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EXPRESIÓN DEL GEN ISOPENTENILTRANSFERASA (IPT) Y SU ACCIÓN SOBRE LA DEFICIENCIA DE NITRÓGENO EN PLANTAS DE TABACO

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Expresión del gen isopenteniltransferasa (IPT) y su acción sobre la deficiencia de nitrógeno en plantas de tabaco

Memoria de Tesis Doctoral presentada por la licenciada en Biología
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"El conocimiento es poder,
pero solo la sabiduría es libertad"

Will Durant

Índice

Resumen

El gran reto del Siglo XXI es la creación de una “segunda revolución verde” basada en una mejora de la producción de los cultivos que asegure alimento a la población mundial junto con una aplicación reducida de fertilizantes. La alternativa más razonable para conseguir este objetivo es la utilización de especies o genotipos de plantas que muestren capacidad para crecer y producir de forma óptima en suelos o medios de cultivo con una baja disponibilidad de nutrientes, especialmente de nitrógeno (N), uno de los factores que determina gran parte de la producción vegetal. La utilización de estas plantas con una fertilización reducida es la estrategia más lógica para mantener y desarrollar una agricultura sostenible, rentable económicamente y respetuosa con el medioambiente. Teniendo en cuenta que las plantas tienen una dependencia fundamental del N para su crecimiento se aplican aproximadamente entre 85-90 millones de toneladas de fertilizantes nitrogenados al suelo anualmente, por lo que en la actualidad se hace imprescindible la reducción de este tipo de fertilizantes y especialmente en forma de nitratos (NO_3^-) que es considerado uno de los principales contaminantes agrícolas.

El N es un macronutriente esencial para las plantas, siendo cuantitativamente uno de los elementos minerales más abundantes en los tejidos vegetales. Por tanto, la deficiencia de N, fundamentalmente de nitrato (NO_3^-), ha sido definida como uno de los factores más limitantes en el crecimiento de los cultivos. Las citoquininas (CKs) son fitohormonas que tradicionalmente han sido relacionadas con procesos como división celular, crecimiento y desarrollo, dominancia apical o senescencia, pero en los últimos

años ha sido estudiada la implicación de las CKs en la señalización del estado nutricional y se conoce que esta fitohormona influye en la homeostasis de algunos nutrientes como N, fósforo (P), azufre (S), hierro (Fe). La relación entre CKs y N ha sido la más ampliamente estudiada y se sabe que actúan como mensajeros a larga distancia que controlan la asimilación de este nutriente y el estatus de éste en la planta, por lo que estas hormonas podría ser buenos candidatos para mejorar la utilización del N plantas y poder así reducirse la aplicación de fertilizantes nitrogenados. Por ello y utilizando como diseño experimental la comparación entre plantas de tabaco wild type (WT) y plantas modificadas geneticamente que expresan el gen *IPT* responsable de la síntesis de CKs, los objetivos de esta tesis doctoral fueron los siguientes:

1. Establecer las respuestas fisiológicas de las plantas a la deficiencia de N y definir la implicación en éstas de la expresión del gen *IPT*.

2. Evaluar si la expresión del gen *IPT* en plantas puede concretarse como una herramienta válida y eficaz para mejorar el uso de N y poder reducir así la aplicación de fertilizantes nitrogenados.

Para lograr dichos objetivos se llevaron a cabo los experimentos que se detallan a continuación y en los que se obtuvieron los siguientes resultados:

- Efecto de las CKs sobre el estrés oxidativo en plantas de tabaco sometidas a deficiencia de N

Plantas de tabaco WT y plantas transgénicas que sobreexpresan el gen *IPT*, enzima que codifica el paso clave de la síntesis de citoquininas (CKs), crecieron bajo condiciones de deficiencia de N (7 mM y 1 mM de NO_3^-). Nuestros resultados muestran que las plantas WT sometidas a deficiencia de N redujeron la biomasa y la tasa de crecimiento relativo (RGR) incrementando el daño oxidativo y reduciendo las concentraciones foliares de las distintas formas de N. Sin embargo, las plantas transgénicas P_{SARK}*::IPT*, a pesar de mostrar un descenso significativo de las formas de N en hoja, no mostró alteración del metabolismo oxidativo manteniendo así la biomasa foliar y el RGR bajo deficiencia de N.

- Formación y asimilación de amonio en plantas transgénicas de tabaco P_{SARK}*::IPT* bajo deficiencia de nitrógeno

Se analizaron las enzimas relativas al metabolismo del N y fotorrespiración junto con las formas de N y los aminoácidos (aas) en plantas de tabaco WT y P_{SARK}*::IPT* sometidas a deficiencia de N. Nuestros resultados indican que las plantas WT redujeron la asimilación de NO_3^-. Sin embargo, en estas plantas incrementaron la producción de amonio (NH_4^+) mediante procesos como la degradación de proteínas y la fotorrespiración lo que conllevó un incremento del ciclo glutamina sintetasa/glutamato sintasa (GS/GOGAT). Además en plantas WT la cantidad de aas disminuyó bajo deficiencia de N, aunque la cantidad relativa de glutamato y glutamina incrementó. A pesar de que en las plantas transgénicas las formas de N disminuyeron bajo deficiencia de N, éstas mantuvieron el ciclo GS/GOGAT a niveles del control. Nuestros resultados parecen indicar que bajo deficiencia de N, las CKs previenen de la

formación y asimilación de NH_4^+ que se genera en procesos como la fotorrespiración o la degradación de proteínas.

- Mejora dependiente de CKs de la eficiencia del uso del N y la calidad de plantas transgénicas de tabaco *P_{SARK}::IPT* sometidas a deficiencia de N

Se evaluó el papel de las CKs en la eficiencia del uso del N (NUE) y diferentes parámetros que determinan la calidad de las hojas de tabaco. Los resultados obtenidos indicaron que las plantas de tabaco WT sometidas a deficiencia de N redujeron el cociente hojas:raíz asociado a un descenso del NUE y de los parámetros de calidad de la hoja de tabaco. Por el contrario, las plantas transgénicas *P_{SARK}::IPT* sometidas a deficiencia de N presentaron un mayor NUE así como mayor calidad de las hojas que las plantas WT, como reducción en la cantidad de nicotina e incremento de azucares reducidos. Por tanto la expresión del gen *IPT* podría ser una herramienta útil para mejorar el cultivo de tabaco, reduciendo la aplicación de fertilizantes, manteniendo la producción e incrementando la calidad de la hoja.

- Respuesta de metabolitos ricos en carbono y N frente a la deficiencia de N en plantas de tabaco *P_{SARK}::IPT*

Se analizó el posible efecto de la deficiencia de N sobre compuestos ricos en carbono, como los compuestos fenólicos así como en compuestos ricos en N como poliaminas (PAs) y prolina (Pro), examinando las rutas implicadas en su síntesis y degradación. La deficiencia de N estimuló el metabolismo fenólico incrementando la cantidad de fenoles en ambas líneas de plantas de tabaco WT y *P_{SARK}::IPT*. Esto sugiere que la concentración NO_3^- en

los tejidos podría actuar como señal favoreciendo la acumulación de compuestos fenólicos bajo deficiencia de N. Además encontramos que en las plantas WT las PAs libres se mantuvieron a pesar de la deficiencia de N, lo que podría estar relacionado con una mayor respuesta al estrés generado por la baja disponibilidad de N en estas plantas. Por el contrario, la reducción de PAs libres y Pro observada en plantas *P_{SARK}::IPT* bajo deficiencia de N podría indicar una activación de mecanismos de reciclaje de N que ayudaría a estas plantas a un uso más eficiente de N.

- La expresión de *P_{SARK}::IPT* causa protección de la fotosíntesis en plantas de tabaco durante la deficiencia de N

En este trabajo realizado en la Universidad de Davis-CA (USA) se midieron parámetros fotosintéticos y del metabolismo de los azucares en plantas de tabaco WT y *P_{SARK}::IPT* sometidas a deficiencia de N. Los resultados obtenidos indican que las plantas WT mostraron una drástica reducción de la tasa fotosintética (*A*) y de parámetros como el cociente máximo de carboxilación de la Rubisco (Vcmax), el cociente máximo de la cadena de transporte electrónico (Jmax) así como del uso de las triosas-P (TPU). Sin embargo, la expresión del gen *IPT* bajo el control del promotor P_{SARK}, inducido por maduración y estrés, conllevó un mantenimiento de la fotosíntesis y de los parámetros Vcmax (manteniendo el nivel de expresión de la subunidad pequeña de la Rubisco (sm-Rubisco)), Jmax y TPU, conservando así la biomasa foliar bajo condiciones de deficiencia de N. El limitado incremento de la concentración de azucares junto con el mantenimiento de la biomasa foliar en las plantas transgénicas observado podrían indicar un posible papel de las

CKs en la limitación del sumidero provocado por la deficiencia de N, mejorando la fuerza del sumidero, formado por las hojas jóvenes, y por tanto mejorando la biomasa foliar bajo condiciones de deficiencia severa de N (1mM).

Por tanto las conclusiones que derivan de los trabajos que acabamos de describir serían las siguientes:

1. La deficiencia de N conlleva una reducción de la biomasa y de la tasa de crecimiento relativo foliar en plantas de tabaco WT, debido a un descenso de las diferentes formas de N y una inducción de especies reactivas de oxigeno (ROS) y por tanto del daño oxidativo. Por el contrario, en las plantas de tabaco *P_{SARK}::IPT* la deficiencia de N no afectó la producción de ROS manteniéndose la biomasa y la tasa de crecimiento relativo foliar a pesar de que también se reduce la concentración de las distintas formas de N. Esta repuesta diferencial nos sugiere que el aumento de CKs en plantas de tabaco *P_{SARK}::IPT* podría actuar como señal impidiéndose la aparición de las respuestas típicas de la deficiencia de N.

2. Ante una deficiencia de N severa las plantas WT activan los procesos de generación de NH_4^+ independientes de la reducción del NO_3^-, como son la fotorrespiración, la degradación de proteínas y el aumento de los niveles relativos de aas de transporte, todo ello con el fin de suministrar N a zonas de crecimiento. Por el contrario, las plantas transgénicas *P_{SARK}::IPT* no inducen estas respuestas típicas de la deficiencia de N lo que les permite en estas

condiciones un mantenimiento de la biomasa foliar y una mejora en la calidad de las hojas de tabaco así como en la eficiencia del uso del N.

3. La deficiencia de N estimula el metabolismo fenólico incrementando la cantidad de fenoles, lo cual podría definirse como respuesta a la baja concentración NO_3^- existentes en los tejidos. El mantenimiento de la cantidad de PAs en plantas de tabaco WT bajo deficiencia de N podría estar relacionado con el mayor estrés oxidativo observado previamente en dichas plantas. Por el contrario la reducción de PAs y Pro en las plantas transgénicas podría representar un mecanismo de reciclaje de N bajo situaciones de baja disponibilidad de dicho nutriente.

4. La expresión del gen IPT en plantas de tabaco sometidas a deficiencia de N conlleva un mantenimiento de la fotosíntesis de tal manera que las CKs podrían actuar protegiendo este proceso fisiológico lo que se demuestra por el mantenimiento de los niveles de transcrito de la sm-Rubisco, la cadena de transporte electrónico y la capacidad del cloroplasto para usar las triosas-P. En relación a la relación fuente-sumidero, la inducción en la síntesis de CKs podría ayudar a potenciar la fuerza del sumidero que representan las hojas jóvenes, mejorando la biomasa foliar bajo condiciones de deficiencia severa de N.

Capítulo 1

Introducción

1. Fisiología del NO_3^- en las plantas

1.1. Absorción y distribución

El N es un macronutriente esencial para las plantas, siendo cuantitativamente uno de los elementos minerales más abundantes en los tejidos vegetales (Horchani et al., 2011). Aunque aproximadamente el 80% de la atmosfera esta formada por N, la forma más abundante (N_2) no se encuentra disponible para las plantas, ya que la mayoría de ellas carecen de la capacidad de fijar el N_2 atmosférico (Maathuis et al., 2009). Por ello, las plantas captan N en forma iónica del suelo mediante absorción por las raíces, preferentemente como NO_3^- y de forma menos frecuente como NH_4^+ (Barceló et al., 2005). En muchos suelos, especialmente en los destinados a la agricultura, el NO_3^- es la forma nitrogenada más abundante, cuya concentración normalmente varía dentro de un rango aproximado de entre 0.5 mM y 10 mM (Benton Jones, 1999). Por el contrario, el NH_4^+ se encuentra en el suelo en una concentración de 10 a 100 veces menor (Marschner, 1995). A diferencia con el NO_3^-, el NH_4^+ predomina en aquellos suelos en los que la nitrificación está inhibida, por ejemplo en suelos encharcados o de climas fríos (Mengel y Kirkby, 2001).

La absorción del NO_3^- ocurre a nivel de la raíz y es un proceso dependiente de energía metabólica. Es llevado a cabo mediante dos sistemas de transporte que coexisten y actúan de forma coordinada para absorber el NO_3^- de la solución del suelo y distribuirlo a nivel de planta completa (Tsay et al., 2007). Generalmente, es asumido que la familia de genes NRT1 media los

transportadores de baja afinidad (LATS), que no presentan cinética de saturación de tal forma que el cociente de absorción incrementa con la concentración de forma lineal. Éstos actúan a elevadas concentraciones de NO_3^- en el medio en un rango de 250 μM hasta 50 mM. Por otro lado, el sistema de transportadores de alta afinidad (HATS) codificado por la familia de genes NRT2, actúa cuando la concentración de NO_3^- en el medio es baja (Masclaux-Daubresse et al., 2010). Dentro de los HATS se han propuesto un sistema constitutivo de alta afinidad (cHATS) inducido rápidamente por la presencia de NO_3^-, y saturable a bajas concentraciones de NO_3^- en el medio (< 1 mM), y un sistema inducible de alta afinidad (iHATS) que son inducidos entre horas y días después de la exposición a los NO_3^- (Crawford y Glass, 1998). Considerando estos dos procesos, Ullrich (1987) demostró un mecanismo de co-transporte $2H^+/NO_3^-$, tanto para los sistemas de alta como de baja afinidad.

La absorción de NO_3^- es sensible tanto a señales externas como internas. La absorción de NO_3^- desde la solución del suelo al simplasto de la raíz depende de la concentración de NO_3^- en dicha solución, mientras que el proceso de carga al xilema depende tanto de la demanda de la parte aérea como del contenido de compuestos de N reducidos en el floema (Saravitz et al., 1998). Aminoacidos (Aas) como la glutamina (Gln) llevan a cabo una regulación “feedback” aunque otros Aas, como glutamato (Glu) o aspartato han sido considerados como inhibidores de la absorción de NO_3^- (Muller y Touraine 1992; Gojon et al., 1998). Algunos factores ambientales también son capaces de influir en la absorción del NO_3^-. Así, el incremento de la temperatura

radicular, los UV-B o la presencia de metales pesados, podrían afectar de forma negativa a la absorción del NO_3^- (Lazof et al., 1994; Hessen-Dago et al., 1997; Vaast et al., 1998).

1.2. Reducción del NO_3^- y formación de NH_4^+

La asimilación del N requiere la reducción del NO_3^- a NH_4^+. El primer paso de dicha reducción es la transformación del NO_3^- a nitrito (NO_2^-) que ocurre en el citoplasma de células tanto de la raíz como de la parte aérea, y que esta catalizada por la enzima clave del proceso de reducción que es la nitrato reductasa (NR) (Fig. 1). Dicha reducción requiere NADH, NADPH o ambos como donadores de electrones. La NR es una enzima homodimérica, donde cada uno de los monómeros esta asociado a tres grupos prostéticos: Flavin adenina dinucleotido (FAD), un grupo hemo y un cofactor de molibdeno (MoCo). La caracterización de mutantes resistentes a clorato identificó dos clases de genes, *Nia* que codificaba la apoenzima y los genes *Cnx* que están implicados en la codificación del cofactor MoCo (Meyer y Stitt, 2001). La actividad de la NR se ha asumido como factor limitante en la vía de asimilación del NO_3^-, siendo considerada como la herramienta que distingue entre diferentes genotipos de plantas (Caba et al., 1995; Bussi et al., 1997). La actividad NR es inducible por NO_3^- aunque la presencia de éste no es indispensable para la expresión de dicha enzima (Kronzucker et al., 1995). También el estado nutricional de N y el flujo de éste controlan la expresión de la NR (Samuelson et al., 1995). Además su inducción requiere luz en los tejidos verdes, aunque el efecto de la luz no esta suficientemente explicada aún y

podría ser reemplazada por glucosa o sacarosa (Gniazdowska, 1998). Como inhibidores de la actividad NR han sido descritos algunos Aas o compuestos reducidos de N como el NH_4^+ (Aslam et al., 1997; Dzuibany et al., 1998). Factores externos como la sequía o la salinidad también pueden reducir la actividad NR (Foyer, 1998; Khan y Srivastra, 1998).

Después de la reducción del NO_3^-, el NO_2^- es trasladado a los plastidios o cloroplastos donde será reducido a NH_4^+ mediante la enzima nitrito reductasa (NiR) (Fig 1.1). Los genes *Nii* codifican la enzima NiR que ha sido clonada en varias especies variando el numero de genes de uno a dos (Meyer y Stitt, 2001). La enzima posee dos centros redox, un grupo sirohemo y un centro hierro-azufre y cataliza la transferencia de seis electrones desde la ferredoxina reducida o desde un donador de electrones similar a ésta (Horchani et al., 2011). La NiR es inducible por NO_3^- y por NO_2^- siendo esta forma la más efectiva (Barneix et al., 1984).

