levels in mycorrhizal plants, especially in the mutant, thus compensating partly the mutant deficiency. Our results highlighted that mycorrhizal colonization protected both genotypes, triggering similar mechanisms (i.e. primed JA-regulated responses and synthesis of secondary metabolites) in WT and *Slwak1* tomato plants.

Remarkably, when exploring the full metabolomic profile, differences related to mycorrhization in WT were only evident under herbivore attack, while in *Slwak1,* the differences were evident even at the basal level. Thus, in *Slwak1* plants, deficient in several aspects of the primary and secondary metabolism (see Chapter 2), mycorrhization had a stronger effect than AM WT plants in absence of stress. Then, it is tempting to speculate that mycorrhizal colonization may contribute to buffering the deficiencies of the mutant even at the basal level, while AM WT plants do not need it, triggering the accumulation of metabolites only under herbivory attack. According to the hierarchical clustering of the metabolic profile (heatmap) Ce separates from Nm and Fm in both genotypes and under both basal and herbivory conditions. We putatively identified 2 compounds constitutively accumulated in Ce-colonized plants, the blumenol (11-carboxyblumenol C-9-O-Glc) and lariciresinol acetate. The blumenol is a metabolite reported recently as a shoot marker of AM symbiosis (Wang et al., 2018; Rivero et al., 2021), and while a subtle increase is observed in Fm plants, there is a clear overacumulation in Ce plants, showing the higher colonization levels. The lariciresinol acetate is a defensive lignan compound with a moderate toxic effect against the caterpillars *Spodoptera littoralis* and *Lymantria dispar* (Brader et al., 1998), and may have contributed to the protection

observed. Although some toxic compounds are constitutively accumulated in mycorrhizal plants, the highest induction occurs after *S. exigua* damage. The main inducible metabolic pathways in response to herbivory in Ce-colonized WT and *Slwak1* belong to phenylpropanoids as lignans, alkaloids and terpenoids. Lignans are described to have antinutritive effects on herbivores (Lattanzio et al., 2006). Alkaloids have toxic effect on insect impairing some biological activities as neuronal signal transduction, DNA replication, protein synthesis, and enzymatic activity (Després et al., 2007; Heidel-Fischer & Vogel, 2015; Züst and Agrawal, 2016). Also terpenoids show toxic effect on insect, inhibiting acetylcholine esterase that cause anoxia and finally insect death (Zunjarrao et al., 2020). Many of these compounds are more accumulated under herbivory in different plant species, showing antifeedant and toxic activities, such as azelaic acid (Mithofer and Maffei, 2016; Marti et al., 2013; Rivero et al., 2021). In tobacco, this compound increase the synthesis of phenylpropanoid-polyamine conjugates and prime defense-related genes expression (Djami-Tchatchou et al., 2017) and it is considered to be a key signal molecule in induced systemic immunity (Jung et al., 2009; Yu et al., 2013; Shine et al., 2019). Interestingly, mycorrhizal tomato showed primed accumulation of azelaic acid under *S. exigua* attack, and this compound has a direct negative effect on larvae fitness (Rivero et al., 2021). Hence, the rearrangement of the metabolic profile supports the primed defenses profile in AM plants.

Mycorrhizal colonization modulates primary metabolism at basal and herbivory conditions

Previously, we showed that *Slwak1* plants have enhanced glycolysis activity and lower energy status than WT plants in basal conditions and that *Slwak1* is altered in the regulation of primary metabolism under herbivory. Here, we explore how mycorrhizal colonization influences the carbohydrate flux in WT and *Slwak1* tomato plants. AMF is dependent on plant carbon compounds, and reprogramming of the carbohydrate metabolism occurs in mycorrhizal plants to increase the CH flux toward roots for the maintenance of symbiosis (Wright et al., 1998; Gavito et al., 2019). Mycorrhizal colonization in WT plants reduced the activity of VacInv and CytInv in leaves, with a corresponding modification in the soluble sugars content. The results are in agreement with the symbiosis regulating plant sucrose biosynthesis and catabolism to transfer the carbon to the fungal symbiont. Indeed, modification of C partitioning in AM plants determines the benefits of the interaction for the host (Salmeron-Santiago et al., 2021). Additionally, it is suggested that the hexoses have a crucial role in regulating sugar signaling pathways during AM symbiosis (Roth & Paszkowski, 2017; Sanmartín et al., 2020), and sugar signaling plays also a role in defense signaling (Kundu et al., 2018). Moreover, it has been

confirmed that invertases play a fundamental role in mycorrhizal colonization development regulating the carbon supply to the AMF (Schaarschmidt et al., 2007). Noteworthy, mycorrhizal colonization lead to more pronounced changes in *Slwak1* plants, with significant increases of all glycolysis-related enzymes (PGI, PFK, FK, Ald, G6PDH and HXK), but also increased starch biosynthesis-related enzymes, promoting starch accumulation to similar levels than in WT plants. In the same line, the expression of sucrose transporters -reduced in Nm *Slwak1* plants as compared with Nm WT ones- slightly increased in mycorrhizal *Slwak1* plants and the invertases -elevated in Nm mutant plants- were significantly reduced in AM *Slwak1* plants. Thus, it is tempting to speculate that the symbiosis buffers the effect of the *Slwak1* deficiency and compensates for the inefficiency of the mutant in C metabolism and partitioning, leading to values closer to those in the WT.

Remarkably there were some differences in sugars depending on the AMF species. For example, the sucrose and glucose content was lower in Ce-colonized plants -but not in Fm- compared to Nm of both genotypes. This different accumulation of soluble sugars between Ce and Fm may be related to the higher Ce-colonization levels. In fact, the dynamics of the carbon distribution in AM plants are in part determined by the genetic background of the symbionts that exert different levels of carbon sink strength (Zhao et al., 2019). In addition, the mycorrhiza-triggered growth promotion in plants is strongly related to the stimulation of sugar transport and sucrose catabolism in the host plant (Salmeron-Santiago et al., 2023). This may explain the higher plant growth promotion in Ce-colonized than Fm plants (compared with non-mycorrhizal ones).

Upon herbivory, mycorrhizal plants showed higher carbohydrate catabolism than Nm, increasing sucrose transport, reducing soluble sugars and increasing the glycosylic activity in injured leaves. Under herbivory, the invertases activities of CwInv and CytInv and the transcriptomic levels of *SUT2* increased in injured leaves of mycorrhizal WT plants, enhancing carbon sink strength. Plants utilize sugars as an energy sources to activate defense mechanisms and as signaling molecules to regulate defense gene expression (Roitsch and González, 2004; Veillet et al., 2016). In fact, plant invertases regulate sucrose partitioning in response to herbivore attacks for energy-gaining reactions and macromolecule and amino acid biosynthesis (Castrillòn-Arbelàez et al., 2012). For example, Arabidopsis plants induce the expression of genes coding for invertases and other enzymes that degrade complex carbohydrates in response to herbivores (Appel et al., 2014). Similarly, local induction of CytInv after foliar herbivory and the relevance of CwInv in the immune response during plant–pest interactions have been reported (Castrillòn-Arbelàez et al., 2012; Proels et al., 2014). Thus, the induction of

invertases activity, the increase of sucrose transporters expression levels and the corresponding higher reduction of soluble sugars in AM plants upon herbivory suggest a more efficient activation of CH catabolism in mycorrhizal plants. The increased activity of sugar transporters seems to be a common plant strategy to face stress (Baena-Gonzalez et al., 2007). The differential C partitioning may redirect sugars in defense-related processes, such as the production of defensive enzymes or molecules (Bhonwong et al., 2009; Arnold and Schultz, 2002). Opposite trends were observed in the *Slwak1* mutant, where mycorrhizal plants triggered changes upon herbivory in the opposite directions than the WT, for example, some CH-related enzymes (PGI, PFK, Ald, HXK, FK) showed reduction upon herbivory in mycorrhizal mutant plants, but an increase in the WT. Opposite patterns were also found for CwInv, CytInv, sucrose, fructose and glucose in the mutant.

Beyond soluble sugars, the starch concentration also decreased in injured leaves differentially in AM and non-mycorrhizal plant, depending on genotype. The reduction upon herbivory of AGP-ase and PGM enzymatic activities, both involved in starch biosynthesis, was common in Nm and AM plants in the WT, leading to reduction in starch. While Nm *Slwak1* mutants increased the activities and starch, mycorrhizal mutant plants behaved as wild type plants, reducing starch upon herbivory. Reduction of starch occurs also in response to pathogen attack (Sanmartín et al., 2020), showing a consolidated response mechanism to different biotic stresses. Starch degradation increases the carbon flux, increasing the plant's energy to respond against the invaders or reducing the plant´s palatability and nutritional content (Dong et al., 2019; Zhou et al., 2015). Hence, WT plants upon herbivory show a higher degree of carbohydrate mobilization in injured leaves that reduce soluble and storage sugars and they activate efficiently the invertases activity. While non-mycorrhizal *Slwak1* display the opposite strategy than WT during an herbivore attack, when the mutant plants are colonized by mycorrhiza the direction of the changes is reverted and AM *Slwak1* plants display similar pattern to those in the WT, being able to redirect carbohydrates movement after herbivore attack. Thus, mycorrhizal colonization seems to buffer the defects of the mutant regarding primary metabolism regulation, as AM *Slwak1* can re-establish a proper C partitioning in response to herbivory as in WT plants.

Finally, after 21 days of herbivory, the mutant showed higher levels of starch in leaves than the wt. Sucrose and fructose were lower in *Slwak*1 shoots, but the levels of glucose and fructose were higher in the mutant roots, regardless of AMF colonization. As a result, the mutant showed higher root/shoot ratio of hexoses. When analyzing the effect of mycorrhiza, the concentration of sucrose and glucose in leaves decreased in both genotypes, while in roots

glucose decreased but fructose increased. This is in agreement as AMF uptake glucose as a carbon source from plant roots enhancing the sink strength and taking up glucose in the roots of AM plants (Bago et al., 2003; Salmeron-Santiago et al., 2021). Mycorrhizal plants showed a higher root-to-shoot ratio of glucose in both genotypes, with Ce showing the strongest effect. Remarkably, the TCA intermediates citric and malic acid were also higher in the roots of Ce plants, and accordingly, they have a higher root-to-shoot ratio in both genotypes. This may positively correlate with the biosynthesis of secondary metabolites. Indeed, many primary metabolites derived from TCA cycle often serve as precursors for the synthesis of different secondary metabolites (Pott et al., 2019), and a higher accumulation of multiple secondary metabolites have been shown in leaves (this work) and in roots (Rivero et al 2018) of Ce colonized plants.

AM symbiosis amplifies the sink-strength response upon herbivory and buffers the susceptibility of *Slwak1* plants

Taking into account all the results, the reduction in some defensive metabolites -pointing to a lower defense capacity- and the highest starch concentrations in *Slwak1* plants -that may increase the nutritional content and palatability of the mutant-, explain the enhanced susceptibility of the mutant. As this highest concentration of starch in the mutant is maintained in mycorrhizal plants, this may lead to similar *S. exigua* weight in both AM and Nm *Slwak1* plants. However, the primed accumulation of defensive secondary metabolites in AM plants of both genotypes seems to explain the highest larval mortality when feeding in mycorrhizal plants regardless of the genotype, and thus, MIR is also effective in *Slwak1*.

In conclusion, our study indicates that mycorrhizal colonization in tomato roots increases plant resistance against *S. exigua* in both genotypes. Our results show that AM symbiosis triggers a higher accumulation of basal and induced secondary metabolites and primes plant defenses in response to herbivory in both WT and *Slwak1* plants. Mycorrhizal colonization also leads to the modulation of the plant's primary metabolism in both genotypes. In WT plants, AM amplifies the response of sink strength after herbivore attack, and in the mutant, AM reverts the direction of sugars movement. Thus, *Slwak1* inability to properly regulate C partitioning is redirected by mycorrhizal colonization, with mycorrhizal *Slwak1* plants behaving as the WT at basal level and even more under *S. exigua* attack. Finally, mycorrhizal colonization buffers the highest susceptibility of *Slwak1,* and efficiently enhanced the mutant resistance. Thus, the results do not support a major role of SlWAK1 in MIR against herbivore pests. Future studies will aim to uncover the different strategies applied by diverse AMF species

in response to herbivory and how mycorrhizal colonization drives the metabolic flux for an efficient C partitioning between source and sink organs.