1.3. Asimilación de NH_4^+

El NH_4^+ procedente de la reducción del NO_3^-, fotorrespiración o reciclaje de Aas, va a ser asimilado mediante el conocido como ciclo Glutamina sintetasa/Glutamato sintasa (GS/GOGAT), tanto en plastidios como en cloroplastos (Lea y Forde, 1994). La GS fija NH_4^+ en Gln a partir de una molécula de Glu, seguidamente la GOGAT cataliza la transferencia del grupo amino de la Gln al 2-oxoglutarato produciendo dos moléculas de Glu (Fig. 1)

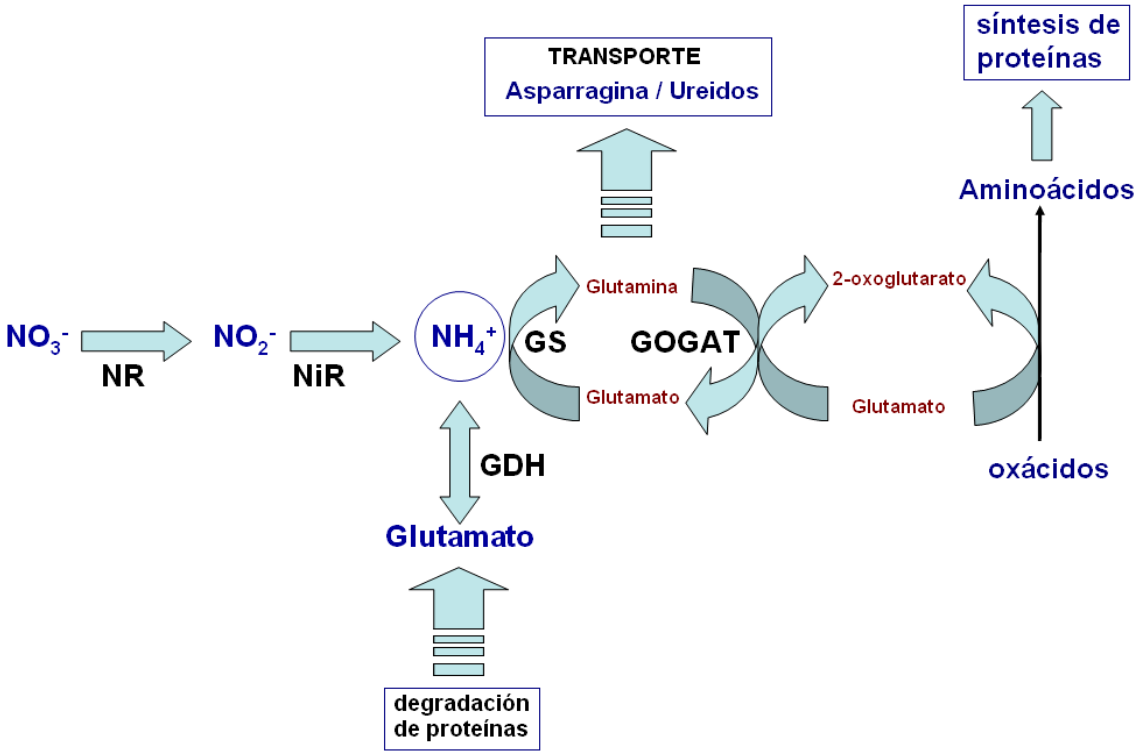

Figura 1. Reducción del nitrato (NO_3^-) y asimilación del amonio (NH_4^+) en las plantas

Se han descrito dos clases de genes nucleares que codifican para la GS. El *GLN1* es una familia de genes que codifica las isoforma GS1 citosólica, envuelta en el reciclaje de NH_4^+ durante determinados estadios del desarrollo como la senescencia de la hoja (Bernard y Habash, 2009). Por otro lado, el gen que codifica las isoforma GS2 cloroplastidial es denominado *GLN2*. Dicha isoforma esta implicada en la asimilación primaria del NH_4^+ y en la reasimilación del NH_4^+ producido en la fotorrespiración en plantas C_3 (Masclaux- Daubresse et al., 2010). En cuanto a la enzima GOGAT se han descrito dos formas presentes en plantas, Fd-GOGAT que aparece preferentemente en cloroplastos de hojas, y NADH-GOGAT que aparece en

plastidios no fotosintéticos. Cada una de las formas utiliza ferredoxina o NADH respectivamente como donador de electrones (Vanoni et al., 2005).

Por otro lado, gran parte de los Aas procedentes de la degradación de proteínas, son convertidos en Glu que es desaminado por la NADH-glutamato deshidrogenasa (GDH) (Fig. 1). La GDH también puede llevar a cabo la reacción inversa, aunque esta vía de asimilación de NH_4^+ es escasa en plantas bajo condiciones normales (Fig.1). No obstante, se ha sugerido que la GDH podría jugar un papel importante en la reasimilación de NH_4^+ bajo condiciones de estrés (Barneix, 2007).

Finalmente, los esqueletos carbonados y especialmente los keto-acidos son esenciales para la formación de compuestos de N orgánico como los Aas. La disponibilidad de éstos así como de ATP, ferredoxina y NADH necesarios para la asimilación del N dependerá de la fotosíntesis, fotorrespiracion y respiración, procesos éstos en los que se forman dichos compuestos (Masclaux- Daubresse et al., 2010).

2. El NO_3^- como fisiopatía en las plantas: deficiencia

La deficiencia de N, fundamentalmente de NO_3^-, ha sido definida como uno de los factores más limitantes en el crecimiento de los cultivos (Glass, 2003), ya que este nutriente forma parte de ácidos nucleicos y aminoácidos, llevando a

cabo su función en la señalización y regulación proteica. Además es de gran importancia en la bioquímica de compuestos como co-enzimas, pigmentos, metabolitos secundarios o poliaminas (Maathuis, 2009). Dada su implicación en numerosos procesos, los síntomas de deficiencia de NO_3^- son múltiples afectando a la fisiología de la planta completa. Así, como síntomas generales las plantas deficientes en NO_3^- muestran una reducción del crecimiento y un incremento del cociente raíz/parte aérea, debido a un redireccionamiento de fotoasimilados hacia la raíz con el objetivo de mejorar la absorción (Kováčik y Bačkor, 2007). Pero el síntoma visual más característico de la deficiencia de NO_3^- en las plantas es la clorosis y senescencia de la hoja que normalmente se inicia en hojas maduras totalmente desarrolladas (Fig. 2), lo que responde a la removilización del NO_3^- hacia las hojas jóvenes con el objetivo de mantener el crecimiento (Mengel et al., 2001). Ambos síntomas de deficiencia de NO_3^- están provocados por la peroxidación de lípidos y la pérdida de pigmentos y proteínas. Dado que se ha descrito que la enzima más importante del proceso fotosintético, la ribulosa 1,5 bifosfato carboxilasa/oxigenasa (Rubisco) representa entre el 30-50% del total de las proteínas solubles, la perdida de éstas unido a la reducción de pigmentos, conlleva un descenso en la capacidad fotosintética y por tanto de la fijación de dióxido de carbono (CO_2) (Long et al., 2006; Casano et al., 1994)

Figura 2. Síntomas foliares de deficiencia de NO_3^- en Mulberry (Tewari et al., 2007).

3. Importancia agronómica y ambiental del NO_3^-

Las plantas tienen una dependencia fundamental del N inorgánico, por lo que su uso en la agricultura actual esta muy extendido. Anualmente se aplican 85-90 millones de toneladas de fertilizantes nitrogenados al suelo en el mundo (Good et al., 2004). Por lo tanto en la mayoría de las regiones de elevada actividad agrícola la producción de los cultivos es altamente dependiente de la aplicación exógena de NO_3^- (Garnett et al., 2009).

Tradicionalmente, el uso de dichos fertilizantes, utilizados para restaurar el N perdido en el suelo, se ha traducido en una mejora del estado de la planta y por tanto en un incremento de la producción (Garnett et al., 2009), pero el N es uno de los nutrientes más caros de suministrar por lo que los fertilizantes comerciales representan uno de los mayores costos en la producción de los cultivos (Masclaux-Daubresse et al., 2010). Recientemente, desde el punto de

vista económico, el incremento de la demanda mundial por las reservas limitadas de combustibles fósiles, ha elevado los precios de los fertilizantes nitrogenados y por tanto el coste de la producción agrícola a nivel mundial (USDA_ERS, 2008; Garnett et al., 2009).

Además de los costes económicos, la aplicación de fertilizantes nitrogenados, principalmente de NO_3^-, implica un coste medioambiental importante ya que solo entre el 30-40% del N aplicado al suelo es utilizado por la planta (Lea y Azevedo, 2006). El NO_3^- restante en el suelo va a sufrir procesos como lixiviación a aguas subterráneas, volatilización a la atmósfera o pérdidas por actividad microbiana (Vitousek et al., 1997). La Unión Europea (UE) presta especial atención a la contaminación por lixiviación de NO_3^- de las aguas. La Directiva de Nitratos de la UE (1991) tiene por objeto proteger la calidad del agua en toda Europa evitando que los NO_3^- procedentes de fuentes agrícolas contaminen las aguas superficiales y subterráneas, y fomentando el uso de buenas prácticas agrícolas. En este sentido esta directiva europea ha recomendando que la cantidad de NO_3^- en agua potable no exceda los 50 mg L^{-1}, pidiendo a los estados miembros políticas de reducción del uso de NO_3^- (Alva et al., 2006). Este efecto medioambiental de la contaminación de agua se ve incrementado por el impacto de los gases de efecto invernadero liberados a la atmósfera en la producción de fertilizantes nitrogenados, los cuales en términos cuantitativos son representativos ya que el 1.2% de la energía consumida en el mundo es utilizada para la producción de dichos fertilizantes (Ahlgren et al., 2008).

Además de la importancia económica y medioambiental del NO_3^- hay que considerar también su importancia en la salud humana. Los vegetales ocupan un lugar especialmente importante en nuestra dieta, pero desafortunadamente constituyen el grupo de alimentos que aporta mayor cantidad NO_3^- para los humanos (Anjana et al., 2007). Algunas especies vegetales tienen gran capacidad de acumulación de NO_3^- (los productos de hoja fundamentalmente), dependiendo el grado de acumulación en parte de la especie y variedad genética, pero fundamentalmente de la cantidad de N disponible. Debido a la aplicación excesiva de fertilizantes nitrogenados, los vegetales pueden acumular elevadas cantidades de NO_3^- en sus hojas, cuando la absorción del ión excede la capacidad de reducción y asimilación de éste (Ruiz et al., 2006). El incremento de la cantidad de NO_3^- en los productos vegetales causa serios problemas en la salud humana ya que éstos pueden ser fácilmente transformados en NO_2^-, los cuales causan enfermedades peligrosas en niños como la metahemoglobinemia por oxidación del Fe^{+2} de la hemoglobina. Además los NO_2^-, pueden reaccionar con aminas y amidas dando lugar a nitrosaminas y nitrosamidas, las cuales son sustancias carcinogénicas y mutagénicas causantes de enfermedades como cáncer del tracto digestivo (Mirvish, 1993).

Por tanto, y dados los costes tanto ambientales, económicos y sanitarios ha despertado recientemente el interés por diversas practicas agronómicas que permitan una mejora en la en la eficiencia del uso del N (NUE) que favorezca la reducción en la aplicación de fertilizantes nitrogenados, y por lo tanto un ahorro

en los costes económicos y medioambientales, favoreciendo la sostenibilidad de la agricultura moderna.

4. Eficiencia en el uso del NO_3^- por las plantas: alternativas para su mejora

La eficiencia nutricional de un genotipo se define como la capacidad de adquirir nutrientes del medio de cultivo y/o incorporarlos y utilizarlos para la generación de biomasa o producción. Por tanto, una planta eficiente en el uso del NO_3^-, es aquella que produce más materia seca o un mayor incremento en la producción por unidad de tiempo, área o N aplicado. Además estas plantas presentaran menores síntomas de deficiencia y/o mayores concentraciones de N que otras plantas crecidas en el mismo medio (Fageria et al., 2008). El NUE puede ser dividido en dos procesos fundamentales, por un lado la habilidad de la planta de extraer el N del suelo, y por otro la eficiencia en la utilización del N absorbido, es decir, la capacidad de las plantas de transferir y utilizar este elemento en los órganos vegetales (Lea y Azevedo, 2006; Ruiz et al., 2006). La variabilidad genética en el NUE se ha demostrado en varias especies como el arroz, la alfalfa, el maíz o el tomate (Harrison et al., 2004; Lawlor, 2002; Machado y Fernandes, 2001; Sánchez-Rodríguez, 2011a) y son numerosos los mecanismos fisiológicos, metabólicos y genéticos que están implicados o asociados al NUE. Además son cuantiosos los trabajos que describen técnicas, tanto agronómicas como moleculares, que persiguen una mejora del NUE de tal manera que plantas con elevado NUE limitarían el uso de fertilizantes nitrogenados, retrasando la degradación medioambiental y mejorando y manteniendo la productividad de la agricultura moderna.

Algunos de los factores principales que afectan al NUE son la eficiencia de absorción y asimilación del NO_3^-, por lo que numerosos esfuerzos por mejorar el NUE se han centrado en la manipulación genética de ambos procesos. Algunos experimentos han modificando la absorción del NO_3^- a través de la sobreexpresión de los transportadores HATS (Fraisier et al., 2000), o han intentado mejorar el NUE a través de la manipulación de la asimilación de NO_3^-, sobreexpresando las enzimas NR y NiR (Pathak et al., 2009). Sin embargo en ninguno de ellos se observó una mejora del NUE, debido a que en ambos casos existe una compleja regulación postranscripcional. Respecto a la sobreexpresión de enzimas como la GS o GOGAT se ha demostrado que pueden incrementar la biomasa y la cosecha de grano en plantas como la judia o el arroz, pero aún se requiere demostrar sus efectos beneficiosos en condiciones de campo (Migge et al., 2000; Habash et al., 2001; Yamaya et al., 2002). Trabajos que manipulan factores de transcripción, los cuales regulan genes implicados en el metabolismo de los ácidos orgánicos, indican que el NUE podría ser mejorado mediante la manipulación del metabolismo del carbono (Masclaux-Daubresse et al., 2010).

Por otro lado, algunas técnicas agrícolas han sido utilizadas con el fin de incrementar el NUE en plantas hortícolas. Diferentes estudios muestran que el uso de injertos puede mejorar el NUE de determinados genotipos, ya que ciertos portainjertos favorecen la absorción y asimilación de N en la parte aérea, evitando la perdida de producción de dichos genotipos (Ruiz et al., 2006; Sánchez-Rodríguez et al., 2011b). También la fertilización con otros macronutrientes esenciales como el azufre (S) ha demostrado un aumento del

NUE ya que un buen estado nutricional de este nutriente en la planta incrementa la absorción y asimilación del N (Salvagiotti et al., 2009).

Trabajos recientes evidencian la relación entre algunas hormonas vegetales y la nutrición mineral. De todos los tipos de hormonas vegetales o compuestos reguladores del crecimiento (auxinas, citoquininas (CKs), ácido abcisico, giberelinas, brasinosteroides, ácido jasmonico o etileno), los genes implicados en la síntesis y degradación de CKs y sus receptores parecen estar implicados de forma más directa que otras hormonas en la regulación del N y otros nutrientes (Forde, 2002; Sakakibara, 2003). Trabajos a nivel molecular con maíz y *Arabidopsis* muestran como la señal de transducción del estatus de N en la planta esta mediada por CKs (Takei et al., 2004), por tanto se ha hipotetizado que el manejo de la ingeniería genética relacionada con las CKs podría tener una posible aplicación en agricultura en este sentido (Ma, 2008).

5. CKs y nutrición vegetal

El funcionamiento de los organismos pluricelulares requiere mecanismos precisos de regulación que permitan la coordinación de las actividades de sus células, tejidos y órganos. Además dichos organismos han de ser capaces de percibir y responder a las fluctuaciones de su ambiente. Entre los mecanismos de regulación, el principal en las plantas se basa en una serie de mensajeros químicos conocidos como hormonas. El control de la respuesta hormonal se lleva a cabo a través de cambios de la concentración y sensibilidad de los tejidos a dichas hormonas (Segura, 2000). Existen varios tipos de hormonas

vegetales como las auxinas, CKs, ácido abcisico, giberelinas, brasinosteroides, Acido jasmónico o etileno, que cumplen numerosas funciones relacionadas fundamentalmente con el desarrollo (Segura, 2000). Sin embargo, en los últimos años, se han encontrado evidencias de la participación de estos compuestos en funciones de señalización, controlando y regulando la respuesta a las fluctuaciones del medio externo, principalmente la variación en la disponibilidad de nutrientes (Schachtman y Shin, 2007).

Las CKs son fitohormonas que tradicionalmente han sido relacionadas con procesos como división celular, crecimiento y desarrollo, dominancia apical o senescencia (Mok, 1994). Las CKs naturales son derivadas de la adenina y llevan unido al N^6-terminal un derivado del isopreno (CKs isoprenoides) o bien un compuesto aromático (CKs aromáticas) (Fig. 3).

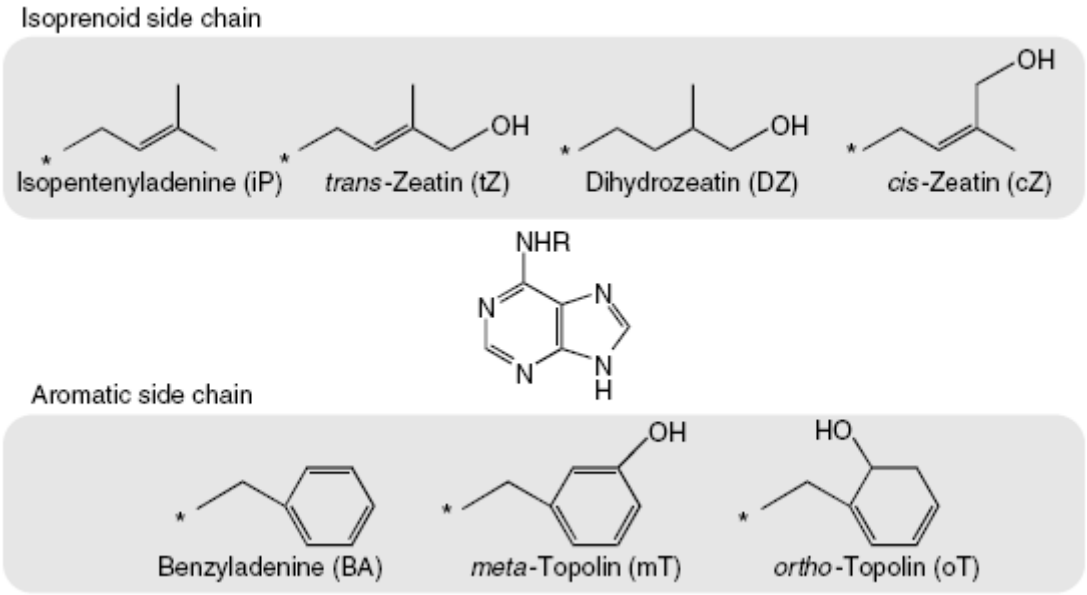

Figura 3. Estructura de las CKs, el sitio de conjugación con el anillo de adenina se representa como * (Hwang y Sakakibara, 2006).

El primer paso de la síntesis de CKs es catalizado por la enzima adenosina fosfato isopenteniltransferasa (IPT) usando adenosina 5`-fosfato (principalmente ATP, ADP pero también AMP) y dimetilalil bifosfato (DMAPP). Según el tipo de adenosina fosfato utilizado se formará isopenteniladenina ribosa 5`difosfato (iPRTP) o isopenteniladenina ribosa 5` trifosfato (iPRTP). La enzima P450 monooxigenasa (CYP735A) mediante una trans-hydroxilación de las formas iP formará transzeatina (tZRTP, tZRDP). Ha sido propuesta una vía alternativa de síntesis de tZ en *Arabidopsis* que utiliza compuestos de la vía del mevalonato aunque su contribución a la síntesis de CKs aún no esta caracterizada (Åstot et al., 2000). En cuanto a la degradación de las CKs, fundamentalmente es llevada a cabo por la enzima CK oxidasa/hidroxilasa (CKX) (Fig. 4) (Hwang y Sakakibara, 2006).