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SUPPLEMENTARY DATA

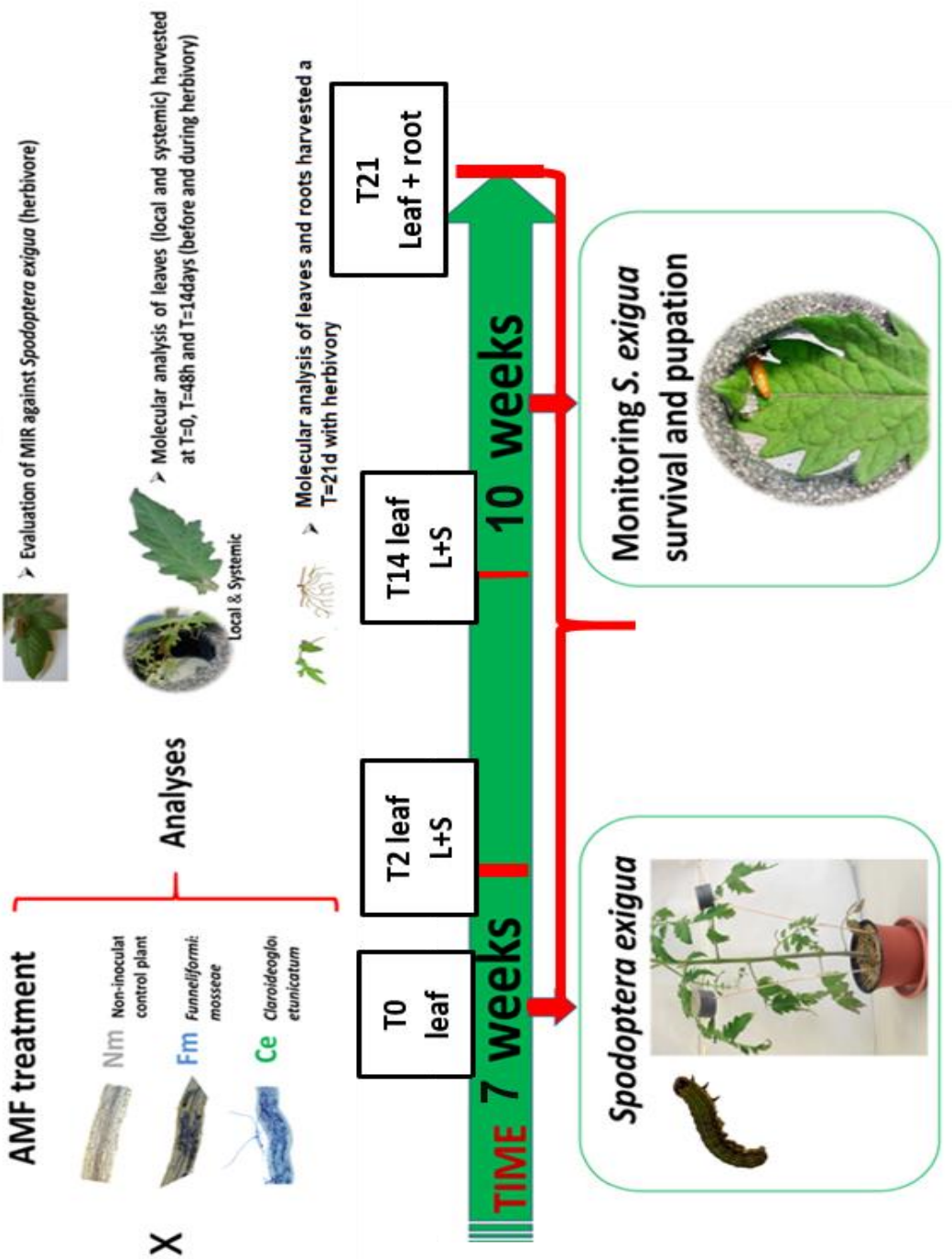

Figure S1. Scheme of experimental design

TABLE S1. Plant growth promotion by mycorrhizal colonization is not impaired in *Slwak1* plants. Plant biomass (Shoot, n=15) (Root, n =6) (g FW) and mycorrhizal colonization (%) were determined in WT and mutant (Slwak1) in non-myccorhizal (Nm) and mycorrhizal (Fm or Ce) plants at harvest (ten-week-old plants). One-way ANOVA was done for each specific plant tissue (Shoot or Root or Shoot/Root) with Nm and AM plants of both genotypes. For mycorrhizal colonization one-way ANOVA was done with AM plants of both genotypes. Data not sharing a letter in common differ significantly according to Fisher's Least Significant Difference (LSD) test ($p<0.05$).

Variable	WT			Slwak1		
	Nm	Fm	Ce	Nm	Fm	Ce
Shoot	126.7 b	131.2 c	137.9 de	119.5 a	134.7 cd	141.5 e
Root	28.6 ab	25.4 a	27.3 a	25.4 a	28.6 ab	32.7 b
Shoot/Root	4.4 a	5.2 c	5.1 bc	4.7 ab	4.7 abc	4.3 a
Mycorrhization	--	24.7% b	52.7% c	--	8.0% a	46.4% c

TABLE S2. Mycorrhizal colonization changes in nutrients content in both genotypes. Macro and micronutrients in leaves of wild type and Slwak1 were determined before *S. exigua* infestation (T0) in leaves, and after 21 days (T21) of herbivory in leaves and roots. Data represent the mean values of 5 biological replicates (ppm). Colors indicate up (red) or down (blue) accumulation of nutrients. The intensity of the color indicates a significant (red or blue) or marginally significant (light red or light blue) accumulation. Asterisks indicate significant effects of each mycorrhizal treatment respect Nm (AM vs Nm) for each time and tissue within the same genotype according to t-test: +:p < 0.1; *:p < 0.05; **:p < 0.01; ***:p < 0.001.

Wild type									
Herbovory	T0			T21 Leaf			T21 Root		
Nutrient (ppm)	Nm	Fm	Ce	Nm	Fm	Ce	Nm	Fm	Ce
C	398286	389482 *	396425	420620	423219	419507	328525	306388	392841 **
N	43701	41462	40881	25274	26559	25977	17476	16644	19904 +
Al	41	44	43	20	19	18	12101	13180	1753 ***
Ca	17088	18591	17337	14204	12398 +	11871 *	4619	3994	3812
Cr	1.7	2.7	2.0	0.0	1.1 +	0.3	619.7	612.3	18 ***
Cu	16	22 *	23 *	7	9 **	12 ***	37	66 *	101 ***
Fe	264	270	225 +	102.4	103.5	99.4	6793	7545	3052
K	17022	20588 *	20421 *	12539	14704 *	16228 **	2809	8998 **	10628 ***
Li	8.0	15 *	40.1 ***	2.2	4 **	14 ***	3	7 *	4
Mg	11651	12600 +	11607	7236	5811 **	5774 **	27531	30363	9471 ***
Mn	163	179	196 **	66	80 *	105 ***	131	180 *	190 *
Na	618	588	655	641	459 **	546 *	3973	2188 *	780 **
Ni	4.6	4.5	2.7	2.9	3.1	0.3 ***	192	191	9 ***
P	2063	1953	1950	1189	1254	1205	949	1088	1161
S	6369	6821	6656	5122	5365	4918	1566	1588	1324 +
Si	472	564	629 +	352	342	312	104	96	164 *
Sr	47	68 **	72 **	28	27	32 +	19	20	22
Ti	1	4	3	0	0	0	1053	1125	154 ***
Zn	70	52	50	36	38	35	35	27 *	22 **
Slwak1									
Herbovory	T0			T21 Leaf			T21 Root		
Nutrient (ppm)	Nm	Fm	Ce	Nm	Fm	Ce	Nm	Fm	Ce
C	389552	392380	394379	426264	425185	419191	338576	288179	376826 +
N	40534	39314	43668	27522	26404	26718	18967	15584 +	20406
Al	34	26	28	16	14	16	9961	8687	3392 *
Ca	19239	13959 *	14936 *	12555	9673 **	11225 **	4752	3473 **	3734 *
Cr	4	1	2	0	0	0	520	306	55 +
Cu	14	20 *	20 *	5	9 **	10 ***	29	103 **	121 ***
Fe	273	202 *	253	72	81	96 *	5492	5626	2774 *
K	15526	16488	17139	8753	12861 ***	13451 ***	1785	9878 ***	11745 ***
Li	9	28 *	32 ***	2	8 *	12 ***	3	6 **	5.6 *
Mg	13242	10144 +	10118 +	5980	4901 **	5389 *	22719	21470	13028 *
Mn	180	176	175	46	80 **	97 ***	132	162 +	194 **
Na	551	497	467	456	425	449	4422	1311 **	1032 **
Ni	5	2 **	1 **	2	0 ***	0 ***	162	99	22 **
P	1563	1423	1615	906	954	974 *	1029	915	1253 *
S	5898	4839	5198	4092	3795	4113	1609	1257 **	1366 **
Si	412	383	375	287	347 +	401 *	96	111 *	119 **
Sr	55	56	60	23	25	30 ***	20	19	24 +
Ti	0	0	0	0	0	0	845	781	341 *
Zn	55	43	41	23	15 **	17 *	32	30	50 +

FIGURE S2. Transcriptional regulation of flavonoid-related genes. Gene expression analysis by real-time qPCR was determined in wild type (filled bars, WT) and mutant *Slwak1* (stripped bars, wak1) leaves before (T0) and after 2 days of local herbivory (T2L) in non-mycorrhizal (Nm, grey) and mycorrhizal (Fm, blue or Ce, green) plants. Expression values are means of four biological replicates, normalized to the housekeeping gene actin. Flavonoid-related marker genes coding for chalcone isomerase 1 (*CHI1*), flavonoid-3'-hydroxylase (*F3'H*) and flavonol synthase (*FLS*). Data not sharing a letter in common differ significantly according to ANOVA, Fisher's Least Significant Difference (LSD) test (p<0.05).