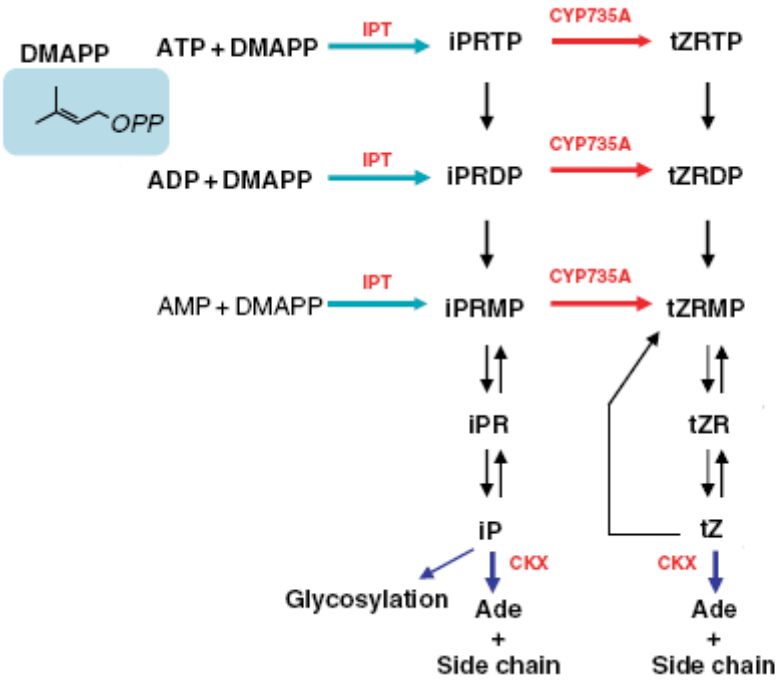

Figura 4. Vía de síntesis de CKs, resumida de Hwang y Sakakibara, 2006.

La biosíntesis y homeostasis de CKs esta controlada por otras fitohormonas y por las fuentes de N inorgánico, lo cual describimos a continuación.

Debido a que las plantas están constituidas por numerosos órganos con diferentes funciones y requerimientos nutricionales se necesita una coordinación a nivel de planta completa, y por tanto una señalización tanto a nivel local como a larga distancia del estado nutricional de cada órgano (Kiba et al., 2011). La implicación de las CKs en la señalización del estado nutricional ha sido ampliamente estudiada y se conoce que esta fitohormona influye en la homeostasis de algunos nutrientes como N, fósforo (P), azufre (S) o hierro (Fe).

Las CKs regulan negativamente la respuesta a deficiencia de P (PSR) en raíces de *Arabidopsis*, así como la expresión de genes inducibles por la deficiencia de P que codifican transportadores de alta afinidad de dicho elemento. Aún no esta claro como ocurre, pero se ha propuesto que podrían actuar como señal a larga distancia durante la deficiencia de P aminorándola (Martín et al., 2000). También se ha sugerido que el efecto negativo del aumento de las CKs en la señalización de la deficiencia de P podría ser debida al incremento de P intracelular que se observa en plantas sometidas a deficiencia de P y tratadas con CKs (Wang et al., 2006). Por tanto, se sabe que la deficiencia de P reprime la acumulación de CKs aunque el mecanismo por el que esto ocurre aún no esta claro (Franco-Zorrilla et al., 2002).

Son menos los trabajos que describen el efecto de las CKs sobre la asimilación del S, pero sí se sabe que la adición de CKs reprime la expresión de genes que codifican para transportadores de alta afinidad de sulfato, los cuales se inducen bajo condiciones de deficiencia de S (Maruyama-Nakashita et al., 2004). Por otro lado, se han realizado estudios que describen como las CKs son capaces de inducir la expresión de genes que responden a la deficiencia de S en *Arabidopsis* (Ohkama et al., 2002). Por tanto aún esta por determinar el papel de las CKs en el control de la señalización de S.

Las plantas responden a la deficiencia de Fe incrementando la proliferación de pelos radiculares e induciendo genes que codifican transportadores de Fe o reductasas férricas. Se ha descrito como las CKs son capaces de reprimir genes que codifican transportadores de Fe y reductasas férricas bajo condiciones de deficiencia de Fe (Séguéla et al., 2008). Sin embargo, el mismo estudio muestra como las CKs pueden mediar la represión de dichos genes, independientemente de la aplicación de Fe, por lo que queda abierta la cuestión de la función de la CKs tanto en la homeostasis de dicho nutriente bajo condiciones normales como bajo condiciones de deficiencia.

La relación entre CKs y N ha sido la más ampliamente estudiada y se sabe que actúan como mensajeros a larga distancia que controlan la asimilación de N y el estatus de éste en la planta (Sakakibara et al., 2006). A continuación describiremos algunos de los estudios más recientes que relacionan las CKs con la homeostasis y señalización del estado nutricional en plantas de N.

5.1. CKs y fisiología del nitrógeno

Los primeros indicios de la relación entre la aplicación de N y la síntesis de CKs se encontraron en cebada y tabaco, plantas en las que se observó que el suministro de N estaba fuertemente relacionado con el contenido de CKs (Singh et al., 1992; Samuelson y Larsson, 1993). Singh et al. (1992) demostraron con plantas de girasol y tabaco que la aplicación de N inducía la acumulación de CKs en la hoja, lo que demostraba que la síntesis de CKs esta regulada por N. Posteriormente se comprobó que la aplicación de N causa un incremento en el contenido de CKs en el xilema pero también en raíces y parte aérea de maíz indicando una posible función de las CKs como señal a larga distancia de la suplementación de N (Takei et al., 2001). Plántulas de *Arabidopsis* crecidas con elevadas concentraciones de N contenían elevados niveles de CKs mientras que aquellas crecidas a bajas concentraciones de N presentaban niveles de CKs menores, lo que evidenció que las CKs no solo actuaban como señal de la cantidad de N existente en el medio, sino que también actuaban como señal del estatus de N (Takei et al., 2004). En *Arabidopsis* se sabe que existen siete genes que codifican la enzima IPT en diferentes tejidos y entre dichos genes se conoce que *At*IPT3 es inducible por NO_3^- (Takei et al., 2004). Además trabajos recientes confirman que las CKs pueden actuar como señal local además de cómo señal a larga distancia (Matsumoto-Kitano, 2008).

Además de los datos descritos anteriormente, la relación N-CKs es reciproca ya que la aplicación externa de estas hormonas podía parcialmente

reducir los efectos de reducción del crecimiento causados por un bajo suministro de N en *Plantago mayor* (Kuiper, 1988). Existen numerosos trabajos que avalan la hipótesis de que las CKs actúan como señal inhibiendo la absorción de NO_3^- en la raíz, así como de otros nutrientes (Li et al., 2007; Lin et al., 2008). Por tanto, las CKs podrían actuar como señal a larga y corta distancia comunicando a la parte aérea si la aplicación de N en la raíz es adecuada y regulando así los sistemas de absorción del N y de otros nutrientes del suelo (Sakakibara et al., 2006).

Las plantas detectan el estado nutricional de N tanto externo como interno y se adaptan a cambios en éste modificando tanto expresión génica como la actividad de enzimas y el contenido de otros metabolitos. Algunos estudios que indican que la señal producida por el N puede ser reemplazada por CKs, las cuales como mensajero a larga y corta distancia podría regular gran cantidad de genes implicados en el metabolismo y desarrollo así como la adquisición de otros macronutrientes (Fig. 5) (Sakakibara et al., 2006).

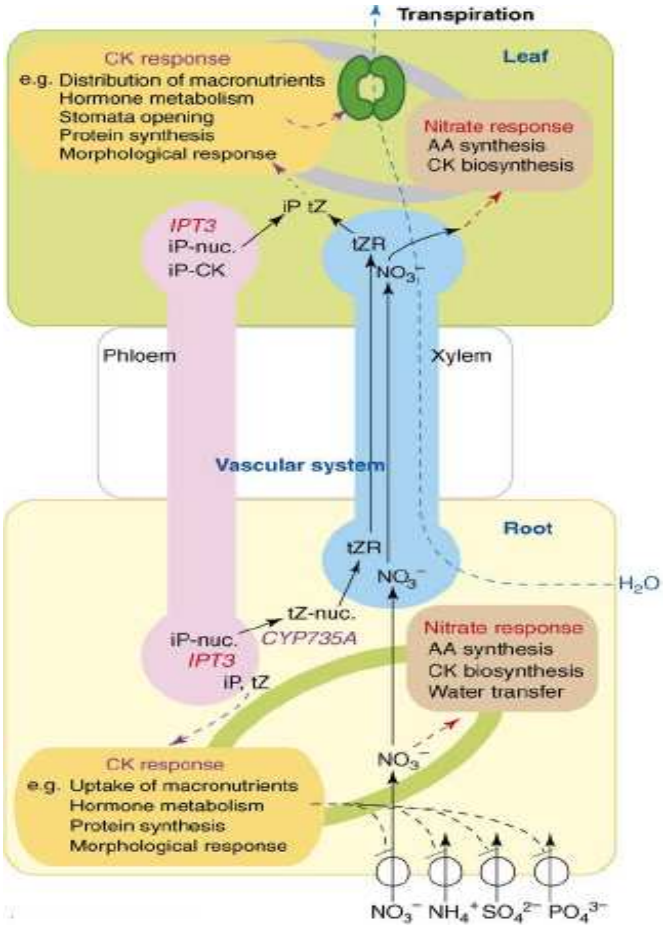

Figura 5. Interacción entre CKs-N y su posible implicación en la regulación del metabolismo y desarrollo (Sakakibara et al., 2006).

REFERENCES

Ahlegren S, Baky A, Bernesson S, Nordberg A, Norén O, Hansson PA. 2008. Ammonium nitrate fertiliser production based on biomass-environmental effects from a life cycle perspective. Bioresource Technology, 99, 8034-8041.

Alva AK. 2006. Sustainable nutrient management in sandy soils-fate and transport of nutrients from animal manure versus inorganic sources. Journal of Sustainable Agriculture, 28, 139-155.

Anjana Umar S, Iqbal M. 2007. Identification of characteristics affecting nitrogen utilization efficiencies in wheat cultivars. Archives of Agronomy and Soil Science, 53, 459-472.

Aslam M, Travis RL, Rains DW, Huffaker RC. 1998. Differential effect of ammonium on the induction of nitrate and nitrite reductase activities in roots of barley (*Hordeum vulgare*) seedlings. Physiologia Plantarum, 101, 612-619.

Barceló J, Nicolás G, Sabater B, Sanchez R. 2005. Reducción asimiladora del nitrógeno. En: Fisiología vegetal. (Eds.) Piramide. Madrid.

Barneix AJ, James DM, Watson EF, Hewitt EJ. 1984. Some effects of nitrate abundance and starvation on metabolism and accumulation of nitrogen in barley (*Hordeum vulgare* L. cv. Sonja). Planta, 162, 469-476.

Barneix AJ. 2007. Physiology and biochemistry of source-regulated protein accumulation in the wheat grain. Journal of Plant Physiology, 164, 581-590.

Benton Jones JJr. 1999. Plant Nutrition Manual. CRC Press Boca Raton, Washington.

Bernard SM, Habash DZ. 2009. The importance of cytosolic glutamine synthetase in nitrogen assimilation and recycling. New Phytologist, 182, 608-620.

Bussi C, Gojon A, Passama L. 1997. In situ nitrate reductase activity in leaves of adult peach trees. Journal of Horticultural Science, 72, 347-353.

Caba JM, Lluch C, Ligero F. 1995. Distribution of nitrate reductase activity in *Vicia faba*: Effect of nitrate and plant genotype. Physiologia Plantarum, 93, 667-672.

Casano LM, Martin M, Sabater B. 1994. Sensitivity of superoxide dismutase transcript levels and activities of oxidative stress is lower in mature-senescent than in young barley leaves. Plant Physiology, 106, 1033-1039.

Crawford NM, Glass ADM. 1998. Molecular and physiological aspects of nitrate uptake in plants. Trends in Plant Science, 3, 389-395.

Dzuibany C, Haupt S, Fock H, Biehler K, Migge A, Becker TW. 1998. Regulation of nitrate reductase transcript levels by glutamine accumulating in the leaves of a feredoxin-dependent glutamate synthase-deficient gluS mutant of *Arabidopsis thaliana*, and by glutamine provided via the roots. Planta, 206, 515-522.

Fageria NK, Baligar VC, Li YC. 2008. The role of nutrient efficient plants in improving crop yields in the twenty first century. Journal of Plant Nutrition, 31, 1121-1157.

Forde BG. 2002. Local and long-range signalling pathways regulating plant responses to nitrate. Annual Review of Plant Biology, 53, 203-224.

Foyer CH, Valadier MH, Migge A, Becker TW. 1998. Drought-induced effects on nitrate reductase activity and mRNA and on the co-ordination of nitrogen and carbon metabolism in maize leaves. Plant Physiology, 117, 283-292.

Fraisier V, Gojon A, Tillard P, Daniel-Vedele F. 2000. Consitutive expression of a putative high-affinity nitrate transporter in *Nicotiana plumbaginifolia*:

evidence for a post-transcriptional regulation by a reduced nitrogen source. Plant Journal, 23, 489-496.

Franco-Zorrilla JM, Cubas P, Jarrillo JA, Fernández-Calvín B, Salinas J, Martinez-Zapater JM. 2002. Mutations at CRE1 impair cytokinin-induced repression of phosphate starvation responses in *Arabidopsis*. Plant Journal, 32, 353-360.

Garnett T, Conn V, Kaiser BN. 2009. Root based approaches to improving nitrogen use efficiency in plants. Plant Cell and Environment, 32, 1272-1283.

Glass ADM. 2003. Nitrogen use efficiency of crop plants: physiological constrains upon nitrogen absorption. Critical Reviews in Plant Science, 22, 453-470.

Gniazdowska M, Mikulska M, Rychter AM. 1998. Growth, nitrate uptake and repiration rate in bean roots under phosphate deficiency. Biologia Plantarum, 41, 217-226.

Gojon A, Dapoigny L, Lejay L, Tillard P, Rufty TW. 1998. Effects of genetic modification of nitrate reductase expression on $^{15}NO_3$-uptake and reduction in *Nicotiana* plants. Plant Cell and Environment, 21, 43-53.

Good AG, Shrawat AK, Muench DG. 2004. Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production?. Trends in Plant Science, 9, 597-605.

Habash D, Massiah A, Rong H, Wallsgrove R, Leigh R. 2001. The role of cytosolic glutamine synthetase in wheat. Annals of Applied Biology, 138, 83-89.

Harrison J, Hirel B, Limani AM. 2004. Variation in nitrate uptake and assimilation between two ecotypes of *Lotus japonicus* and their recombinant inbred lines. Physiologia Plantarum, 120, 124-131.

Hessen-Dago O, De-Lange-Hendrike J, Van-Donk E. 1997. UV-induced changes in phyto-plankton cells and its effects on grazers. Freshwater-Biology, 38, 513-524.

Horchani F, R`bia O, Hajri R, Aschi-Smiti S. 2011. Nitrogen nutrition and ammonium toxicity in higher plants. International Journal of Botany, 7, 1-16.

Hwang I, Sakakibara H. 2006. Cytokinin biosynthesis and perception. Physiologia Plantarum, 126, 528-538.

Khan MG, Srivastava HS. 1998. Changes in growth and nitrogen assimilation in maize plants induced by NaCl and growth regulators. Biologia Plantarum, 41, 93-99.

Kiba T, Kudo T, Kojima M, Sakakibara H. 2011. Hormonal control of nitrogen acquisition: roles of auxin, abcisic acid, and cytokinin. Journal of Experimental Botany, 62, 1399-1409.

Kováčik J, Bačkor M. 2007. Changes of phenolic metabolism and oxidative status in nitrógeno-deficient *Matricaria chamomilla* plants. Plant and Soil, 297, 255-265.

Kronzucker H, Glass ADM, Siddiqi MY. 1995. Nitrate induction in spruce: An approach using compartmental analysis. Planta, 196, 683-690.

Kuiper D. 1988. Growth reponses of *Plantago major* L. ssp. *pleiosperma* to changes in mineral supply. Plant Physiology, 87, 555-557.

Lawlor DW. 2002. Carbon and nitrogen assimilation in relation to yield: mechanisms are the key to understanding production systems. Journal Experimental Botany, 53, 773-787.

Lazof DB, Rincon M, Rufty TW, Mackown CT, Carter TE. 1994. Aluminium accumulation and associated effects on $^{15}NO_3$ influx in roots of two soybean genotypes differing in Al tolerance. Plant and Soil, 164, 291-297.

Lea PJ, Azevedo RA. 2006. Nitrogen use efficiency. 1. Uptake of nitrogen from the soil. Annals of Applied Biology, 149, 243-247.

Lea PJ, Forde BG. 1994. The use of mutants and transgenic plants to study amino acids metabolism. Plant Cell and Environment, 17, 541-556.

Li W, Wang Y, Okamoto M, Crawford NM, Siddiqi MY, Glass AD. 2007. Dissection of the AtNRT2.1:AtNRT2.2 inducible high-affinity nitrate transporter gene cluster. Plant Physiology, 143, 425–433.

Lin SH, Kuo HF, Canivenc G, Lin CS, Lepetit M, Hsu PK, Tillard P, Lin HL, Wang YY, Tsai CB, Gojon A, Tsay YF. 2008. Mutation of the Arabidopsis NRT1.5 nitrate transporter causes defective root-to-shoot nitrate transport. Plant Cell, 20, 2514–2528.

Long SP, Zhu XG, Naidu SL, Ort DR. 2006. Can improvement in photosynthesis increase crop yield?. Plant Cell and Environment, 29, 315-330.

Ma Q-H. 2008. Genetic engineering of cytokinins and their application to agriculture. Critical Reviews in Biotechnology, 28, 213-232.

Maathuis FJM. 2009. Physiological funcions of mineral macronutrients. Current Opinion in Plant Biology, 12, 250-258.

Machado AT, Fernandes MS. 2001. Participatory maize breeding for low nitrogen tolerance. Euphytica, 122, 567-573.

Marschner H. 1995. Mineral nutrition of higher plants. Second Edition. Academic Press Inc., San Diego CA.

Martín AC, del Pozo JC, Iglesias J, Rubio V, Solano R, de la Peña A, Leyva A, Paz-Ares J. 2000. Influence of cytokinins on the expression of phosphate starvation responsive genes in *Arabidopsis*. Plant Journal, 24, 559-567.

Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gauichon L, Suzuki A. 2010. Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Annals of Botany, 105, 1141-1157.

Matsumoto-Kitano M, Kusumoto T, Tarkowski P, Kinoshita-Tsujimura K, Vaclavikova K, Miyawaki K, Kakimoto T. 2008. Cytokinins are central regulators of cambial activity. Proceedings of the National Academy of Sciences, USA, 105, 20027–20031.

Mengel K, Kirkby EA. 2001. Nitrogen. In: Principles of plant nutrition. (Eds.) Kluwer Academic Publishers. The Netherlands.

Meyer C, Stitt M. 2001. Nitrate reductase and signalling. In: Plant nitrogen. (Eds.) Lea, PJ., Morot-Gaudry, JF. Springer, New York.

Migge A, Carrayol E, Hirel B, Becker T. 2000. Leaf-specific overexpression of plastidic glutamine synthetase stimulates the growth of transgenic tobacco seedlings. Planta, 210, 252-260.

Mirvish SS. 1993. Vitamin C inhibition of N-nitroso compounds formation. The American Journal of Clinical Nutrition, 57, 598-599.

Mok MC. 1994. Cytokinins and plant development-an over-view. En: Cytokinins: Chemistry, Activity, and function. (Eds) Mok DWS., Mok MC. CRC Press, Boca Raton, Florida.

Muller B, Touraine B. 1992. Inhibition of NO_3^- uptake by various phloem translocated amino acids in soybean seedlings. Journal of Experimental Botany, 43, 617-623.

Ohkama N, Goto DB, Fujiwara T, Naito S. 2002. Differencial tissue-specific response to sulfate and methionine of a soybean seed storage protein promoter region in transgenic *Arabidopsis*. Plant Cell Physiology, 43, 1266-1275.

Pathak RR, Ahmad A, Lochab S, Raghuram N. 2009. Molecular physiology of plant nitrogen use efficiency and biotechnological options for its enhancement. Current Science, 94, 1394-1403.

Ruiz JM, Rivero RM, Cervilla LM, Castellano R, Romero L. 2006. Grafting to improve nitrogen-use efficiency traits in tobacco plants. Journal of the Science of Food and Agriculture, 86, 1014-1021.

Sakakibara H, Takei K, Hirose N. 2006. Interactions between nitrogen and cytokinin in the regulation of metabolism and development. Trends in Plant Science, 11, 440-448.

Sakakibara H. 2003. Nitrate-specific and cytokinin-mediated nitrogen signalling pathways in plants. Journal of Plant Research, 116, 253-257.

Salvagiotti F, Castellarín JM, Miralles DJ, Pedrol HM. 2009. Sulfur fertilization improves nitrogen use efficiency in wheat by increasing nitrogen uptake. Field Crop Research, 113, 170-177.

Samuelson ME, Larsson CM. 1993. Nitrate regulation of zeatin riboside levels in barley roots. Effects of inhibitors of N-assimilation and comparison with ammonium. Plant Science, 93, 77–84.

Samuelson ME, Ohlen E, Lind M, Larsson CM. 1995. Nitrate regulation of nitrate uptake and nitrate reductase expression in barley grown at different nitrate: Ammonium ratios at constant relative nitrogen addition rate. Physiologia Plantarum, 94, 254-260.

Sánchez-Rodríguez E, Rubio-Wilhelmi MM, Blasco B, Constán-Aguilar C, Romero L, Ruiz JM. 2011a. Variation in the use efficiency of N under moderate water deficit in tomato plants (*Solanum lycopersicum*) differing in their tolerance to drought. Acta Physiologiae Plantarum, 33, 1861-1865.

Sánchez-Rodríguez E, Rubio-Wilhelmi MM, Blasco B, Leyva R, Romero L, Ruiz JM. 2001b. Grafting between two cultivars of Cherry tomato differing in their tolerance drought: Effect in nitrogen use efficiency. Acta Horticulturae. (In press).

Saravitz CH, Devienne BF, Raper Jr CD, Chaillou S, Lamaze T. 1998. Nitrate uptake rate by soybean and wheat plants determined by external nitrate concentration and shoot mediated demand. International Journal of Plant Sciences, 159, 305-312.

Schachtman DP, Shin R. 2007. Nutrient sensing and signaling: NPKS. Annual Review of Plant Biology, 58, 47-69.

Séguéla M, Briat JF, Vert G, Curie C. 2008. Cytokinins negatively regulate the root iron uptake machinery in *Arabidopsis* through a growth-dependent pathway. Plant Journal, 55, 289-300.

Segura J. 2000. Introducción al desarrollo. Concepto de hormona vegetal. En: Fundamentos de fisiología vegetal (Eds.) Azcón-Bieto, J., Talón, M. McGraw-Hill, Madrid.

Singh S, Letham DS, Zhang R, Palni LMS. 1992. Cytokinin biochemistry in relation to leaf senescence: IV. Effect of nitrogenous nutrient on cytokinin levels and senescence of tobacco leaves. Physiologia Plantarum, 84, 262-268.

Takei K, Sakakibara H, Taniguchi M, Sugiyama T. 2001. Nitrogen dependent accumulation of cytokinins in root and the translocation to leaf: implication of cytokinin species that induces gene expression of maize response regulator. Plant Cell Physiology, 42, 85–93.

Takei K, Ueda N, Aoki K, Kuromori T, Hirayama T, Shinozaki K, Yamaya T, Sakakibara H. 2004. AtIPT3 is a key determinant of nitrate-dependent cytokinin biosynthesis in Arabidopsis. Plant Cell Physiology, 45, 1053-1062.

Tsay YF, Chiu CC, Tsai CB, Ho CH, Hsu PK. 2007. Nitrate transporter and peptide transporters. FEBS Letters, 581, 2290-2300.

Ullrich WR. 1987. Nitrate and ammonium uptake in green algae and higher plants: Mechanism and relationship with nitrate metabolism. In: Inorganic nitrogen metabolism. (Eds.) Ullrich, WR., Aparicio, PJ., Syrett, PJ., Castillo, F. Springer, Berlin, Heidelberg, New York.

USDA_ERS. 2008. Average US farm prices of selected fertilizers, 1960-2008. United States Department of Agriculture, Economics Research Service.

Vaast P, Zasoski RJ, Bledsoe CS. 1998. Effect of solution pH, temperature, nitrate/ammonium ratios, and inhibitors on ammonium and nitrate uptake

by Arabica coffee in short-term solution culture. Journal of Plant Nutrition, 21, 1551-1564.

Vanoni M, Dossena L, van den Heuvel R, Curti B. 2005. Structure-function studies on the complex iron-sulfur flavoprotein glutamate synthase: the key enzyme of ammonia assimilation. Photosynthesis Research, 83, 219-238.

Vitousek PM, Mooney HA, Lubchenco J, Melillo JM. 1997. Human domination of earth`s ecosystems. Science, 227, 494-499.

Wang X, Yi K, Tao Y, Wang F, Wu Z, Jiang D, Chen X, Zhu L, Wu P. 2006. Cytokinin represses phosphate-starvation response through increasing of intracellular phosphate level. Plant Cell and Environment, 29, 1924-1935.

Yamaya T, Obara M, Nakajima H, Saski S, Hayakawa T, Sato T. 2002. Genetic manipulation and quantitative trait loci mapping for nitrogen recycling in rice. Journal of Experimental Botany, 53, 917–925.

Capítulo 2

Objetivos

El gran reto del Siglo XXI es la creación de una “segunda revolución verde” basada en una mejora de la producción de los cultivos que asegure alimento a la población mundial junto con una aplicación reducida de fertilizantes. La alternativa más razonable para conseguir este objetivo es la utilización de especies o genotipos de plantas que muestren capacidad para crecer y producir de forma óptima en suelos o medios de cultivo con una baja disponibilidad de nutrientes, especialmente de N, uno de los factores que determina gran parte de la producción vegetal. La utilización de estas plantas con una fertilización reducida es la estrategia más lógica para mantener y desarrollar una agricultura sostenible, rentable económicamente y respetuosa con el medioambiente. Teniendo en cuenta que las plantas tienen una dependencia fundamental del N para su crecimiento se aplican aproximadamente entre 85-90 millones de toneladas de fertilizantes nitrogenados al suelo anualmente, por lo que en la actualidad se hace imprescindible la reducción de este tipo de fertilizantes y especialmente en forma de NO_3^- que es considerado uno de los principales contaminantes agrícolas. Por ello y utilizando como diseño experimental la comparación entre plantas de tabaco wild type (WT) y plantas modificadas genéticamente que expresan el gen *IPT* responsable de la síntesis de CKs, los objetivos de esta tesis doctoral fueron los siguientes:

1. Establecer las respuestas fisiológicas de las plantas a la deficiencia de N y definir la implicación en éstas de la expresión del gen *IPT*.

2. Evaluar si la expresión del gen *IPT* en plantas puede concretarse como una herramienta válida y eficaz para mejorar el uso de N y poder reducir así la aplicación de fertilizantes nitrogenados.

Capítulo 3

Effect of cytokinins on oxidative stress in tobacco plants under nitrogen deficiency

ABSTRACT

Wild type (WT) and transgenic tobacco plants expressing isopentenyltransferase (IPT), a gene coding the rate-limiting step in cytokinins (CKs) synthesis, were grown under limited nitrogen (N) conditions. Our results indicated that the WT plants subjected to N deficiency displayed reduced biomass and relative growth rates (RGR), increased levels of oxidative damage and reduced foliar concentrations of the different N forms. However, the transgenic plants expressing P_{SARK}*::IPT*, in spite of showing a significant decline in all the N forms in the leaf, avoided the alteration of the oxidative metabolism and maintained biomass and the RGR at control levels, under suboptimal N conditions. These results suggest that the increased CKs synthesis in the transgenic plants is an effective mechanism to improve N-use efficiency.

Keywords: Antioxidant system; cytokinins; nitrogen deficiency; oxidative stress; tobacco plants.

Abbreviations: AA, ascorbate; APX, ascorbate peroxidase; CAT, catalase; CKs, cytokinins; GPX, guaiacol peroxidase; GSH, glutathione; H_2O_2, hydrogen peroxide; IPT, isopentenyltransferase; MDA, malondialdehyde; N, nitrogen; NH_4^+, ammonium; NO_3^-, nitrate; $O_2^{\cdot -}$, superoxide ion; RGR, relative growth rate; ROS, reactive oxygen species; SOD, superoxide dismutase.

INTRODUCTION

N is an essential macronutrient for plants, forming part of nucleic and amino acids, serving its function in protein signalling and regulation. In addition, it is of great importance in the biochemistry of compounds such as enzymes, pigments, secondary metabolites, and polyamines (Maathuis, 2009). The main symptom of N deficiency in plants is leaf senescence provoked by lipid peroxidation and pigment loss as well as protein degradation that leads to the inhibition of photosynthetic capacity (Casano et al., 1994). Thus, during N-deficiency-induced senescence, the rise in reactive oxygen species (ROS), such as superoxide ion (O_2^-) or hydrogen peroxide (H_2O_2) trigger oxidative stress (Grossman and Takahashi, 2001).

Plants can respond to this stress through their antioxidant system, which is composed of non-enzymatic antioxidants such as ascorbate (AA) or glutathione (GSH) and enzymatic antioxidants, including the enzymes superoxide dismutase (SOD) capable of detoxifying the O_2^- and of transforming it into H_2O_2, which later will be eliminated by the action of catalase (CAT), guaiacol peroxidase (GPX) or enzymes belonging to the AA-GSH or Halliwell-Asada cycle, such as ascorbate peroxidase (APX), which reduces H_2O_2 by AA oxidation (Jaleel et al., 2009). The induction of the plant antioxidant system during moderate N-deficiency has been reported (Polesskaya et al., 2004; Tewari et al., 2007). Polesskaya et al. (2004) showed greater activity of SOD, APX, or CAT in wheat plants exposed to N deficiency. Also, Tewari et al. (2007) demonstrated a higher H_2O_2 concentration in leaves of Mulberry subjected to N

deficiency, which prompted a rise in lipid peroxidation, in the concentration of antioxidant compounds such as AA or GSH, and in the activity of enzymes in charge of detoxification.

CKs are phytohormones that control the plant developmental programme. CKs can also regulate plant responses against abiotic stress (Haberer and Kieber, 2002; Rivero et al., 2007). In addition, a relationship between CKs and macronutrient acquisition has been postulated (Franco-Zorrilla et al., 2002; Brenner et al., 2005). Recent studies indicated that CKs act as long-distance messengers signalling the N status of the plant (Forde, 2002; Takei et al., 2002). Therefore, CKs can act as a signal communicating to the shoot if the N application of the root is adequate, thereby regulating the nutrient uptake systems (Sakakibara et al., 2006). Gan and Amasino (1995) showed that leaf senescence could be delayed in transgenic plants overexpressing IPT, an enzyme that catalyses the limiting step in CKs synthesis. Therefore, the aim of the present work was to evaluate the effect of N deficiency on oxidative metabolism in WT and transgenic tobacco plants expressing P_{SARK}*::IPT*.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of WT (*Nicotiana tabaccum* cv.SR1) and transgenic plants expressing P_{SARK}*::IPT* were germinated and grown in soil for 30 days (d) in a tray with wells (each well 3 cm x 3 cm x 10 cm). During this time, no differences in germination

or plant development between WT and the transgenic plants were observed. Afterwards, the seedlings were transferred to a growth chamber under controlled conditions with relative humidity of 50±10%, at 28ºC/20ºC (day/night), and a 16h/8h photoperiod with a PPFD (photosynthetic photon-flux density) of 350 µmol $m^{-2}s^{-1}$ (measured with an SB quantum 190 sensor, LI – COR Inc., Lincoln, NE, USA). Under these conditions, plants were grown in individual pots (25 cm upper diameter, 17 cm lower diameter, and 25 cm high) of 8 L in volume and filled with a 1:1 perlite:vermiculite mixture. During 30 d, the plants were grown in a complete nutrient solution containing: 10 mM $NaNO_3$, 2 mM NaH_2PO_4, 5 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 2 mM Na_2SO_4, 2 µM $MnCl_2$, 0.75 µM $ZnCl_2$, 0.25 µM $CuCl_2$, 0.1 µM $(NH_4)_6Mo_7O_{24}$, 5 µM Fe-EDDHA, and 50 µM H_3BO_3, pH 5.8. The nutrient solution was renewed every 3 d and the soil was rinsed with distilled water to avoid nutrient accumulation. The N treatments began 60 d after sowing (DAS) and was maintained for 30 d. The treatments were 10 mM (Control), 7 mM and 1 mM $NaNO_3$. The experimental design was a randomized complete block with six treatments, arranged in individual pots with six plants per treatment, and three replicates. The experiment was repeated three times under the same conditions (n = 9).

Sampling and determination of the relative growth rate (RGR)

All plants were at the late vegetative stage when harvested. Middle leaves (positions 7^{th} and 8^{th}) were harvested, frozen immediately in liquid N_2, and kept at -80ºC until used. To determine the RGR, leaves from three plants per line were sampled at 60 DAS, before starting the N treatment. The leaves were

dried in a forced-air oven at 70ºC for 24 h, and the dry biomass (DB) was recorded. The remaining plants were sampled at 90 DAS. The relative growth rate was calculated from the increase in leaf DW at the beginning and at the end of N-treatment, using the equation RGR = (ln DWf – ln DWi)/(Tf – Ti) where T is the time and the subscripts denote the final and initial sampling (i.e. d 0 and 30, respectively, after nitrogen treatment) (Cervilla et al., 2007).

Analytical Methods

Total reduced N concentration was analysed as described by Baethgen and Alley (1989). NO_3^- was measured by spectrophotometry following Cataldo et al. (1975), and NH_4^+ was determined as described by Krom (1980). Malondialdehyde (MDA) concentration in leaves was determined as described before (Sanchez-Rodriguez et al., 2010).

H_2O_2 content of leaf samples was determined as described by Mukherje and Choudhuri (1983) and the detection of $O_2^{\cdot -}$ was based on its ability to reduce nitro blue tetrazolium (NBT) as described by Kubis (2008). The extraction and quantification of total and reduced ascorbic acid and dehydroascorbate (DHA) was perforemed according to Okamura (1980) with the modifications by Law et al. (1992). GSH was measured by the recycling assay initially described by Tietze (1969) and modified by Noctor and Foyer (1998).

Pyridine nucleotides were extracted from liquid N-frozen leaves material in 1 mL of 100 mM NaOH for NADPH or 5% TCA for $NADP^+$. Nucleotides were

quanified by the enzyme-cycling method (Matsumura and Miyachi, 1980) with some modification (Gibon and Larher, 1997).

SOD (EC 1.15.1.1) activity was assayed according to the methods of Giannopolitis and Ries (1977) and Beyer and Fridovitch (1987), with some modifications (Yu et al. 1998). CAT (1.11.1.6) activity was determined according to Nakano and Asada (1981). GPX (EC 1.11.1.7) activity were determined following a modified version of Cara et al. (2002) using 100 mM K-phosphate buffer (pH 7) for extraction.

APX (EC 1.11.1.11) and glutathione reductase (GR; EC 1.6.4.1) were assayed following Rao et al. (1996). Dehydroascorbate reductase activity (DHAR; EC 1.8.5.1) was measured at 265 nm for 3 min following the change in absorbance resulting from the formation of AA (Nakano and Asada, 1981). The free radical scavenging capacity of extracts was determined as described by Re et al. (1999).

The Ferric Reducing Ability of Plasma (FRAP) assay was made with 1 mM 2,4,6-tripyridyl-2-triazine (TPTZ) and 20 mM $FeCl_3$ in 0.25 M CH_3COONa, pH 3.6 (FRAP reagent). An aliquot of 100 mL of extract (1 g per 10 mL in methanol) was added to 2 mL of FRAP reagent and mixed thoroughly. After the mixture was left at room temperature (20 ºC) for 5 min, absorbance at 593 was measured. Calibration was against a standard curve (25–1600 mM Fe^{3+}) using freshly prepared ammonium ferrous sulphate (Benzie and Strain, 1996). The Trolox Equivalent Antioxidant Capacity (TEAC) value of an extract represents

the concentration of Trolox solution that has the same antioxidant capacity as the extract. TEAC was expressed as mg Trolox g DW^{-1}.

The protein concentration of the extracts was determined according to the method of Bradford (1976), using BSA as the standard.

Statistical analysis

The data compiled were submitted to an analysis of variance (ANOVA) and the differences between the means were compared by Duncan's multiple-range test ($P > 0.05$).

RESULTS

Effects of reduced NO_3^- on plant biomass and RGR

Nitrogen deficiency resulted in reduced foliar biomass and RGR in the WT plants (Table 1). The application of 7 mM and 1 mM NO_3^- resulted in reductions of 20-33% and 21-33% in foliar biomass and RGR, respectively. Besides, neither biomass nor growth rates of the transgenic plants were affected by the reduction in NO_3^- (Table 1).

Table 1: Foliar biomass and foliar RGR in two lines of Tobacco plants subjected to N deficit.

	Foliar Biomass (g DW)		**Foliar RGR** (g day)	
NO_3^- treat.	**WT**	**IPT**	**WT**	**IPT**
Control	6.77 ± 0.36 a	9.15 ± 0.23	0.042 ± 0.002 a	0.048 ± 0.001
7 mM	5.41 ± 0.01 b	7.88 ± 0.07	0.033 ± 0.001 b	0.044 ± 0.001
1 mM	4.53 ± 0.08 c	8.22 ± 0.62	0.028 ± 0.001 c	0.045 ± 0.001
P-value	**	NS	***	NS
$LSD_{0.05}$	0.745	1.338	0.004	0.004

Values are means ± SE (n = 9) and differences between means were compared using LSD (P = 0.05). Means followed by the same letter in the same column do not differ significantly. Levels of significance are represented by *P < 0.05, **P < 0.01, ***P < 0.001 and ns (not significant) P > 0.05.

N-deficiency affected the concentrations of the different N-forms in both WT and transgenic plants. Both lines showed the minimum concentrations of total reduced N, NO_3^- and total N under the 1 mM NO_3^- treatment (Table 2). The reduction in total N was more pronounced in the WT plants (36%) than in the *P_{SARK}::IPT* plants (19%) (Table 2). A treatment of 7 mM NO_3^- had no effect on the different N-forms with exception of a reduction of 20% in NO_3^- content in the transgenic plants (Table 2).

Table 2: Concentration of total reduced N, NO_3^- and total N in two lines of tobacco plants subjected to N deficit.