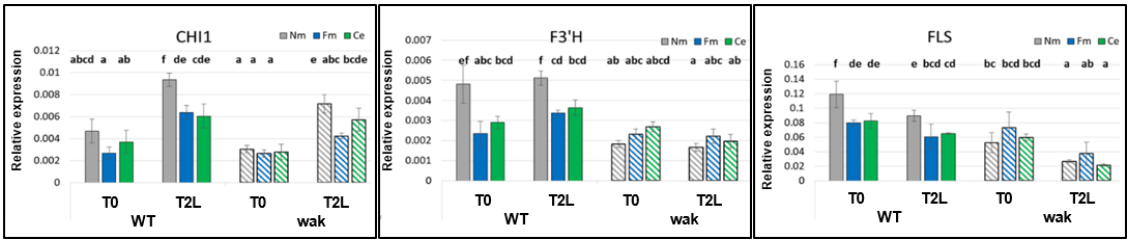

TABLE S3. Metabolites accumulated in Ce-colonized plants under local herbivory in both WT and *Slwak1*

ESI+			
Pathway		Compound	Mass exact
Phenypropanoids	Flavonoid biosintesis	Isoliquiritigenin/ 7;4'-Dihydroxyflavanone; Liquiritigenin/ Pinocembrin ch	256.12
	Phenylpropanoid biosynthesis	Chavicol / (E)-4-Hydroxypropenylbenzene; (E)-4-Propenylphenol; Isochav	134.11
alkaloids	Isoquinoline alkaloid biosynthesis	Cephaeline	466.26
	Phenylalanine//alkaloid	(S)-alpha-Amino-beta-phenylpropionic acid// D-Phenylalanine//Ephedrine	165.08
	Tropane, piperidine and pyridine alkaloid biosynthesis	3,6-Dihydronicotinic acid	125.08
	Ubiquinone and other terpenoid-quinone biosynthesis	3;4-Dihydroxy-5-polyprenylbenzoate;	290.19
	Biosynthesis of alkaloids derived from terpenoid and polyketide	Deoxynupharidine	233.20
terpenoids	Terpenoid	Solavetivone / Albaflavenone /Costunolide	218.17
	Diterpenoid biosynthesis	Momilactone A; 2,3-Dehydro-gibberellin A9	314.18
others	Biosynthesis of secondary metabolites	3-(3-Amino-3-carboxypropyl)-N6-(delta2-isopentenyl)-adenine; Discadeni	304.17
	Porphyrin metabolism	Bilirubin / (3Z)-Phytochromobilin / 15;16-Dihydrobiliverdin	584.28
	Carotenoid biosintesis // 2-Oxocarboxylic acid metabolism	2-cis;4-trans-Xanthoxin // 2-(5'-Methylthio)pentylmalate	250.12
	Folate biosintesis	THF-polyglutamate; Tetrahydrofolyl-[Glu](n); Tetrahydrofolyl-[Glu](n+1); T	574.26
	Steroid hormone biosynthesis	Estradiol	272.17
	Opioid receptor agonists/antagonists	Deltorphin B	782.39
	ND	ND	566.27
	ND	ND	321.25
	ND	5-Oxoavermectin "2a" aglycone	600.28

ESI-			
Pathway		Compound	Mass exact
Alkaloids	Alkaloid	Irinotecan	586.25
	Isoquinoline alkaloid biosynthesis	(1S)-1;2;3;4-Tetrahydro-1-[[4-[2-hydroxy-5-[[(1R)-1;2;3;4-tetrahydro-7-hydroxy-6-methoxy-2-methyl-1-; Berbamunine / Guattegaumerine; N;N'-Dimethyllindoldhamine	596.27
	Biosynthesis of alkaloids derived from shikimate pathway	Quinidinone	322.18
terpenoids	Diterpenoid biosynthesis	Gibberellin A37 open lactone	364.20
	Diterpenoid biosynthesis	Stevioside	804.38
JA metabolism	Jasmonic acid biosynthesis	OPDA internal library	292.21
	Jasmonic acid metabolism	(-)-Methyl jasmonate; Methyl jasmonate / (+)-7-Isomethyljasmonate	224.14
Others	Lignanes	Lariciresinol-diglucoside	684.22
	Lignanes	Lariciresinol acetate	402.19
	Porphyrin and chlorophyll metabolism	Red chlorophyll catabolite	626.26
	Porphyrin and chlorophyll metabolism	3-Vinylbacteriochlorophyllide	616.24
	Carbon metabolism	5;10-Methenyl-5;6;7;8-tetrahydromethanopterin	786.27
	Fatty acid biosynthesis	(9Z)-Hexadecenoic acid/ Palmitoleic acid	254.19
	Alpha-Linolenic acid metabolism	Stearidonic acid / (6Z;9Z;12Z;15Z)-Octadecatetraenoic acid	276.21
	ND	ND	610.27
	ND	ND	496.25
	ND	ND	238.16

Discussion

DISCUSSION

Plants are sessile organisms that, in the course of evolution, have developed a plethora of specific strategies and mechanisms to cope with stresses. To unravel these strategies and to design strategies to boost plant defenses in an environmentally friendly manner is crucial. The application of beneficial microorganisms is emerging as a very valuable alternative to chemical inputs in agriculture, as they could address key crop production challenges by enhancing plant nutrient and water uptake efficiency and, at the same time, improving plant tolerance to unfavorable conditions (Mącik et al., 2020; Pozo et al., 2021). Among beneficial microorganisms, soil fungi, and particularly Arbuscular Mycorrhiza Fungi (AMF) are able to increase plant fitness, improving plant production and strengthening plant defenses, without compromising environmental health.

However, plant-microbe interactions are complex, and the outcome of symbiotic interactions may vary according to the partner's genetic background and the environmental conditions, particularly the biotic and abiotic stresses they have to face. Among the multitude of menaces that plants have to deal with, insects are one of the most critical challenges to plant survival, in particular, chewing herbivores that provoke strong mechanical damage in plant tissues. It is crucial that the plant recognizes the herbivore in order to reprogram its own metabolism and trigger an efficient signaling defense response.

Several studies have shown that mycorrhizal plants display an enhanced resistance (mycorrhizal induced resistance; MIR) against chewing herbivores and necrotrophic pathogens that is mainly attributed to a potentiation of plant defense mechanisms regulated by the jasmonic acid (JA) signaling pathway (Jung et al., 2012; Song et al., 2013; Mora-Romero et al., 2015). Although MIR has been described in multiple herbivore-plant systems (Schoenherr et al., 2019; Selvaraj et al., 2020; Rivero et al., 2021; Jiang et al, 2021; Shafiei et al, 2022), opposite effects have also been reported (Bennett et al., 2009; Hoffmann et al, 2009; Bernaola et al., 2018; Zeng et al., 2022; Ramirez et al., 2022). Despite priming of plant defenses, changes in the nutrition status of the plant can alter the nutritional value of the plant tissues for the herbivore, and thus, may benefit the herbivore (Hartley and Gange, 2008; Pozo et al., 2021). The final outcome of the interaction depends on the balance of the different effects -nutritional and defensive-, and the relative contribution of each of them also depend on the environmental conditions, for example, on nutrient availability. These different outcomes of AM symbiosis

highlight that the variability of MIR in natural systems is highly context-dependent and the need for a deeper knowledge of the molecular mechanisms underlying priming and MIR.

The present Doctoral Thesis has focused on exploring the reprogramming of the plant's primary and secondary metabolism during MIR in different contexts. We first analyzed how Phosphorus (P), a key element in plant nutrition and an essential regulator of the mycorrhizal symbiosis may influence this reprogramming and MIR against different aggressors, including the fungal necrotrophic pathogen *Botrytis cinerea* and the chewing herbivore *Spodoptera exigua*. Then, we explored the molecular mechanisms leading to the increased resistance of mycorrhizal plants to herbivore attack by using tomato plants altered on damage perception.

In **Chapter 1** we explore how P, as a key element in mycorrhizal symbiosis, plant nutrition and immunity, may influence the context dependency of MIR.

P affects *S. exigua* performance

In our study we found that P levels affect both *B. cinerea* lesion development and *S. exigua* performance, displaying reduced pathogen infection and reduced larval performance (lower weight and higher mortality) at the lowest P fertilization as compared to the other fertilization regimes. The worst larval performance in P-deficient plants found in our system agrees with previous studies in different plant-herbivore systems where a reduction of P nutrition was related to a reduction of larval fitness (Khan et al., 2016; Qu et al., 2021). Then, we explored whether this effect on larval performance was associated with nutritional or plant immunity aspects.

P *per se* is an essential element not only for plants but also for caterpillars (Perkins et al., 2004), but in addition, we found that P levels influenced also other plant macro- and micro-nutrients, such as C and N, which are key limiting nutrients for insect herbivores (Mattson, 1980). Then, P availability strongly affects the nutritional value of the plant tissues for caterpillar development.

Besides its role in the nutritional status of the plant, pieces of evidence show that P has a regulatory function in plant immunity (Campos-Soriano et al., 2020; Chan et al., 2021; Val-Torregrosa et al., 2022). Under conditions of P starvation, the jasmonic acid pathway can be activated and potentially increase the plant resistance to eventual attackers (Khan et al., 2016; Luo et al., 2021). Although we found higher JA-related gene expression in plants with low P, it was not correlated with higher JA content. The results suggest that the worst larval fitness depended, instead of a stronger activation of defenses, more likely on a poor quality of the

leaves in this growing condition. Indeed, plants upon the lowest P regime showed reduced biomass, generally lower nutrients and higher anthocyanin content.

P influences the outcome of MIR

Although the influence of P was also evident in plants colonized by *F. mosseae*, the AM symbiosis buffered its effects on *S. exigua*. We observed that the effects of mycorrhizal colonization on larval biomass ranged from moderately positive when P levels were lowest to negative when P levels were moderate. Then, only in the latter condition, MIR was significant, with herbivore development adversely affected, resulting in increased mortality rates, reduced biomass, and fewer individuals reaching the pupal stage. This is in accordance with the fact that the outcome of plant-beneficial microbe interaction can range to have a positive, neutral or negative impact on plant growth and resistance to stresses depending on the context (Pozo et al., 2020). Indeed, in the lowest P fertilization, mycorrhiza promoted nutritional benefits to reduce the negative effects of nutrient deficiency. Then, when nutrition deficiency was not a major challenge, the symbiosis promoted defensive benefits. This is in agreement with previous results in which, during double stress due to transient N depletion and pathogen attack, mycorrhizal plants reprogram their defensive mechanisms towards prioritizing abiotic stress tolerance, (Sánchez-Bel et al., 2016), confirming that the effective protection of MIR is context-dependent. Then, MIR is highly dependent on the growing conditions and the stresses the plant has to face, and its effectiveness may vary depending on the prevailing environmental factor.

In order to gain insight into the mechanisms that influenced the outcome of MIR, we initially examined the nutritional plant aspects. Mycorrhizal colonization positively enhances the plant's ability to uptake P from the soil (Smith and Smith, 2011), and accordingly, we found that AM symbiosis led to higher P levels in plants at both low and sufficient P fertilization. In addition, at the lowest P, AM symbiosis increases carbon (C) concentrations, while it does not influence the nitrogen (N) plant concentrations. Then, when considering if the better plant nutritional status, due by mycorrhizal colonization, may favor the herbivore performance (Koricheva et al, 2009; Pozo et al., 2020), only the C –but not P nor N– increase could have a positive effect on larvae (thus, the changes may explain higher larval biomass in AM than non-mycorrhizal plants only at the lowest P fertilization). However, under moderate P levels, no appreciable plant nutritional differences may explain the worst larval performance in AM plants. Then, we explored if the differences in MIR were associated with plant immunity aspects.

AM symbiosis primes plant defenses in a P-dependent manner

In order to explore the regulation of plant defenses, we analyzed the capacity of the plant for defense by studying early defense responses upon the application of the damage-associated molecules oligogalacturonides (OGs). Then, we searched for key regulators that may explain the observed enhanced resistance.

The JA signaling plays a crucial role in coordinating plant defense responses against herbivores and necrotrophic pathogens, and it is a central modulator in three-way interactions between plants, microbes, and arthropods (Gruden et al., 2020). Remarkably, priming of plant defenses by beneficial microorganisms typically occurs through the JA signaling pathway (Pieterse et al., 2014), and this is the case also with MIR (Mora-Romero et al., 2014). In accordance with this, we found higher induction of well-characterized anti-herbivory JA-related defense marker genes as *Pin II*, *MC*, *LapA* and *TD* (Uppalapati et al, 2005; Yan et al, 2013) in response to OG. Remarkably, priming of plant defenses in mycorrhizal plants occurred only under sufficient P levels, but not under the lowest P conditions. Then, our research findings confirm that the availability of P is a crucial factor influencing the context-dependent nature of MIR and the associated priming of plant defenses.