Lines/NO_3^- Treatment	**Total reduced N** ($mg \cdot g^{-1}$ DW)	**NO_3^-** ($mg \cdot g^{-1}$ DW)	**Total N** ($mg \cdot g^{-1}$ DW)
WT			
Control	30.60±2.22 a	24.94±1.24 a	55.61±4.02 a
7 mM	31.74±1.23 a	27.52±1.30 a	59.27±1.29 a
1 mM	19.65±0.98 b	3.52±0.18 b	23.18±1.18 b
P-value	***	***	***
$LSD_{0.05}$	4.596	3.157	8.760
IPT			
Control	30.41±2.03 a	29.44±1.09 a	59.86±2.08 a
7 mM	29.87±1.53 a	23.28±1.36 b	53.16±2.81 a
1 mM	24.77±0.83 b	3.71±0.28 c	28.48±1.16 b
P-value	*	***	***
$LSD_{0.05}$	4.525	3.079	7.376

Values are means ± SE (n = 9) and differences between means were compared using LSD (P = 0.05). Means followed by the same letter in the same column do not differ significantly. Levels of significance are represented by *P < 0.05, **P < 0.01, ***P < 0.001 and ns (not significant) P > 0.05.

Effects of reduced NO_3^- on lipid peroxidation, ROS and $NADP^+$, NADPH concentration

MDA concentrations, a measure of lipid peroxidation, together with the concentration of ROS are good indicators of tissue oxidative stress. In WT plants, N-deficiency caused a significant increase in the $O_2^{\cdot-}$ (Fig. 1A) and H_2O_2 (Fig. 1B) concentrations in the leaves, raising the foliar MDA concentration (Fig. 1C). On the contrary, no increase was seen MDA concentration in the transgenic plants subjected to N deficiency (Fig. 1C). Furthermore, neither the $O_2^{\cdot-}$ (Fig. 1A) nor the H_2O_2 (Fig.1B) concentrations displayed significant differences with N deficiency, both values remaining close to those from the control treatments (10 mM). Besides, N-deficiency affected the concentrations of $NADP^+$ and NADPH (Table 3). In WT plants $NADP^+$ decreased significantly under N treatments, although no differences were observed in $NADP^+$ concentration in transgenic plants. NADPH increased with 1 mM treatments in the WT and transgenic plants.

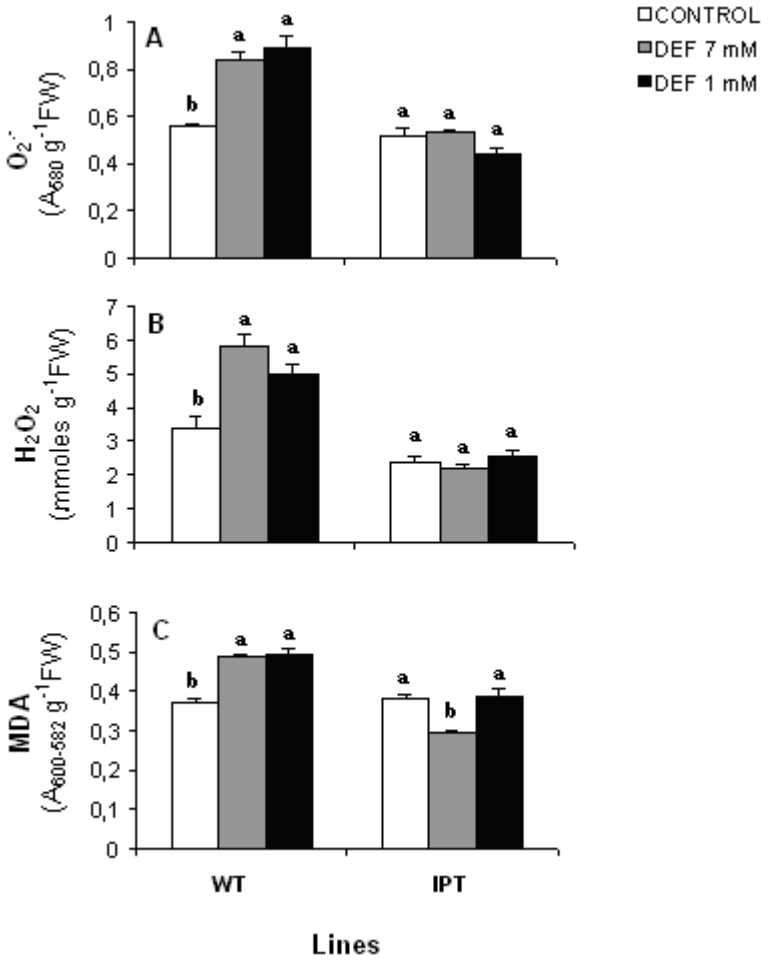

Fig. 1 Effect of 10 mM NO_3^- (control) and NO_3^- deficiency (7 and 1 mM) on (A) Superoxide ($O_2^{\cdot-}$) (B) Hydrogen peroxide (H_2O_2) and (C) Malondialdehyde (MDA) concentration in leaves of two tobacco lines: 'WT' and 'IPT'. Bars represent means+s.e. (n=9); for each lines.

Table 3: Effect of N deficit on $NADP^+$, NADPH and $NADP^+$:NADPH ratio in two lines of tobacco plants.

Lines/NO_3^- Treatment	**$NADP^+$** (mmol g^{-1}FW)	**NADPH** (mmol g^{-1}FW)	**NADPH/$NADP^+$**
WT			
Control	0.554±0.021 a	0.364±0.017 b	0.656±0.030 c
7 mM	0.467±0.017 b	0.345±0.007 b	0.739±0.016 b
1 mM	0.374±0.011 c	0.412±0.011 a	1.101±0.030 a
P-value	***	**	***
$LSD_{0.05}$	0.051	0.037	0.078
IPT			
Control	0.391±0.017	0.333±0.006 b	0.853±0.017
7 mM	0.338±0.020	0.328±0.030 b	0.969±0.089
1 mM	0.395±0.027	0.404±0.013 a	1.024±0.033
P-value	NS	*	NS
$LSD_{0.05}$	0.065	0.056	0.173

Values are means ± SE (n = 9) and differences between means were compared using LSD (P = 0.05). Means followed by the same letter in the same column do not differ significantly. Levels of significance are represented by *P < 0.05, **P < 0.01, ***P < 0.001 and ns (not significant) P > 0.05.

Effects of N-deficiency on antioxidant activities

During N-deficiency treatments, the activities of some of the foliar antioxidant enzymes increased significantly in the WT plants. SOD, CAT and GPX activities (Figs. 2A, B and C) increased under both the 1 and 7 mM NO_3^- treatments. While SOD increased with N-deficiency in the WT plants, the activity remained constant in the transgenic plants. A large increase in CAT was observed in the WT plants under low N. Besides, in the transgenic plants, CAT activity remained constant under 7 mM N and was even lower in the 1 mM treatment. GPX activity was higher under 7 mM N (Fig. 2C).

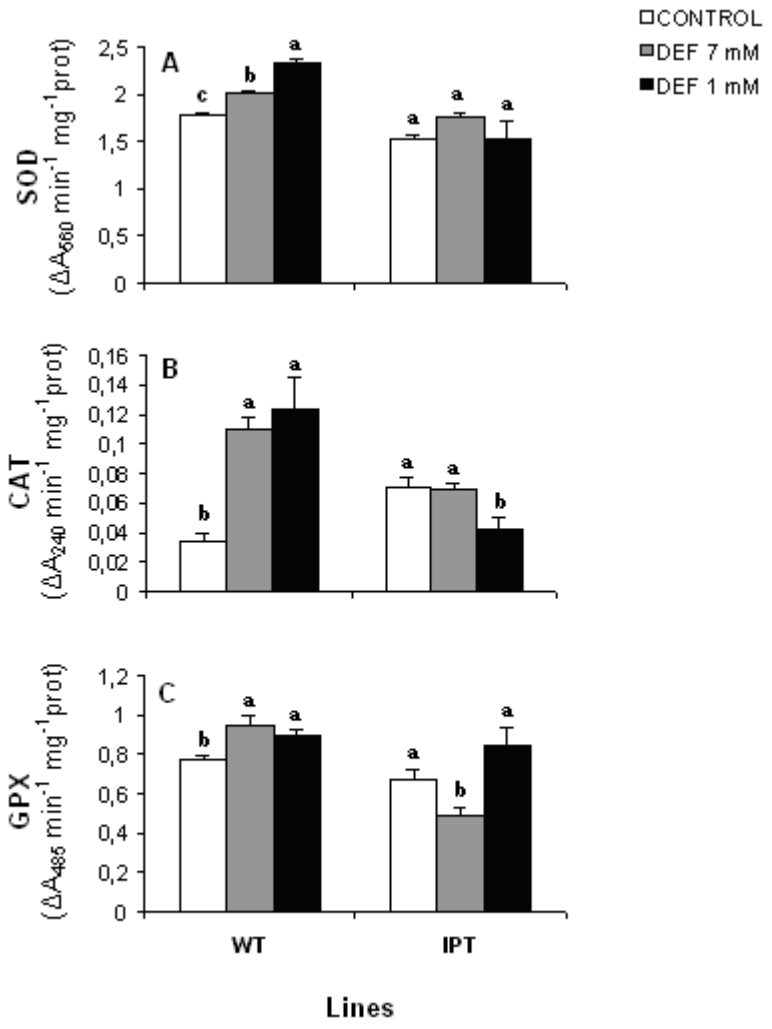

Fig. 2 Effect of 10 mM NO_3^- (control) and NO_3^- deficiency (7 and 1 mM) on (A) Superoxide dismutase (SOD) (B) Catalase (CAT) and (C) Guaiacol peroxidase (GPX) activity in leaves of two tobacco lines: 'WT' and 'IPT'. Bars represent means+s.e. (n=9); for each lines.

Although no differences were observed in the total AA concentrations in WT plants, there was a significant increase in reduced AA content and a decrease in DHA in the WT plants in the 7 mM and 1 mM treatments (Table 4). As a consequence, the reduced AA to foliar DHA increased significantly during N-deficiency treatments. The transgenic plants showed no differences in total AA concentrations during N-deficiency, although DHA declined in the 1 mM

treatment with the concomitant increase in the reduced AA to DHA ratio (Table 4).

Table 4: Concentration of reduced AA, DHA, total AA and the ratio of reduced AA:DHA in the leaves of two lines of tobacco plants subjected to 3 treatment of N deficit.

Lines/NO_3^- Treatment	**AA red** (mmol.g^{-1}FW)	**DHA** (mmol.g^{-1}FW)	**Total AA** (mmol.g^{-1}FW)	**AA red/DHA** (mmol.g^{-1}FW)
WT				
Control	5.99±0.06 b	2.69±0.20 a	8.69±0.26	2.34±0.19 c
7 mM	7.14±0.20 a	1.48±0.06 c	8.62±0.15	4.93±0.33 a
1 mM	7.11±0.06 a	2.07±0.10 b	9.19±0.15	3.50±0.19 b
P-value	***	***	NS	***
$LSD_{0.05}$	0.372	0.409	0.575	0.731
IPT				
Control	4.93±0.04	3.41±0.17a	8.34±0.21 ab	1.47±0.06 b
7 mM	5.06±0.08	3.59±0.17a	8.66±0.20 a	1.43±0.06 b
1 mM	4.99±0.05	2.75±0.22b	7.75±0,21 b	1.91±0.17 a
P-value	NS	*	*	**
$LSD_{0.05}$	0.185	0.553	0.615	0.327

Values are means ± SE (n = 9) and differences between means were compared using LSD (P = 0.05). Means followed by the same letter in the same column do not differ significantly. Levels of significance are represented by *P < 0.05, **P < 0.01, ***P < 0.001 and ns (not significant) P > 0.05.

N-deficiency induced a decrease in total foliar GSH in both WT and transgenic plants (Table 5), and a reduction of GSSG in the WT plants, but not in the transgenic plants.

With regard to the enzymes involved in the Halliwell-Asada cycle, a decrease in the foliar activities of APX, DHAR, and MDHAR (Figs. 3A, B and C) was found in the WT grown under lower N-regimes. However, GR activity was lower under the 7 mM treatment (Fig. 3D). Notably, while an increase of foliar APX activity was seen in the *P_{SARK}::IPT* plants under the 1 mM dosage of N (Fig. 3A), MDHAR activity remained unchanged (Figs. 3C and D) and an increase in DHAR activity was detected only with the 7 mM (Fig. 3B).

Table 5: Concentration of reduced GSH, GSSG, total GSH and the relationship of reduced GSH/GSSG in the leaves of two lines of tobacco plants subjected to 3 treatment of N deficit.

Lines/NO_3^- Treatment	**GSH red** (mmol g^{-1}FW)	**GSSG** (mmol g^{-1}FW)	**GSH total** (mmol g^{-1}FW)	**GSH red/GSSG**
WT				
Control	0.16±0.03	0.28±0.01 a	0.44±0.03 a	0.60±0.11
7 mM	0.13±0.04	0.12±0.00 c	0.25±0.02 b	1.06±0.12
1 mM	0.15±0.02	0.18±0.01 b	0.33±0.04 b	0.86±0.29
P-value	NS	***	**	NS
$LSD_{0.05}$	0.101	0.036	0.107	0.569
IPT				
Control	0.17±0.03 a	0.15±0.00 b	0.32±0.04 a	1.11±0.25 a
7 mM	0.10±0.02 ab	0.21±0.00 a	0.31±0.09 a	0.53±0.12 b
1 mM	0.09±0.00 b	0.15±0.00 b	0.23±0,00 b	0.66±0.06 ab
P-value	*	**	*	*
$LSD_{0.05}$	0.075	0.037	0.077	0.483

Values are means ± SE (n = 9) and differences between means were compared using LSD (P = 0.05). Means followed by the same letter in the same column do not differ significantly. Levels of significance are represented by *P < 0.05, **P < 0.01, ***P < 0.001 and ns (not significant)

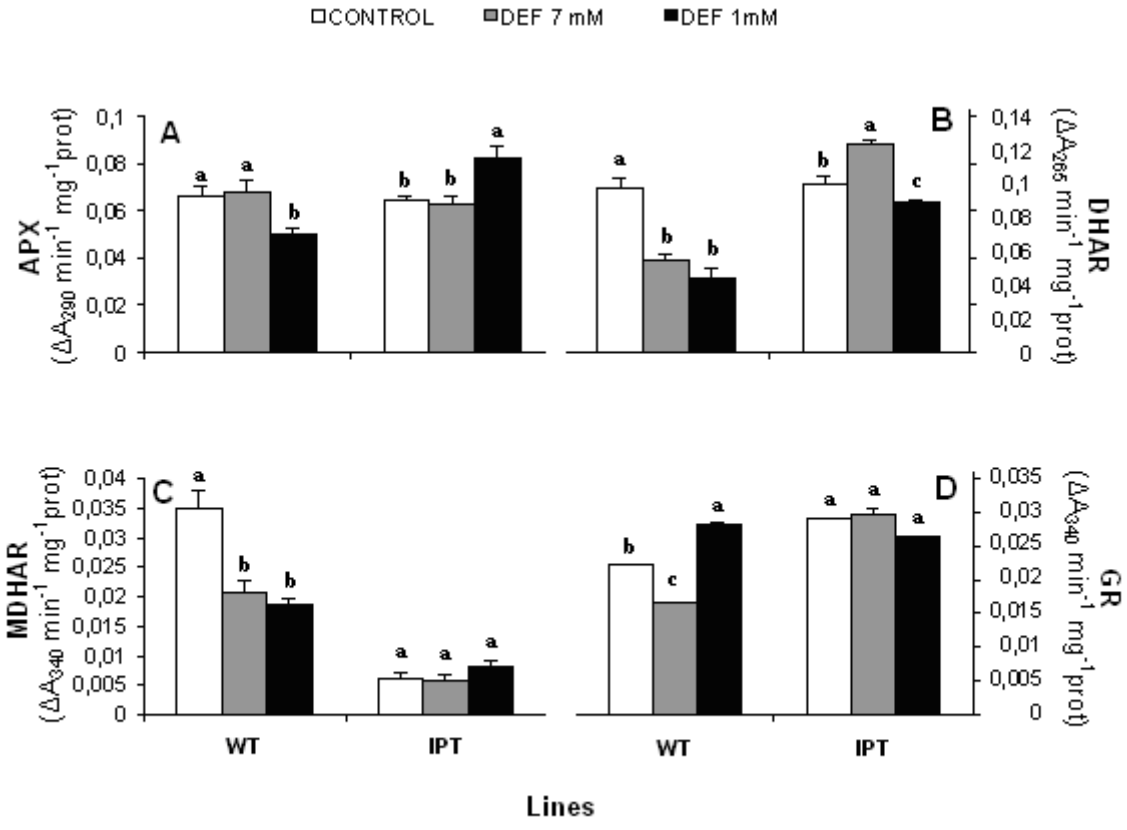

Fig. 3 Effect of 10 mM NO_3^- (control) and NO_3^- deficiency (7 and 1 mM) on (A) Ascorbate peroxidase (APX) (B) Dehydroascorbate reductase (DHAR), (C) Monodehydreascorbate reductase (MDHAR) and Glutation reductase (GR) activity in leaves of two tobacco lines: 'WT' and 'IPT'. Bars represent means+s.e. (n=9); for each lines.

The antioxidant activity in tobacco leaves was studied by the analysis of the FRAP and TEAC tests. Whereas in the WT plants, there was a significant increase in both tests for plants subjected to 1 mM of N deficiency, the transgenic plants did not display significant differences among the N treatment (Table 6).

Table 6: Effect of N deficit on antioxidant test FRAP and TEAC in two lines of tobacco plants.

Lines/NO_3^- Treatment	**FRAP** ($mg.g^{-1}FW$)	**TEAC** ($mg.g^{-1}FW$)
WT		
Control	2.84±0.03 b	1.10±0.02 b
7 mM	2.36±0.05 c	1.09±0.02 b
1 mM	4.28±0.06 a	1.42±0.03 a
P-value	***	***
$LSD_{0.05}$	0.381	0.076
IPT		
Control	1.72±0.06	0.97±0.01
7 mM	2.18±0.10	1.00±0.02
1 mM	2.17±0.33	1.00±0.05
P-value	NS	NS
$LSD_{0.05}$	0.597	0.109

Values are means ± SE (n = 9) and differences between means were compared using LSD (P = 0.05). Means followed by the same letter in the same column do not differ significantly. Levels of significance are represented by *P < 0.05, **P < 0.01, ***P < 0.001 and ns (not significant) P > 0.05.

DISCUSSION

N deficiency reduced the RGR as well as the biomass in the leaves of WT tobacco plants (Table 1). Similar results have been reported in *Triticum*, *Morus* or *Matricaria* subjected to N deficiencies (Tewari et al., 2004, 2007; Kovacik and Backor, 2007). These reductions in RGR and biomass have been explained based on the numerous functions of this element within the plant (Maathuis, 2009). Furthermore, the N-deficiency induced decline in biomass and RGR,

particularly in the case of severe deficiency (1 mM of N) appeared to be directly related to the diminished foliar concentration of total N, total reduced N and NO_3^- (Table 2). In addition, N deficiency, like other abiotic stresses such as drought or heavy metals (Arora et al., 2002) is capable of altering oxidative metabolism. During this stress conditions, the rate of the Calvin cycle is disminished, which prevents oxidation of NADPH and restoration of $NADP^+$. The insufficient electron acceptor $NADP^+$ pool carried accumulation of ROS (Chaves et al., 2009). In our work, the N deficiency increased NADPH:$NADP^+$ ratio (Table 3), this could explain the accumulation of ROS, $O_2^{\cdot -}$ and H_2O_2 , in the WT plants, resulting in greater lipid peroxidation (Fig. 1A, B, and C). It is known that under conditions of N deficiency, an accumulation of ROS, result an increase activity of ROS-detoxifying enzymes (Huang et al., 2004). Our result shown the induction of enzymes involved in detoxification of $O_2^{\cdot -}$ and H_2O_2, such as SOD (Fig. 2A), CAT (Fig. 2B), or GPX (Fig. 2C), in WT plants under the 1 and 7 mM N treatments. These results agree with those of Polesskaya et al. (2004), who reported increased activity of these enzymes in wheat plants subjected to N deficiency. However, the increased CAT and GPX activity in tobacco leaves of the WT plants was not sufficient to detoxify all the H_2O_2 accumulated in the leaf during the N deficient treatments (Fig. 1B). This might be due to the diminished activity in the enzymes of the AA regeneration cycle or the Halliwell-Asada cycle, particularly of APX, the enzyme that detoxify H_2O_2 via the oxidation of reduced AA (Jaleel et al., 2009). High APX endogenous levels have been defined as essential for the maintenance of the antioxidant system that protects against damage due to abiotic stress (Shigeoka et al., 2002). The APX activity in WT plants was reduced with N-deficiency (Fig. 3A), leading to the

accumulation of reduced AA (Table 4) which was not used by APX. This, together with the failure to induce enzymes such as MDHAR or GR under N deficiency could be determinant in the accumulation of toxic levels of H_2O_2 in the WT plants, since APX in combination with the rest of the enzymes of the Halliwell-Asada cycle are essential in the detoxification of H_2O_2 in photosynthetic organisms (Asada, 1999).

The non-enzymatic antioxidant levels also play an essential role against the deleterious effects of abiotic stress (Jaleel et al., 2009). Prior studies demonstrated that AA and/or GSH increases could be the result of increased *de novo* synthesis as a response to oxidative stress provoked by the N deficiency (Logan et al., 1999; Kandlbinder et al., 2004). Also, Szalai et al. (2009) postulated that greater GSH synthesis under different types of abiotic stress would prompt stronger tolerance of the plant against stress. The inability of the WT plants to increase the concentrations of AA and GSH during stress correlated with their sensitivity to N-deficiency stress. However, the tests for antioxidant capacity (FRAP and TEAC) would suggest that phenols, tocoferols and/or carotenoids could increased in the WT plants during N-deficiency (Table 6). Similar results have been reported during the effects of stress on lettuce and tomato (Cervilla et al., 2007; Blasco et al., 2008).

Nitrogenous nutrition has been associated with the synthesis of CKs, since CKs are believed to act as N-sensing because of their role in the regulation of genes involved in the uptake of nutrients (Sakakibara et al., 2006; Rubio et al., 2009). It has been postulated that CKs play a role in the NO_3^--

regulated plant growth response (Samuelson et al., 1992), and that the amounts of available NO_3^- regulates plant CK levels in plants, thus, NO_3^- deficiency would lower CKs endogenous levels while an application of NO_3^- would raise CK levels in the shoot, thereby activating genes associated with nutrient uptake and homeostasis (Argueso et al., 2009). Kuiper (1988) argued for the importance of CKs in growth regulation, demonstrating that the decrease in CKs contents in *Plantago mayor* plants subjected to nutrient deficiencies was due to diminished endogenous cytokinins and not a decrease in the nutrient concentration in plant tissues. The expression of *IPT* under the control of the maturation- and stress-induced *SARK* promoter induced CKs synthesis during stress and drought tolerance in tobacco plants (Rivero et al., 2007, 2009). The higher CK content of the transgenic P_{SARK}*::IPT* plants used in this study would explain the ability of the transgenic plants to maintain plant biomass and RGR during N-deficit (Table 1), despite the decrease in foliar concentrations of total N, total reduced N, and NO_3^- seen when plants were grown under 1 mM treatment (Table 2). The P_{SARK}*::IPT* plants displayed low H_2O_2 content during a pronounced drought stress (Rivero et al., 2007). A decrease in the concentration of radical hydroxyl and MDA formation was also observed in wheat plants exposed to N-deficiency and treated with exogenous CKs (Stoparic and Maksimovic, 2008). These results, together with the results presented here, showing the lack of induction of lipid peroxidation in the transgenic plants during N-deficiency, support the notion of a role of CKs in the plant response to N-deficiency.

The absence of oxidative stress in the transgenic plants under N-deficient conditions was also indicated by the unchanged total AA, reduced AA

and in other antioxidant compounds as shown by the FRAP and TEAC tests. The decrease in the concentrations of total GSH, reduced GSH, and the reduced-GSH:GSSG ratio under these conditions would correlate with the sharp decrease in the N forms under N-limited conditions and the need of N for GSH synthesis. Nonetheless, the normal growth displayed by the transgenic plants indicated that the decrease in GSH content was not harmful for the plants.

In conclusion, our results showed that the tobacco WT plants displayed reduced biomass during N-limited conditions that produced a decrease in all of the different N forms and the generation of ROS that induced a damaging oxidative stress. In contrast, under N-deficiency transgenic P_{SARK}*::IPT* did not produce high ROS concentrations and maintained biomass and RGR values similar to the control plants. Our results suggest that the expression of P_{SARK}*::IPT* in plants could improve N use efficiency, favouring a reduction in the application of nitrogenous fertilizers, without losses in crop productivity.

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REFERENCES

Argueso CT, Ferreira FJ, Kieber JJ. 2009. Environmental perception avenues: the interaction of cytokinin and enviromental response pathways. Plant Cell and Environment, 32, 1147-1160.

Arora A, Sairam RK, Srivastava GC. 2002. Oxidative stress and oxidative system in plants. Current Science, 82, 1227-1238.

Asada K. 1999. The water–water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. Annual Review of Plant Physiology and Plant Molecular Biology, 50, 601–639.

Baethgen WE, Alley MM. 1989. A manual colorimetric procedure for measuring ammonium nitrogen in soil and plant. Communications in Soil Science and Plant Analysis, 20, 961–969.

Benzie IEF, Strain JJ. 1996. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. Annual Review Biochemistry, 239, 70–76.

Beyer WF, Fridovitch I. 1987. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Analytical Biochemistry, 161, 559–566.

Blasco B, Rios JJ, Cervilla LM, Sánchez–Rodríguez E, Ruiz JM, Romero L. 2008. Iodine biofortification and antioxidant capacity of lettuce: Potential benefits for cultivation and human health. Annals of Applied Biology, 152, 289-299.

Bradford MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. Analytical Biochemistry, 72, 248–254.

Brenner WG, Romanov GA, Kölmer I, Brürkle L, Schmülling T. 2005. Immedite-early and delayed cytokinin response genes of *Arabidopsis thaliana* identificied by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascade. Plant Journal, 44, 314-333.

Cara FA, Sanchez E, Ruiz JM, Romero L. 2002. Is phenol oxidation responsible for the short-term effects of boron deficiency on plasma-membrane permeability and function in squash roots? Plant Physiology and Biochemistry, 40, 853–858.

Casano LM, Martin M, Sabater B. 1994. Sensitivity of superoxide dismutase transcript levels and activities of oxidative stress is lower in mature-senescent than in young barley leaves. Plant Physiology, 106, 1033- 1039.

Cataldo DA, Haroon M, Schrader LE, Young VL. 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Communications in Soil Science and Plant Analysis, 6, 71–80.

Cervilla LM, Blasco B, Rios JJ, Romero L, Ruiz JM. 2007. Oxidative stress and antioxidants in tomato (*Solanum lycopersicum*) plants subjected to Boron toxicity. Annals of Botany, 100, 747-756.

Chaves MM, Flexas J, Pinheiro C. 2009. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Annals of Botany, 103, 551-560.

Forde BG. 2002. Local and long-range signalling pathways regulating plant responses to nitrate. Annual Review of Plant Biology, 53, 203-224.

Franco-Zorrilla JM, Cubas P, Jarrillo JA, Fernández-Calvín B, Salinas J, Martinez-Zapater JM. 2002. Mutations at CRE1 impair cytokinin-induced repression of phosphate starvation responses in *Arabidopsis*. Plant Journal, 32, 353-360.

Gan SS, Amasino RM. 1995. Inhibition of leaf senescence by autoregulated production of cytokinin. Science, 270, 1986-1988.

Giannopolitis CN, Ries SK. 1977. Superoxide dismutase occurrence in higher plants. Plant Physiology, 59, 309–314.

Gibon Y, Larher F. 1997. Cycling assay of nicotidamine adenine dinucleotides: NaCl precipitation and ethanol solubilisation of the reduced tetrazolium. Analytical Biochemistry, 251(2), 153-157.

Grossman A, Takahashi H. 2001. Macronutrient utilisation by photosynthetic eukaryotes and the fabric of interactions. Annual Review of Plant Physiology and Plant Molecular Biology, 52, 163-210.

Haberer G, Kieber JJ. 2002. Cytokinins: new insights into a classic phytohormone. Plant Physiology, 128, 354–362.

Huang ZA, Jiang DA, Yang Y, Sun JK, Jin SH. 2004. Effects of nitrogen deficiency on gas exchange, chlorophyll fluorescence, and antioxidant enzymes in leaves of rice plants. Photosynthetica, 42, 357-364.

Jaleel CA, Riadh K, Gopi R, Manivannan P, Inés J, Al-Juburi HJ, Chang-Xing Z, Hong-Bo S, Panneerselvam R. 2009. Antioxidant defense responses: physiological plasticity in higher plants under abiotic contraints. Acta Physiologia Plantarum, 31, 427-436.

Kandlbinder A, Finkemeier I, Wormuth D, Hanitzsch M, Dietz KJ. 2004. The antioxidant status of photosynthesizing leaves under nutrient deficiency: Redox regulation, gene expression and antioxidant activity in *Arabidopsis thaliana*. Physiologia Plantarum, 120, 63-73.

Kováčik J, Backor M. 2007. Changes of phenolic metabolism and oxidative status in nitrogen-deficient *Matricaria chamomilla* plants. Plant and soil, 297, 255-265.

Krom MD. 1980. Spectrophotometric determination of ammonia: study of a modified Berthelot reaction using salicylate and dichloroisocyanurate. Analyst, 105, 305–316.

Kubis J. 2008. Exogenous spermidine differentially alters activities of some scavening system enzymes, H_2O_2 and superoxide radical levels in water-stressed cucumber leaves. Journal of Plant Physiology, 165, 397-406.

Kuiper D. 1988. Growth reponses of *Plantago major* L. ssp. *pleiosperma* to changes in mineral supply. Plant Physiology, 87, 555-557.

Law MY, Charles SA, Halliwell B. 1992. Glutathione and ascorbic acid in spinach (*Spinacea oleracea*) chloroplast: the effect of hydrogen peroxide and paraquat. Biochemical Journal, 210, 899–903.

Logan BA, Demming-Adams B, Rosenstiel TN, Adams WW. 1999. Effect of nitrogen limitation on foliar antioxidants in relationship to other metabolic characteristics. Planta, 209, 213-220.

Maathuis FJM. 2009. Physiological funcions of mineral macronutrients. Current Opinion in Plant Biology, 12, 250-258.

Matsumura H, Miyachi S. 1980. Cycling assay for nicotinamide adenine dinucleotides. Methods in Enzymology, 69, 465- 470.

Mukherje SP, Choudhuri MA. 1983. Implications of water stress-induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in Vigna seedlings. Physiologia Plantarum, 58, 166–170.

Nakano Y, Asada K. 1981. Hydrogen peroxide scavenged by ascorbate specific peroxidise in spinach chloroplast. Plant and Cell Physiology, 22, 867–880.

Noctor G, Foyer CH. 1998. Simultaneous measurement of foliar glutathione, γ–glutamylcysteine, and amino acids by high-performance liquid chromatography: Comparison with the two other assay methods for glutathione. Analytical Biochemistry, 264, 98–110.

Okamura M. 1980. An improved method for determination of L-ascorbic acid and L-dehydroascorbic acid in blood plasma. Clinica Chimica Acta, 103, 259–268.

Polesskaya OG, Kashirina EI, Alekhina ND. 2004. Changes in the activity of antioxidant enzymes in wheat leaves and roots as a function of nitrogen source and supply. Russian Journal of Plant Physiology, 51, 686-691.

Rao MV, Paliyath G, Ormrod DP. 1996. Ultraviolet-B radiation and ozone-induced biochemical changes in the antioxidant enzymes of *Arabidopsis thaliana*. Plant Physiology, 110, 125–136.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine, 26, 1231–1237.

Rivero RM, Kojima M, Gepstein A, Sakakibara H, Mittler R, Gepstein S, Blumwald E. 2007. Delayed leaf senescence induces extreme drought tolerance in a flowering plant. Procceding of the National Academy of Science USA, 104, 19631–19636.

Rivero RM, Shulaev V, Blumwald E. 2009. Cytokinin-dependent photorespiration and the protection of photosynthesis during water deficit. Plant Physiology, 150, 1530-1540.

Rubio V, Bustos R, Irioyen ML, Cardona-López X, Rojas-Triana M, Paz-Ares J. 2009. Plant hormones and nutrient signaling. Plant Molecular Biology, 69, 361-373.

Sakakibara H, Takei K, Hirose N. 2006. Interactions between nitrogen and cytokinin in the regulation of metabolism and development. Trends in Plant Science, 11, 440-448.

Samuelson ME, Eliasson L, Larsson CH. 1992. Nitrate-regulated growth and cytokinin responses in seminal roots of barley. Plant Physiology, 98, 309-315.

Sanchez-Rodriguez E, Rubio-Wilhelmi MM, Cervilla LM, Blasco B, Rios JJ, Rosales MA, Romero L, Ruiz JM. 2010. Genotypic differences in some physiological parameters symptomatic for oxidative stress under moderate drought in tomato plants. Plant Science, 178, 30-40.

Shigeoka S, Ishikawa T, Tamoi M, Miyagawa Y, Takeda T, Yabuta Y, Yoshimura K. 2002. Regulation and function of ascorbate peroxidase isoenzymes. Journal of Experimental Botany, 53, 1305-1319.

Stoparic G, Maksimovic I. 2008. The effect of cytokinins on the concentration of hydroxyl radicals and the intensity of lipid peroxidation in nitrogen deficient Wheat. Cereal Research Communications, 36, 601-609.

Szalai G, Kellös T, Galiba G, Kocsy G. 2009. Glutathione as antioxidant and regulatory molecule in plants under abiotic stress conditions. Journal of Plant Growth Regulation, 28, 66-80.

Takei K, Takahashi T, Sugiyama T, Yamaya T, Sakakibara H. 2002. Multiple routes communicating nitrogen availability from roots to shoots: a signal transduction pathway mediated by cytokinin. Journal of Experimental Botany, 53, 971-977.

Tewari RK, Kumar P, Sharma PN. 2007. Oxidative stress and antioxidant responses in young leaves of mulberry plants grown under nitrogen, phosphorus or potassium deficiency. Journal of Integrative Plant Biology, 49, 313-322.

Tewari RK, Kumar P, Tewari N, Srivastava S, Sharma PN. 2004. Macronutrient deficiencies and differential antioxidant responses-influence on the activity and expression of superoxide dismutase in maize. Plant Science, 166, 687-694.

Tietze F. 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues. Analytical Biochemistry, 27, 502–522.

Yu Q, Osborne L, Rengel Z. 1998. Micronutrient deficiency changes activities of superoxide dismutase and ascorbate peroxidase in tobacco plants. Journal of Plant Nutrition, 21, 1427–1437.

Capítulo 4

Ammonium formation and assimilation in P_{SARK}::IPT tobacco transgenic plants under low N

ABSTRACT

Wild type (WT) and transgenic tobacco plants expressing isopentenyltransferase (IPT), a gene encoding the enzyme regulating the rate-limiting step in cytokinin (CKs) synthesis, were grown under limited nitrogen (N) conditions. We analyzed nitrogen forms, nitrogen metabolism related-enzimes, aminoacids and photorespiration related-enzymes in WT and *P_{SARK}::IPT* tobacco plants. Our results indicate that the WT plants subjected to N deficiency displayed reduced nitrate (NO_3^-) assimilation. However, an increase in the production of ammonium (NH_4^+), by the degradation of proteins and photorespiration, led to an increase in the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle in WT plants. In these plants, the amounts of amino acids decreased with N deficiency, although the relative amounts of glutamate and glutamine increased with N deficiency. Although the transgenic plants expressing *P_{SARK}::IPT* growing under suboptimal N conditions displayed a significant decline in the N forms in the leaf, they maintained the GS/GOGAT cycle at control levels. Our results suggest that under N-deficiency CKs prevented the generation and assimilation of NH_4^+ by increasing such processes as photorespiration, protein degradation, the GS/GOGAT cycle, and the formation of glutamine.

Keywords: Aminoacids, cytokinins, *Nicotiana tabaccum*, nitrate reductase, photorespiration.

Abbreviations: AAs, amino acids; AAT, aspartate aminotransferase; CKs, cytokinins; GDH, glutamate dehydrogenase; GGAT, glutamate:glyoxylate aminotransferase; GO, glyoxylate oxidase; Gln, glutamine; Gly, glycine; Glu, glutamate; GOGAT, glutamate synthase; GS, glutamine synthetase; HR, hydroxypyruvate reductase; IPT, isopentenyltransferase; NH_4^+, ammonium; NO_3^-, nitrate; NR, nitrate reductase; NiR, nitrite reductase; 2-PG, glycolate-2-phosphate; Rubisco, RuBP carboxylase/oxygenase; RuBP, ribulose-1,5-biphosphate; Ser, serine; SGAT, serine:glyoxylate aminotransferase.

INTRODUCTION

N is an essential macronutrient that can frequently act as a limiting factor for growth (Antal et al., 2010). Under normal growth conditions, NO_3^- is the main source of N and its assimilation is essential for normal plant growth and development (Cruz et al., 2004). The reduction of NO_3^- NH_4^+ involves the sequential action of nitrate reductase (NR) (EC.1.6.6.) and nitrite reductase (NiR) (EC.1.7.2.1). The resulting NH_4^+ is then assimilated by GS (EC.6.3.1.2) and GOGAT (EC.1.4.1.13) to organic forms such as glutamate (Glu) and glutamine (Gln). These amino acids (AAs) are precursors in the synthesis of other AAs, nucleic acids, chlorophylls or hormones (Oliveira et al., 2001). NO_3^- nutrition changed metabolite levels and enzyme activities considerably. Under nitrogen limiting conitions, several authors have described, in various plants such as *Arabidopsis* and barley a decrease in nitrate reductase activity while GS activity was increased due to a higher accumulation of the cytosolic enzyme (Barneix et al., 1984; Lemaître et al., 2008). In addition, compounds such as

amino acids, directly related to the N metabolism, reducing their concentration in plants subjected to low N (Cruz et al., 2004; Kováčik et al., 2006).