AM symbiosis modulates the growth-defence trade-off

The balance between growth and defense and their trade-offs is fundamental for plant development and survival. Upon biotic attack, plants reallocate their resources from growth to defense, downregulating the expression of genes involved in growth and increasing those related to defense (He et al., 2022). If nutrition is most limiting, resources are required for growth and the activation of defenses against attackers is impaired. In our study, mycorrhizal colonization improves the plant growth–defense trade-offs tailoring the plant response to improve growth under the most limiting nutrient conditions and to defenses when nutrition was not so restricted.

Indeed, only when plants were in a status of P deficiency, AM symbiosis seemed to "direct the effort" to increase plant growth and improve the plant nutritional status (e.g. increase plant biomass, C, P) supporting that mycorrhizal plants prioritized the improvement of plant growth. Under this growing condition, mycorrhizal plants upregulate genes related to carbohydrate metabolism and coding for the jasmonic acid repressors JAZs, compared with non-mycorrhizal ones. These repressors inhibit the activity of key transcription factors involved in JA signaling, thereby repressing the expression of downstream JA-responsive genes and

downregulating defenses (Wang et al., 2021; Liu and Timko, 2021). On the other hand, when plants had sufficient P there were no differences in *JAZs* expression between mycorrhizal and non-mycorrhizal plants suggesting that when no deficiency was present, AM symbiosis does not downregulate JA signaling to promote growth.

Instead, under these conditions of sufficient P, mycorrhizal plants prioritized the activation of defenses increasing the expression of defense-related genes upon herbivore attack. This is in agreement with an optimization of the use of the resources depending on the specific needs when facing multiple stresses (Smakowska et al., 2016). In accordance, we demonstrate in **Chapter 1** that under P deficiency mycorrhizal plants prioritize the improvement in nutrition, while under damage perception AM plants efficiently prioritize the defense activation whether their nutritional status is not compromised. On the contrary, at the lowest P fertilization, non-mycorrhizal plants displayed a higher expression level of JA-related marker genes before and lower expression after the OG elicitation, showing a growth- and defense-related gene expression imbalance. It suggests that these plants activated JA-dependent defenses without a biotic challenge, -probably just as a general stress response to the nutritional stress- but fail to trigger plant defenses after perceiving a real damage, while in the same P condition, the mycorrhizal colonization buffers this growth-defence imbalance. On the other hand, both, non-mycorrhizal and AM-plants properly activate their defenses upon damage perception when sufficient P is available. Nonetheless, the defense responses in this moderate P condition, were boosted in AM plants, showing a stronger induction (priming). Also, in a N-deficiency context, mycorrhizal plants exhibited a differential regulation of growth-defense balance as shown in the study by Sanchez-Bel et al. (2018). Our study supports that the AM symbiosis fine-tunes the plant growth-defense balance according to nutrient availability.

Plant response to OG and herbivory

Although OGs have not been described in response to chewing herbivory, it has been postulated that these molecules are released under herbivore attack (Aljbory et al., 2018; Erb and Reymond, 2019). These molecules are among the best-characterized plant DAMPs released in plant cells (De Lorenzo et al., 2011; Ferrari et al., 2013; Benedetti et al., 2015; Pontiggia et al., 2020). It is well established that wounding or pathogen attack provokes the release of OGs through the activity of endogenous or exogenous poligalacturonases, triggering defense responses and promoting immunity in different plant species, including tomato (Cervone et al., 1989; De Lorenzo and Ferrari, 2002; D'Ovidio et al., 2004; Bergey et al., 1999; Orozco-Cardenas

et al., 1999; Ferrari et al., 2013; Benedetti et al., 2015). The results in **Chapter 1** –higher expression of JA-related genes coding for anti-feedant proteins that impair herbivore fitness upon OGs elicitation– and the fact that chewing herbivores, for their feeding guild, may release OGs, triggering wound responses and inducing endogenous PG, support this assumption. Hence, to explore whether damage perception, likely through OG perception, has a function in plant response to herbivory, we analyzed the role of SlWAK1 in tomato plants with altered expression levels of this gene, the mutant *Slwak1* (Meco et al., 2020) (**Chapter 2**). WAK1 has been described as a major OGs receptor in Arabidopsis, and docking analyses point to a high affinity of the SlWAK1 for pectin oligomers in tomato plants (Kohorn, 2016; Kurt et al., 2020). Our results confirm that *SlWAK1* is transcriptionally upregulated by OG application (**Chapter 1**) and in response to local herbivory (**Chapter 2**). Then, the upregulation of *SlWAK1*, upon both stimuli, supports the possible relation between chewing herbivory and OGs release.

In addition, to explore the function of the gene, the *Slwak1* tomato mutant was used (Meco et al., 2020). We found that the mutant had reduced expression levels of *SlWAK1* compared with the wild-type in the absence or presence of herbivory, suggesting an impairment in damage perception. Then, in **Chapter 2**, we tried to elucidate the role of SlWAK1 in the regulation of plant defense responses to herbivory.

Alteration of SlWAK1 impairs plant defense responses to herbivore attack

A correct perception of the challenge is fundamental for the plant to trigger the most appropriate defense response to limit damage and re-establish homeostasis conditions. Then, signal perception represents the most important step to initiate any defense response (Mostafa et al., 2022). The cell wall represents the first layer of defense for the plant cell and it is fundamental for the plant to monitor cell wall integrity (Hamann, 2015). Because of their location in the cell wall, WAKs are among the first receptors operating in damage perception, triggering a chain of reactions that leads to plant defense responses (Stephens et al., 2022).

In our research, we tried to elucidate the role of SlWAK1 in the regulation of plant defense responses to herbivory. It has been demonstrated an increased expression of the gene coding to this receptor in response to the PAMPs flg22 and flg28 and plants silenced in *SlWAK1* display enhanced disease symptoms against bacterial infection in tomato (Zhang N. et al., 2020; Rosli et al., 2013). However, to our knowledge, no studies of the SlWAK1 receptor role against herbivores have been described. Then, in **Chapter 2** we explored the role of SlWAK1 in plant

defences against *S. exigua* using the tomato mutant plants *Slwak1* altered in the promoter region of this receptor.

Our results demonstrated that SlWAK1 has an important role in plant defenses as the mutant resulted more susceptible to *S. exigua* infestation than wild-type (WT) plants. In order to analyze whether this different larval performance was attributable to compromised plant defenses, we explored whether this effect on larval performance was associated with plant immunity aspects. We observed that, upon herbivore attack, JA and ethylene (ET) transcription levels increased in both genotypes following similar patterns. Then, the results do not suport a role of SlWAK1 in regulating these responses and that deficiencies in JA and ET signaling are not responsible for the reduced susceptibility of the mutant to *S. exigua.*

However, upon herbivory, *Slwak1* mutants showed reduced expression -compared with WT- of genes involved in auxin signaling. This hormone has been described to increase in response to herbivory and to be involved in carbohydrate (CH) reallocation during the response (Machado et al., 2013; 2016). Moreover, its accumulation promotes the production of secondary metabolites as phenolics and flavonoids (Lulu et al., 2015; Mahdieh et al., 2015). Accordingly, in response to herbivory, mutant plants also showed a reduced induction of genes involved in the biosynthesis of secondary metabolites, such as phenylpropanoid, flavonoid and alkaloid compared with the WT.