Another process involved in NH_4^+ formation is photorespiration, a process that is a consequence of the oxygenation of ribulose-1,5-biphosphate (RuBP). This reaction is catalysed by RuBP carboxylase/oxygenase (Rubisco) (EC.4.1.1.39), which generates one molecule of glycerate-3-phosphate and one of glycolate-2-phosphate (2-PG) in the chloroplast. This 2-PG is hydrolysed by phosphoglycolate phosphatase (EC.3.1.3.18) to glycolate, and converted to glyoxylate in the peroxisome by glyoxylate oxidase (GO) (EC.1.2.3.5). Glyoxylate is transaminated to glycine (Gly) by the reaction catalysed by glutamate:glyoxylate aminotransferase (GGAT) (EC.2.6.1.4) and is transported to the mitochondria. Subsequently, Gly is transformed into serine (Ser) by the action of the enzymes and hydroxymethyltransferase. This reaction forms NH_4^+, which is integrated in the N-assimilation pathway through GS. Ser formed in the mitochondria is transported to the peroxisome, where it is transformed by serine:glyoxylate aminotransferase (SGAT) (EC.2.6.1.45) into hydroxypyruvate, which in turn is reduced to glycerate by hydroxypyruvate reductase (HR) (EC.1.1.1.81). Finally, the glycerate enters the chloroplast, where it is phosphorylated by glycerate kinase, giving rise to a molecule of 3-PGA, which enters the Calvin-Benson cycle (Wingler et al., 2000). Although photorespiration involves a reduction of photosynthesis and CO_2 assimilation, photorespiration allows the supply of RuBP to the Calvin-Benson cycle (Osmond, 1981; Wu et al., 1991) and also can generate Gly and Ser (Madore and Grodzinksi, 1984). Moreover, photorespiration play roles in N assimilation, since the production of

NH_4^+ during photorespiration can be significant, in particular during NO_3^- reduction (Keys et al., 1978). The reassimilation of NH_4^+ caused during photorespiration by the enzymes GS and GOGAT have been defined as essential in the maintenance of the N status in the plant (Wingler et al., 2000).

The N status of the whole plant can regulate the plant N assimilation (Dluzniewska et al., 2006). NO_3^- can also induce the accumulation of mRNA and enzymes such as NR, GS, and GOGAT (Fan et al., 2002; Redinbaugh and Campbell, 1993). In addition to external factors such as temperature or atmospheric CO_2, the signalling of the internal status of N is essential to coordinate the absorption and assimilation of this element (Imsande and Touraine, 1994). It is known that in addition to reduced N compounds such as AAs, CKs are involved in N-dependent signalling, since in response to NO_3^-, CKs accumulate in the plant tissues and induce the response of protein and enzyme regulators involved in assimilation (Takei et al., 2002; Sheen, 2002).
The rate-limiting step of cytokinin biosynthesis, the transfer of an isopentenyl moiety from dimethylallyl diphosphate (DMAPP) to the N^6 position of ATP/ADP, is catalysed by the enzyme IPT (Argueso et al., 2009). In Arabidopsis, IPT is encoded by seven genes that are differentially expressed in various tissues. Among these seven genes, *At*IPT3 is NO_3^- inducible (Kiba et al., 2011).

Here, we aim at the characterization of the effects of N deficiency on N-assimilation pathways and on photorespiration of WT tobacco plants and transgenic tobacco expressing P_{SARK}*::IPT* (Rivero et al., 2007, 2009; Rubio-Wilhelmi et al., 2011).

MATERIAL AND METHODS

Plant material, growth conditions and plant growth

Seeds of WT (*Nicotiana tabaccum* cv.SR1) and transgenic plants expressing P_{SARK}*::IPT* were germinated and grown in soil for 30 days in a tray with wells (each well 3 cm x 3 cm x 10 cm). During this time, no differences in germination or plant development between WT and the transgenic plants were observed. Afterwards, the seedlings were transferred to a growth chamber under controlled conditions with relative humidity of 50±10%, at 28ºC/20ºC (day/night), and a 16h/8h photoperiod with a PPFD (photosynthetic photon-flux density) of 350 µmol $m^{-2}s^{-1}$ (measured with an SB quantum 190 sensor, LI – COR Inc., Lincoln, NE, USA). Under these conditions, plants were grown in individual pots (25 cm upper diameter, 17 cm lower diameter, and 25 cm high) of 8 L in volume and filled with a 1:1 perlite:vermiculite mixture. During 30 days, the plants were grown in a complete nutrient solution containing: 10 mM $NaNO_3$, 2 mM NaH_2PO_4, 5 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 2 mM Na_2SO_4, 2 µM $MnCl_2$, 0.75 µM $ZnCl_2$, 0.25 µM $CuCl_2$, 0.1 µM $(NH_4)_6Mo_7O_{24}$, 5 µM Fe-EDDHA, and 50 µM H_3BO_3, pH 5.8. The nutrient solution was renewed every 3 days and the soil was rinsed with distilled water to avoid nutrient accumulation. The N treatments began 60 d after sowing (DAS) and was maintained for 30 d. The treatments were 10 mM (Control), 7 mM and 1 mM $NaNO_3$. The experimental design was a randomized complete block with six treatments, arranged in individual pots with six plants per treatment, and three replicates. The experiment was repeated three times under the same conditions (n = 9).

Plant sampling

All plants were at the late vegetative stage when harvested. Middle leaves (positions 7^{th} and 8^{th}) were harvested, frozen immediately in liquid N_2, and kept at -80ºC until used. A part of the plant material was used for the determination of fresh weight (FW), NR (EC.1.6.6.1), NiR (EC.1.7.2.1), GS (EC. 6.3.1.2), GOGAT (EC.1.4.1.13), aspartate aminotransferase (AAT) (EC. 2.6.1.1), GO (EC.1.2.3.5), GGAT (EC.2.6.1.4), HR (EC.1.1.1.81) and glutamate dehydrogenase (GDH) (EC.1.4.1.2) enzymatic activities. The rest of the plant material was lyophilised and used to determine AAs, NO_3^- and NH_4^+.

Analysis of N forms and free AAs concentration

NO_3^- was measured by spectrophotometry following Cataldo et al. (1975), and NH_4^+ was determined as described by Krom (1980).

AAs were measured by high performance liquid chromatography (HPLC, Agilent 1100). Fresh leaf tissue was homogenized in methanol containing DL-3-aminobutyric acid as an internal standard and samples were agitated. Subsequently, 200 µL of chloroform were added and samples were agitated again for 5 min. Finally, 400 µL of ultra-pure water were added and samples were then vortexed and centrifuged at 13 000 g for 5 min. The upper phase was transferred to a microtube and dried under vacuum over-night. Dry residues were resuspended in an appropriate volume of ultra-pure water and 10 µL of the

resulting extract were sampled for amino acid derivatization according to the AccQ Tag Ultra Derivitization Kit protocol (Waters Corp., Milford, USA).

Enzyme extractions and assays

Leaves were homogenized in 50 mM buffer KH_2PO_4 (pH 7.5) containing 2 mM EDTA, 1.5% (w/v) soluble casein, 2 mM dithiothreitol (DTT) and 1% (w/v) insoluble polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 30 000 g for 20 min. Supernatant was used to measure enzyme activity of NR, NiR, GOGAT and GDH (Groat and Vance, 1981; Kaiser and Lewis, 1984; Lillo 1984; Singh and Srivastava, 1986). The NR assay followed the methodology of Kaiser and Lewis (1984). The NO_2^- formed was determined colorimetrically at 540 nm after azocoupling with sulphanilamide and naphthylethylenediamine dihydroclhoride according to the method of Hageman and Hucklesby (1971). NiR activity was measured by the disappearance of NO_2^- from the reaction medium (Lillo, 1984). After incubation at 30ºC for 30 min, the NO_2^- content was determined colorimetrically. GOGAT and GDH activities were assayed spectrophotometrically at 30ºC by monitoring the oxidation of NADH at 340 nm, essentially as indicated by Groat and Vance (1981) and Singh and Srivastava (1986). GS and AAT was determined by an adaptation of the hydroxamate synthetase assay by Kaiser and Lewis (1984) and according to Gonzalez et al. (1995) respectively. Leaves were homogenized in 50 mM of maleic acid-KOH buffer (pH 6.8), containing 100 mM sucrose, 2% (v/v) β- mercaptoethanol and 20% (v/v) ethyleneglycol. The homogenate was centrifuged at 30 000 g for 20 min. For GS, the formation of glutamylhydroxamate was colorimetrically

determined at 540 nm after complexing with acidified ferric chloride (Wallsgrove et al., 1979). Supernanatant was used to measure AAT activity spectrophotometrically at 340 nm according to Gonzalez et al. (1995). For the determination of GO activity, fresh leaf tissue was homogenized with PVPP and 1 mL of 50 mM Tris-HCl buffer (pH 7.8) with 0.01% Triton X-100 and 5 mM DTT. The homogenate was centrifuged at 30 000 g for 20 min. GO activity was assayed as described by Feierabend and Beeevers (1972). For determination of GGAT and HR activities, leaves were homogenized in 100 mM Tris-HCl buffer (pH 7.3) containing 0.1% (v/v) Triton X-100 and 10 mM DTT. The homogenate was centrifuged at 20 000 g for 10 min. Supernantant was used to measure GGAT activity by coupling the reduction of 2-oxoglutarate by NADH in a reaction catalyzed by GDH (Igarashi et al., 2006). HR activity was assayed spectrophotometrically by monitoring NADH oxidation at 340 nm (Hoder and Rej, 1983).

The protein concentration of the extracts was determined according to the method of Bradford (1976), using BSA as the standard.

Statistical analysis

The data compiled were submitted to an analysis of variance (ANOVA) and the differences between the means were compared by Duncan's multiple-range test ($P > 0.05$).

RESULTS

Effects of N-deficiency on foliar biomass and foliar NO_3^- and NH_4^+ concentrations

N deficiency resulted in reduced foliar biomass in the WT plants (Table 1). The application of 7 mM and 1 mM NO_3^- resulted in reductions of 20-33% in foliar biomass. Besides, foliar biomass of the transgenic plants was not affected by the reduction in NO_3^- (Table 1).

Table 1: Foliar biomass in two lines of Tobacco plants subjected to N deficit.

	Foliar Biomass (g DW)	
NO_3^- treat.	**WT**	**IPT**
Control	6.77 ± 0.36 a	9.15 ± 0.23
7 mM	5.41 ± 0.01 b	7.88 ± 0.07
1 mM	4.53 ± 0.08 c	8.22 ± 0.62
P-value	**	NS
$LSD_{0.05}$	0.745	1.338

Values are means ± SE (n = 9) and differences between means were compared using LSD (P = 0.05). Means followed by the same letter in the same column do not differ significantly. Levels of significance are represented by *P < 0.05, **P < 0.01, ***P < 0.001 and ns (not significant) P > 0.05.

The internal NO_3^- concentrations was severely reduced in both WT and *P_{SARK}::IPT* plants growing at low N-concentrations (i.e. 1 mM) (Fig. 1A). A significant reduction in leaf NH_4^+ concentrations was also seen in plant growing under low N, although the decrease was less pronounced in the *P_{SARK}::IPT* plants (Fig. 1B).

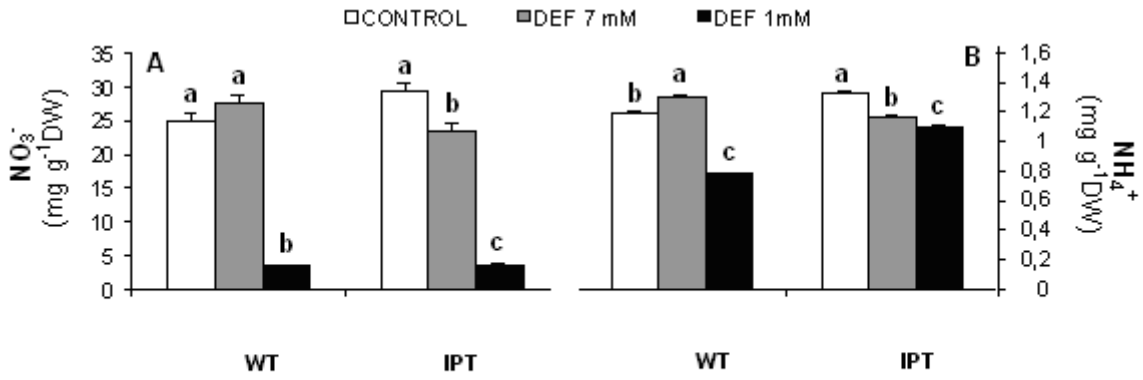

Fig. 1 Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on (A) foliar NO_3^- concentration, (B) foliar NH_4^+ concentration of two tobacco lines: 'WT' and 'IPT'. Bars represent means+s.e. (n=9); for each lines.

Effects of N-deficiency on NH_4^+ formation and assimilation

We measured the activities of enzymes comprising different biochemical pathways associated with the formation of NH_4^+ in plants. The NR activity of WT plants declined with the N-deficiency treatments, while a slight increase was seen in NiR activity (Table 2). At control conditions, the transgenic plants displayed lower NR activity levels as compared to WT plants, but the activity remain constant (or increased at 7 mM N) when grown at N-deficient conditions (Table 2). In contrast to WT plants, the transgenic plants displayed a reduction in NiR activity under N-deficiency.

Table 2: Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on nitrate reductase (NR) and nitrite reductase (NiR) activities in two tobacco lines: 'WT' and 'IPT'.

	NR (µmol $min^{-1}mg^{-1}prot$)		**NiR** (mmol $min^{-1}mg^{-1}prot$)	
NO_3^- treat.	**WT**	**IPT**	**WT**	**IPT**
Control	90.40 ± 9.53 a	34.36 ± 9.43	0.90 ± 0.02 b	1.39 ± 0.12 a
7 mM	33.67 ± 6.64 b	47.17 ± 8.03	1.02 ± 0.06 ab	1.28 ± 0.10 ab
1 mM	7.09 ± 0.77 c	32.75 ± 19.9	1.08 ± 0.03 a	1.05 ± 0.02 b
P-value	***	NS	*	*
$LSD_{0.05}$	19.62	39.56	0.13	0.27

Values are means ± SE (n = 9) and differences between means were compared using LSD (P=0.05). Means followed by the same letter in the same column do not differ significantly. Levels of significance are represented by *P < 0.05, **P < 0.01, ***P < 0.001 and NS (not significant).

We tested the activity of enzymes involved in the photorespiration-mediated NH_4^+ production and NH_4^+ assimilation. GO and GGAT activities increased and HR activity decreased in WT plants grown under N-deficiency (Table 3). On the other hand, GO and HR decrease in *P_{SARK}::IPT* plants grown under 1 mM N, GGAT activity decreased at both 7 and 1 mM N treatments.

Table 3: Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on glyoxylate reductase (GO), glutamate:glyoxylate reductase (GGAT) and hidroxypyruvate reductase (HR) activities in two tobacco lines: 'WT' and 'IPT'.

Lines/NO_3^- Treatment	**GO** (ΔA_{324} $h^{-1}mg^{-1}prot$)	**GGAT** (ΔA_{340} $h^{-1}mg^{-1}prot$)	**HR** (ΔA_{340} $h^{-1}mg^{-1}prot$)
WT			
Control	0.19±0.03 b	1.06±0.09 b	6.56±0.29 a
7 mM	0.25±0.03 b	1.50±0.08 a	4.25±0.31 c
1 mM	0.45±0.05 a	1.51±0.14 a	5.34±0.41 b
P-value	***	**	***
$LSD_{0.05}$	0.12	0.31	1.00
IPT			
Control	0.63±0.04 a	1.17±0.12 a	6.61±0.27 a
7 mM	0.66±0.07 a	0.92±0.05 ab	6.40±0.14 a
1 mM	0.32±0.02 b	0.83±0.11 b	4.62±0.10 b
P-value	***	*	***
$LSD_{0.05}$	0.14	0.29	0.55

Values are means ± SE (n = 9) and differences between means were compared using LSD (P = 0.05). Means followed by the same letter in the same column do not differ significantly. Levels of significance are represented by *P < 0.05, **P < 0.01, ***P < 0.001 and NS (not significant) P > 0.05.

GS, GOGAT, GDH, and ATT increased their activity in the WT plants grown under N deficiency, showing the highest values at 1 mM of N (Fig 2). The transgenic plants did not display differences in their GS and ATT activities (Figs. 2A and D), while in the *P_{SARK}::IPT* plants grown in the presence of 7 mM N, GOGAT and GDH displayed their highest and lowest activity, respectively (Figs. 2B and C), although these values did not differ from those obtained with plants grown under control N conditions.

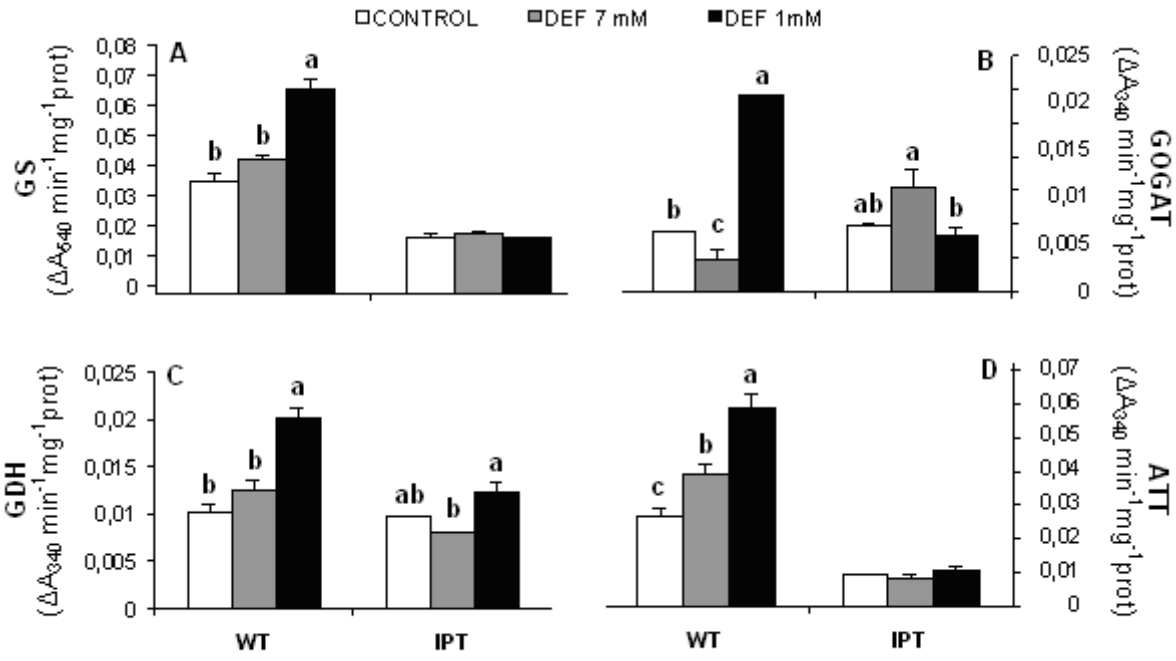

Fig. 2 Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on (A) glutamine sintetase (GS), (B) glutamate sintase (GOGAT), (C) glutamate dehydrogenase (GDH) and (D) aspartate aminetransferase (AAT) activity in leaves of two tobacco lines: 'WT' and 'IPT'. Bars represent means+se.(n=9);for each line.

Effects of N deficiency on free AAs contents

The concentration of total free AAs decreased drastically with the N-deficiency treatments in both WT and transgenic lines (Table 4). In spite of this decrease in AA contents, the relative content of Glu and Gln increased in WT plants (Table 4). In the transgenic plants, the relative content of Gln remained constant with N deficiency, while the relative content of Glu and alanine (Ala) increased. The relative contents of other AAs such as Gly, arginine (Arg), valine (Va), lysine (Lis), isoleucine (Ile), leucine (Leu), and phenylalanine (Phe) slightly increased with N-deficiency in both plant lines (Table 4). A sharp decrease in the relative content of proline (Pro) was seen under the N-deficiency treatments in both tobacco-plant lines (Table 4).