The untargeted metabolomic analysis also evidenced a compromised accumulation of flavonoid, terpene, phenol, lignan and alkaloid compounds in the mutant after herbivory, and some of them also showed lower basal levels. Several studies confirm an accumulation of these secondary metabolites in tomato plants in response to herbivory and to OGs application (Steinbrenner et al., 2011; da Silva Souza et al., 2020; Miklavčič Višnjevec et al., 2021; Rivero et al., 2021; Gamir et al., 2020).

Then our results support a role of SlWAK1 in auxin signaling regulation that may contribute to the alteration in the plant metabolism and the differential accumulation of some secondary metabolites observed in the mutant. The failure of *Slwak1* plants to upregulate the expression of phenylpropanoid, flavonoids and alkaloid-related genes under attack and the corresponding reduced accumulation of defensive compounds, may have contributed to increase *S. exigua* fitness in mutant plants.

SlWAK1 is involved in CH metabolism and its impairment alters carbon partitioning in response to herbivory

The dysfunction of the SlWAK1 receptor led to a basal alteration in primary metabolism. Specifically, the mutant had lower energy status than WT plants due to increased glycolysis activity and lower levels of soluble sugars, TCA cycle intermediates, and starch biosynthesis. Many primary metabolites products derived from glycolysis and TCA cycle often serve as precursors for the synthesis of secondary metabolites (Pott et al., 2019). Then, the reduction in sugars and TCA cycle intermediates may contribute to the reduction of secondary metabolites that we found in *Slwak1* mutants already at basal level.

The *Slwak1* alteration in CH metabolism was more extreme upon *S. exigua* attack. We observed that, under herbivory, the activity of cell wall invertases increased and, consequently, the sucrose content was reduced, as well as the starch accumulation, in WT plants. On the other hand, *Slwak1* plants followed an opposite strategy, increasing starch and sucrose accumulation. When plants are attacked by herbivores, they need more energy to produce inducible defenses, usually mobilizing resources from reservoirs and promoting local catabolism to produce defense-related metabolites (Zhou et al., 2015). The invertases, mainly the cell wall invertase, play a crucial role in CH reallocation and sink strength of the tissues influencing the capacity to import assimilates and leading to a reduction of long-distance sucrose export (Roitsch and Gonzales, 2004; Veillet et al., 2016; Dong et al., 2019). In addition, plants usually turn local-wounded leaves into sink tissue to reduce the level of energetic compounds and synthesize secondary metabolites as chemical defenses (Arnold and Schultz, 2002; Steinbrenner et al., 2011; Ferrieri et al., 2012). Then, it is clear that under *S. exigua* attack the mutant is compromised in the carbohydrate partitioning and this may impair its ability to induce plant defenses.

Overall in **Chapter 2**, we demonstrated that alterations in SlWAK1 compromise the activation of effective defense responses against *S. exigua*. Our findings indicated that the coordination between defense responses, primary carbohydrate metabolism and assimilate partitioning, inducing sink and suppressing source activities, was operational in WT plants in response to herbivores, but was clearly altered in the *Slwak1* mutant. Therefore, our results suggest that SlWAK1, likely through the deficient perception of OGs, plays a role in coordinating tomato defense responses against chewing insects by regulating primary metabolism, carbohydrate reallocation and the biosynthesis of defensive secondary metabolites.

SlWAK1 is not essential for MIR

In **Chapter 2** we demonstrated that SlWAK1 has a fundamental role in plant defense responses against *S. exigua*, and previous results in the group confirmed the ability of mycorrhizal plants for a primed response to OG. Then, we hypothesized that the *SlWAK1* gene, for its function in regulating plant immunity, may have a role in mycorrhizal-induced resistance (MIR). In **Chapter 3** we evaluated the effect *S. exigua* in WT and *Slwak1* mutant plants colonized by *F. mosseae* or *C. etunicatum.* We observed host plant protection in both AMF-colonized genotypes, demonstrating that AM symbiosis significantly impacts herbivore performance, regardless of the levels of the SlWAK1 receptor. However, an aspect of MIR was compromised in the mutant. Although the larval survival and individuals reaching pupae and moths stage were compromised in the AM mutant as well as, or even more than, WT plants, the larval biomass reduction was lost in *Slwak1*. Then, although SlWAK1 may mediate some aspects of MIR, our results do not show an essential role in MIR. Nevertheless, it is worthy to consider that the mutant had lower levels of expression, but still there is some expression and response to herbivory. Because the mutation is in the promoter, the protein should be functional, so we cannot rule out that the reduced levels are sufficient for its function in MIR.

In **Chapter 2** we discover that, upon herbivore attack, the JA signaling pathway followed a similar pattern in both genotypes, suggesting no role of SlWAK1 in regulating JA response. Accordingly, in **Chapter 3** we found that under herbivory, the transcriptional levels of JA-related genes increased in the mycorrhizal plants of both genotypes. The well-established JA-dependent priming of plant resistance (Song et al., 2013; Mora-Romero et al., 2014) occurred in AM WT (as we also see in mycorrhizal plant elicited with OGs in **Chapter 1**) as well as in AM mutant plant, then it is not influenced by SlWAK1.

Attending to the leaf metabolomic profile, mycorrhizal colonization modified the mutant metabolome already under basal conditions (without herbivory), while in WT plants, the differences were only evident under herbivore attack. As demonstrated in previous studies, AM symbiosis can increase plant resistance to herbivores by activating both constitutive and inducible defenses that lead to a higher accumulation of metabolites with toxic effects against insects (Bennett et al., 2009; Rivero et al., 2021). Although, in **Chapter 3** we found some toxic compounds constitutively accumulated in mycorrhizal plants, mainly in Ce-colonized plants, the higher differences in the accumulation of secondary metabolites - lignans, alkaloids and phenylpropanoids- occurred in AM plants subjected to *S. exigua* herbivory. All these secondary metabolites have antinutritive and/or toxic effects on herbivores (Lattanzio et al., 2006; Després et al., 2007; Heidel-Fischer et al. 2015; Züst et al., 2016; Merillon and Ramawat, 2020).

Remarkably, we found a higher accumulation of azelaic acid, previously described to accumulate under herbivory in different plant species (Mithofer and Maffei, 2016; Marti et al., 2013) and showed a primed accumulation in mycorrhizal tomato under herbivory (Rivero et al., 2021) his compound has a direct negative effect on *S. exigua* fitness (Rivero et al. 2021). This compound is a key signal molecule in induced systemic immunity, priming defense-related genes expression and showing antifeedant and toxic activities (Djami-Tchatchou et al., 2017; Jung et al., 2009; Yu et al., 2013; Shine et al., 2019). Thus, the herbivory led to a rearrangement of the metabolic profile increasing the accumulation of secondary metabolites in Ce-colonized plants of both genotypes. It, again, highlights that AM symbiosis primes plant defenses independently of SlWAK1.

Mycorrhizal colonization influences the carbohydrates flux within the plant

In **Chapter 2** we showed that the mutant had lower energy status than WT plants. In the following **Chapter 3,** we found that AM symbiosis modified the carbohydrate flux compensating for the deficient energy status of *Slwak1*. In both genotypes, mycorrhizal colonization reduces the activity of cytosolic invertase in leaves. In WT there was a corresponding change in soluble sugar content, suggesting a possible carbon transfer to the fungal symbiont. Changes in carbohydrate flux are in accordance with that the symbiotic relationship between plants and AMF depends on the availability of plant carbon compounds and that the presence of the fungal symbiont requires an extra demand of energy (Salmeron-Santiago et al., 2021). In *Slwak1* plants, mycorrhizal colonization led to a significant increase of all glycolysis-related and starch biosynthesis-related enzymes, promoting starch accumulation to levels similar to those in WT plants. Then, the symbiosis buffers the effects of *Slwak1* deficiency and compensates for the inefficient carbohydrate metabolism and partitioning of the mutant.

In response to herbivory, the AM symbiosis improves the source-sink relations and redirects the altered carbon partitioning in *Slwak1*

Under herbivore attack, mycorrhizal plants displayed a more efficient carbohydrate metabolism in both genotypes. Although in some aspects AM symbiosis showed opposite trends in WT and *Slwak1* plants (such as the activity of glycolysis-related enzymes and invertases), mycorrhizal plants increased sucrose biosynthesis and transport and decreased the activity of enzymes related to starch biosynthesis in both genotypes. Plants use sugars as an energy source to activate defense mechanisms as well as signaling molecules to regulate defenses (Roitsch and

González, 2004; Veillet et al., 2016). Increasing the activity of sugar transporters appears to be a common strategy for plants to face stress and the corresponding C partitioning may redirect sugars toward defense-related processes (Baena-Gonzalez et al., 2007; Bhonwong et al., 2009; Arnold and Schultz, 2002). Thus, a reduction of starch production and a higher sucrose biosynthesis and transport in mycorrhizal plants is in agreement with a better mobilization of resources to activate defenses.

We demonstrated in **Chapter 2** that, when non-mycorrhizal *Slwak1* plants were attacked by herbivores, they exhibited an opposite strategy compared to WT. However, when the mutant plants were colonized by AMF, the direction of the changes was reverted, displaying a similar pattern to that of WT. This suggests that mycorrhizal colonization can mitigate the defects of the mutant's primary metabolism regulation, restoring proper C partitioning in response to herbivory, similar to WT plants.

Overall our results showed that the mycorrhizal colonization (**Chapter 3**) increases plant resistance against *S. exigua* in WT and *Slwak1* mutant plants and induced higher accumulation of both basal and induced secondary metabolites. These findings point out that MIR is also effective in plants compromised in SlWAK1 and that AM symbiosis primes plant defenses in response to herbivory regardless of plant genotype (WT or *Slwak1*). In addition, mycorrhizal colonization modulates the host primary metabolism in both genotypes. Moreover, in *Slwak1* mutant, the AM symbiosis compensates for the inability to properly regulate C partitioning. After herbivore attack, the AM symbiosis enhances the sink strength in WT and reverts the direction of sugar flux in the mutant. Then, the mycorrhizal colonization buffers the heightened susceptibility of *Slwak1* and efficiently enhances mutant resistance.

Concluding remarks

In summary, the investigation carried out in this Doctoral Thesis illustrates how mycorrhizal colonization helps plants to cope with suboptimal conditions by buffering the potential impact of negative external environmental factors (nutrient deficiency) or even internal imbalances, such as the reduced ability to perceive damage and the deficient regulation of CH metabolism in the *Slwak1* mutant. Our results reveal that when resources are limited, mycorrhizal plants prioritize growth, but when nutrition is not so limiting and an attack occurs (such as herbivory), the symbiotic plants redirect more efficiently plant resources towards defense, providing a more rapid and robust response than non-mycorrhizal plants. Additionally, mycorrhizal colonization may partially compensate for deficiencies in the coordination of plant

responses to an attack. Indeed, we found that mutant plants with reduced expression levels of the *SlWAK1* showed higher susceptibility to herbivores due to a deficient activation of defenses and deregulation of the primary and secondary metabolism trade-off. In such case, AM symbiosis compensated the mutant deficiency, boosting plant defenses by priming defense-related hormone pathways and secondary metabolite biosynthesis, and properly regulating carbon partitioning by reverting the direction of sugars allocation, reverting the phenotype to levels in the WT plants.

Therefore, our research supports a role for mycorrhizal symbiosis in buffering the impact of potentially negative factors, tailoring plant responses through transcriptional and metabolic reprogramming towards the most important challenge. Thus, the symbiosis seems to contribute to the plant prioritization of stress responses.

AMF inoculation represents a powerful solution for mitigating the impact of increasingly extreme environmental conditions associated with climate change. Indeed, besides increasing plant efficiency in obtaining nutrients and water, AMF also enhance plant tolerance and resistance against a wide range of stresses. As such, they are an invaluable tool in the fight against the effects of climate change and can help to improve the future of natural resources and agricultural production.

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Conclusions

CONCLUSIONS

1. Mycorrhiza-induced resistance against the chewing herbivore *Spodoptera exigua* and the fungal pathogen *Botrytis cinerea* is influenced by phosphorus (P) fertilization in tomato plants colonized by *Funneliformis mosseae*.

2. Mycorrhizal plants display primed jasmonate-regulated defenses upon activation of damage signaling by exogenous application of oligogalacturonides, but dependent on P availability. Priming does not occur under the lowest P fertilization level.

3. The growth-defense balance is differentially fine-tuned in mycorrhizal plants according to P availability. The symbiosis tailors the plant to prioritize primary metabolism and nutrition-growth-related responses under P deficiency while promoting priming of plant defenses when P is not limiting.

4. P availability is a key factor contributing to the context dependency of MIR. The mycorrhizal status of the plant modulates the impact of P on JA-dependent defenses and plant resistance.

5. The gene coding for the cell wall-associated kinase receptor SlWAK1 is transcriptionally upregulated upon herbivory. Phenotypic analyses of a tomato line with reduced levels of *SlWAK1* expression revealed that this gene contributes to the proper regulation of plant defense responses against herbivory, as *Spodoptera exigua* larvae performed better when feeding on the *Slwak1* mutant line than in the wild-type.

6. SlWAK1 has a role in the regulation of carbohydrate metabolism and partitioning and the biosynthesis of defensive secondary metabolites: phenylpropanoids (flavonoids, lignin), alkaloids, and terpenes.

7. Tomato wild-type and mutant *Slwak1* plants colonized by *F. mosseae* or *Clareidoglomus etunicatum* show increased resistance against *Spodoptera exigua* compared with non-mycorrhizal ones. Mycorrhizal plants, mainly those colonized by *C. etunicatum*, showed primed induction of jasmonate-regulated defenses and accumulation of defensive

secondary metabolites, in response to herbivory in both wild-type and *Slwak1* genotypes.

8. Mycorrhizal colonization modulates the plant's primary metabolism in both genotypes, amplifying the sink strength after herbivore attack in the wild-type. In the *Slwak1* mutant, the symbiosis alters the direction of sugars movement, compensating for the inability of *Slwak1* mutants for proper carbohydrate partitioning.

9. Mycorrhizal colonization buffers the highest susceptibility of the *Slwak1* mutant, so that MIR against *Spodoptera exigua* is even stronger in this genotype.