DISCUSSION

N deficiency reduced the foliar biomass in the leaves of WT tobacco plants (Table 1). Similar results have been reported in plants as different as *Triticum*, *Morus* or *Matricaria* subjected to N deficiencies (Tewari et al., 2004, 2007; Kovačik and Backor, 2007). This reduction in foliar biomass have been explained based on the numerous functions of this element within the plant (Maathuis, 2009). Furthermore, the N-deficiency induced decline in biomass, particularly in the case of severe deficiency (1 mM of N) appeared to be directly related to the diminished foliar concentration of NO_3^- and NH_4^+ (Fig. 1). In spite of the decrease in NO_3^- and NH_4^+ (Fig. 1), the transgenic plants expressing P_{SARK}*::IPT* maintained active foliar biomass under severe N-deficiency (Table

1). These results suggest that the higher CK content of the transgenic *P_{SARK}::IPT* plants resulted in the ability of the transgenic plants to growth under low N. It has been shown that in plants growing under nutrient deficiency, the decrease in growth was correlated with a decrease in CKs content (Kuiper, 1988), and that NO_3^- deficiency promoted lower CKs levels while NO_3^-

Table 4: Effect of 10 mM NO_3^- (control) and NO_3^- deficiency (7 and 1 mM) on foliar amino acids concentration in two tobacco lines: 'WT' and 'IPT'.

	Amino acids concentration (nmol g^{-1}FW) **and relative proportion** (%)					
Lines	**WT**			**IPT**		
NO_3^- Treatment	Control	7 mM	1 mM	Control	7 mM	1 mM
Aspartate	4657.33 (4.8)	3415.05 (4.8)	1791.46 (3.5)	4221.68 (4.4)	4020.91 (6.2)	1700.02 (3.8)
Serine	7993.42 (8.3)	7456.07 (10.5)	4037.21 (7.9)	10786.62 (11.3)	7104.29 (11.0)	6127.70 (13.7)
Glutamate	13899.41 (14.5)	5940.17 (8.4)	9398.65 (18.5)	9379.66 (9.8)	8452.01 (13.1)	7934.68 (17.1)
Glycine	2599.64 (2.7)	2428.42 (3.4)	1994.56 (3.9)	2186.21 (2.3)	1309.97 (2.0)	2596.99 (5.8)
Histidine	5716.29 (5.9)	4987.94 (7.0)	1456.72 (2.8)	7133.14 (7.5)	3197.57 (5.0)	1465.07 (3.3)
Asparagine	1531.69 (1.59)	1119.34 (1.5)	721.02 (1.4)	1678.91 (1.7)	1420.01 (2.2)	867.09 (1.94)
Glutamine	17658.01 (18.4)	15645.83 (22.1)	15137.40 (29.9)	18742.72 (19.7)	13486.03 (20.9)	8860.63 (19.88)
Arginine	437.53 (0.4)	457.49 (0.6)	389.38 (0.7)	767.65 (0.8)	450.05 (0.7)	433.99 (0.97)
Threonine	3046.95 (3.1)	2306.17 (3.2)	1661.17 (3.2)	4765.50 (5.0)	3659.12 (5.7)	2662.25 (5.9)
Alanine	5427.07 (5.6)	5371.04 (7.6)	1714.20 (3.4)	3930.18 (4.1)	3158.13 (4.9)	3638.80 (8.2)
Proline	27557.23 (28.7)	16384.47 (23.2)	7129.48 (14.1)	25976.87 (27.3)	14048.94 (21.8)	3018.55 (6.7)
Cysteine	nd	nd	nd	nd	nd	nd
Thyroxine	339.46 (0.4)	752.73 (1.1)	210.42 (0.4)	778.77 (0.8)	303.41 (0.4)	735.52 (1.6)
Valine	658.19 (0.7)	1137.06 (1.6)	847.47 (1.7)	1079.13 (1.1)	908.47 (1.4)	1109.99 (2.5)
Methionine	756.60 (0.8)	nd	nd	nd	nd	nd
Lysine	1115.60 (1.2)	744.85 (1.1)	968.23 (1.9)	285.78 (0.3)	279.19 (0.4)	780.57 (1.7)
IsoLeucine	666.59 (0.7)	522.98 (0.7)	647.35 (1.3)	858.16 (0.9)	441.28 (0.7)	781.93 (1.7)
Leucine	930.48 (1.0)	925.64 (1.3)	742.78 (1.5)	917.17 (1.0)	846.69 (1.3)	923.42 (2.0)
Phenylalanine	987.11 (1.0)	996.82 (1.4)	1019.45 (2.0)	1357.33 (1.4)	1184.54 (1.8)	923.39 (2.0)
Total	95970.09 (100)	70592.13 (100)	50688.02 (100)	94845.55 (100)	64270.68 (100)	44560.67 (100)

applications resulted in increase shoot CKs and the activation of genes associated with nutrient uptake and homeostasis (Argueso et al., 2009). NR and NiR are critical steps in NO_3^- assimilation (Lea and Azevedo, 2007). The decrease in NR activity seen in the WT plants is supported by the reported NO_3^- induced NR activity (Cruz et al. 2004). It has been shown that high endogenous CK levels reduced the inducibility of NR by NO_3^- (Lexa, 2002). This observation is in agreement with our results showing that the *P_{SARK}::IPT* plants displayed a low response of NR activity to N deficiency (Table 2).

NH_4^+ can be formed not only by NO_3^- assimilation but also by diverse metabolic pathways such as photorespiration or protein degradation (Temple et al., 1998; Tercé-Lagorgue et al., 2004). The increase in the enzymes GO and GGAT (Table 3) seen in WT plants during N deficiency could lead to a greater NH_4^+ formation and an increase in N-assimilation (Wingler et al., 2000). In addition, the increased GDH activity, observed in the deficient WT plants (Fig. 2C), would result in increased protein degradation, especially in senescent parts of the plant. Thus, increased photorespiration and GDH activity in WT plants would increase NH_4^+ formation. These functions are carried out by the GS/GOGAT cycle, which increased in the WT plants grown under severe N deficiency (1mM) (Figs 2A and B). This strategy would also include the elimination of excess toxic NH_4^+, and contribution to the maintenance of N status during N deficiency.

The transgenic plants did not display the activation of enzymes associated with photorespiration under N deficiency (Table 3). Consequently,

the formation of NH_4^+ by this pathway did not appear to be affected. The relatively low amounts of NH_4^+ in the *P_{SARK}::IPT* plants were paralleled by the maintenance in GDH activity and the low response of the GS/GOGAT cycle in the transgenic plants.

N deficiency lowered the amounts of AAs in both WT and transgenic plants (Table 4). Similar decline in AAs under N-deficiency conditions has been reported in tobacco plants as well as other species such as *Cassava* or *Matricaria* (Terce-Laforgue et al., 2004; Cruz et al., 2004; Kováčik et al., 2006). Despite the general decrease in AAs, there as a relative increase in Glu and Gln during N-deficiency. However, the relative amounts of Pro decreased (Table 4). The degradation of proteins by GDH and the decline in Pro could have favoured the synthesis of Glu and Gln. In addition to their role as donors of the amino group for the synthesis of other AAs or compounds such as ureides or nucleic acids, Glu and Gln constitute the main form of N transported by the phloem towards the young parts in growth, a vital process for plant growth this process under N deficiency (Lea and Azevedo, 2007). In the transgenic plants, the relative amounts of Gln remained constant with N deficiency, while there was a relative increase in Glu and Ala. It has been suggested that CKs may support the AA-mediated communication of N demand from the shoot to the roots and participate in the regulation of N assimilation (Collier, 2003; Gessler et al., 2004). In our work, the transgenic plants showed similar quantities of total AAs as in WT plants, reducing their amount in similar proportions under N deficiency (Table 4). These results are in agreement with Dluzniewska et al.

(2006), who showed that the application of external CKs did not raise the levels of AAs in *Poplar*.

N often limits plant growth and development, and in response to N deficiency, plants have developed a number physiological as well as morphological strategies to adjust and maintain their growth and development. In this work, we demonstrate that in transgenic tobacco expressing P_{SARK}*:: IPT* plants, CKs prevented the generation and assimilation of NH_4^+ by stimulating such processes as photorespiration, protein degradation, the GS/GOGAT cycle, and the formation of AAs such as Gln, responses that were seen in the WT plants grown under N deficiency. In spite of the N-deficient conditions, the transgenic tobacco P_{SARK}*:: IPT* plants, maintained their foliar growth constant, suggesting a role of the CKs in the efficient use of the N available under these conditions.

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REFERENCES

Antal T, Mattila H, Hakala-Yatkin M, Tyystjärvi T, Tyystjärvi E. 2010. Acclimation of photosynthesis to nitrogen deficiency in *Phaseolus vulgaris*. Planta, 232, 887-898.

Argueso CT, Ferreira FJ, Kieber JJ. 2009. Environmental perception avenues: the interaction of cytokinin and enviromental response pathways. Plant Cell and Environment, 32, 1147-1160.

Barneix AJ, James DM, Watson EF, Hewitt EJ. 1984. Some effects of nitrate abundance and starvation on metabolism and accumulation of nitrogen in barley (*Hordeum vulgare* L. cv. Sonja). Planta, 162, 469-476.

Bradford MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. Analytical Biochemistry, 72, 248–254.

Cataldo DA, Haroon M, Schrader LE, Young VL. 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Communication in Soil Science and Plant Analysis, 6, 71–80.

Collier MD, Fotelli NM, Nahm N, Rennenberg H, Hanke DE, Gessler A. 2003. Regulation of nitrogen uptake by Fagus sylvatica on a whole plant level: interaction between soluble nitrogen compounds and cytokinins. Plant Cell and Environment, 26, 1549-1560.

Cruz JL, Mosquim PR, Pelacani CR, Araújo WL, Damatta FM. 2004. Effects of nitrate nutrition on nitrogen metabolism in cassava. Biologia Plantarum, 48, 67-72.

Dluzniewka P, Gessler A, Kopriva S, Strnad M, Novák O, Dietrich H, Rennenberg H. 2006. Exogenous supply of glutamine and active cytokinin to the roots reduces NO_3^- uptake rates in poplar. Plant Cell and Environment, 29, 1284-1297.

Fan XH, Tang C, Rengel Z. 2002. Nitrate uptake, nitrate reductase distribution and their relation to proton release in five nodulated grain legumes. Annals of Botany, 90, 315-323.

Feierabend J, Beevers H. 1972. Developmental studies on microbodies in wheat leaves. I. Conditions influencing enzyme development. Plant Physiology, 49, 28-32.

Gessler A, Rennenberg H, Kopriva S. 2004. Regulation of nitrate uptake on the whole plant level: interaction between nitrogen compounds, cytokinins and carbon metabolism. Tree Physiology, 24, 1313-1321.

Gonzalez EM, Gordon AJ, James CL, Arrese-Igor C. 1995. The role of sucrose synthase in the response of soybean nodules to drought. Journal of Experimental Botany, 26, 1515- 1523.

Groat RG, Vance CP. 1981. Root nodule enzymes of ammonia assimilation in alfalfa (*Medicago sativa* L.). Plant Physiology, 67, 1198–1203.

Hageman RH, Hucklesby DP. 1971. Nitrate reductase. Methods of Enzimology, 23, 497-503.

Hoder M, Rej R. 1983. Alanine aminotransferase. In: Methods of enzymatic analysis, Vol III. (Eds) Bergmeyer, HU, Bergmeyer J, Graβl M. Weinhein: Verlag chemie.

Igarashi D, Tsuchida H, Miyako M, Oshumi C. 2006. Glutamate:Glioxilate aminotrasnferase modulates aminoacid content during photorespiration. Plant Physiology, 142, 901-910.

Imsande J, Touraine B. 1994. N demand and the regulation of nitrate uptake. Plant Physiology, 105, 3-7.

Kaiser JJ, Lewis OAH. 1984. Nitrate reductase and glutamine synthetase activity in leaves and roots of nitrate fed *Helianthus annuus* L. Plant and Soil, 70, 127–130.

Key AJ, Bird IF, Cornelius MJ, Lea PJ, Wallsgrove RM, Miflin BJ. 1978. Photorespiratory nitrogen cycle. Nature, 275, 741-743.

Kiba T, Kudo T, Kojima M, Sakakibara H. 2011. Hormonal control of nitrogen acquisition: roles of auxin, abscisic acid, and cytokinin. Journal of Experimental Botany, 62, 1399-1409.

Kováčik J, Backor M. 2007. Changes of phenolic metabolism and oxidative status in nitrogen-deficient *Matricaria chamomilla* plants. Plant and Soil, 297, 255-265.

Kováčik J, Repčák M, Kron I. 2006. Nitrogen deficiency induced changes of free amino acids and coumarin contents in the leaves of *Matricaria chamomilla*. Acta Physiologia Plantarum, 28, 159-164.

Krom MD. 1980. Spectrophotometric determination of ammonia: study of a modified Berthelot reaction using salicylate and dichloroisocyanurate. Analyst, 105, 305–316.

Kuiper D. 1988. Growth reponses of *Plantago major* L. ssp. *pleiosperma* to changes in mineral supply. Plant Physiology, 87, 555-557.

Lea PJ, Azevedo RA. 2006. Nitrogen use efficiency. 1. Uptake of nitrogen from the soil. Annals of Applied Biology, 149, 243-247.

Lemaître T, Gaufichon L, Bouret-Mercey S, Christ A, Masclaux-Daubresse C. 2008. Enzymatic and metabolic diagnostic of nitrogen deficiency in *Arabidopsis thaliana Wassileskija* accession. Plant Cell Physiology, 49, 1056-1065.

Lexa M, Genkov T, Bzobohatý P. 2002. Inhibitory effects of elevated endogenous cytokinins on nitrate reductase in ipt-expressing tobacco are eliminated by short-term exposure to benzyladenine. Physiologia Plantarum, 115, 284-290.

Lillo C. 1984. Diurnal variations of nitrite reductase, glutamine synthetase, glutamate synthase, alanine aminotransferase and aspartate aminotransferase in barley leaves. Physiologia Plantarum, 61, 214-218.

Maathuis FJM. 2009. Physiological funcions of mineral macronutrients. Current Opinion in Plant Biology, 12, 250-258.

Madore M, Grodzinski B. 1984. Effect of oxygen concentration on ^{14}C-photoassimilate transport from leaves *Salvia splendens* L. Plant Physiology, 76, 782-786.

Oliveira IC, Brenner E, Chiu J, Hsieh MH, Kouranov A, Lam HM, Shin MJ, Coruzzi J. 2001. Metabolite and light regulation of metabolism in plants: lessons from the study of a single biochemical pathway. Brazilian Journal of Medical and Biological Research, 34, 567-575.

Osmond, CB. 1981. Photorespiration and photoihibition. Some implications for the energetic of photosynthesis. Biochimica et Biophysica Acta, 639, 77-98.

Redinbaugh MG, Campbell WH. 1993. Glutamine synthetase and ferredoxin-dependent glutamate synthase expression in the maize (*Zea mays*) root primary response to nitrate (evidence for an organ-specific response). Plant Physiology, 101, 1249-1255.

Rivero RM, Kojima M, Gepstein A, Sakakibara H, Mittler R, Gepstein S, Blumwald E. 2007. Delayed leaf senescence induces extreme drought tolerance in a flowering plant. Proceedings of the National Academy of Science USA, 104, 19631-19636.

Rivero RM, Shulaev V, Blumwald E. 2009. Cytokinin-dependent photorespiration and the protection of photosynthesis during water deficit. Plant Physiology, 150, 1530-1540.

Rubio-Wilhelmi MM, Sanchez-Rodriguez E, Rosales MA, Blasco B, Rios JJ, Romero L, Blumwald E, Ruiz JM. 2011. Effect of cytokinins on oxidative stress in tobacco plants under nitrogen deficiency. Environmental and Experimental Botany, 72, 167-173.

Sheen J. 2002. Phosphorelay and transcription control in cytokinin signal transduction. Science, 296, 1650-1652.

Singh RP, Srivastava HS. 1986. Increase in glutamate synthase activity in maize seedlings in response to nitrate and ammonium nitrogen. Physiologia Plantarum, 66, 413-416.

Takei K, Takahashi T, Sugiyama T, Yamaya T, Sakakibara H. 2002. Multiple routes communicating nitrogen availability from roots to shoots: a signal transduction pathway mediated by cytokinins. Journal of Experimental Botany, 53, 971-977.

Temple SJ, Vance CP, Gantt JS. 1998. Glutamate synthase and nitrogen assimilation. Trends in Plant Science, 3, 51-56.

Tercé-Laforgue T, Mäck G, Hirel B. 2004. New insight towards the function of glutamate dehydrogenase revealed during source-sink transition of tobacco (*Nicotiana tabacum*) plants grown under different nitrogen regimes. Physiologia Plantarum, 120, 220-228.

Tewari RK, Kumar P, Sharma PN. 2007. Oxidative stress and antioxidant responses in young leaves of mulberry plants grown under nitrogen, phosphorus or potassium deficiency. Journal of Integrative Plant Biology, 49, 313-322.

Tewari RK, Kumar P, Tewari N, Srivastava S, Sharma PN. 2004. Macronutrient deficiencies and differential antioxidant responses-influence on the activity and expression of superoxide dismutase in maize. Plant Science, 166, 687-694.

Wallsgrove RM, Lea PJ, Miflin BJ. 1979. Distribution of enzymes of nitrogen assimilation within the pea leaf cell. Plant Physiology, 63, 232-236.

Wingler A, Lea PJ, Quick WP, Leegood RC. 2000. Photorespiration: metabolic pathways and their role in stress protection. Phylosophical Transaction of the Royal Society B, 355, 1517-1529.

Wu J, Neimanis S, Heber U. 1991. Photorespiration is more effective than the Mehler reaction in protecting the photosynthetic apparatus against photoinhibition. Botanica Acta,104, 283-291.

Capítulo 5

Cytokinin-dependent improvement in N-use efficiency and plant quality in transgenic P_{SARK}::IPT tobacco under N deficiency

ABSTRACT

Wild type (WT) and transgenic tobacco plants overexpressing isopentenyltransferase (IPT), a gene coding the rate-limiting step in cytokinin (CKs) synthesis, were grown under limited nitrogen (N) conditions, in order to evaluate the role of CKs in N-use efficiency (NUE) and in different parameters that determine the quality of tobacco leaves. Our results indicate that WT tobacco plants submitted to N deficiency show a decline in the leaves:roots ratio, associated with a decrease in the NUE and in tobacco-leaf quality, defined by an increase in the quantity of nicotine. On the contrary, the transgenic plants submitted to N deficiency maintained the leaves:roots ratio constant, presenting a higher NUE and greater quality of tobacco leaves than the WT plants, as the latter showed reduced nicotine and an increase in reducing sugars under severe N-deficiency conditions. Therefore, the overexpression of CKs under N deficiency could be a useful tool to improve tobacco cultivation, given that it could reduce N-fertilizer application and thereby provide economic savings and environmental benefits, maintaining yield and improving tobacco leaf quality.

Keywords: Cytokinins; nicotine; nitrogen deficiency; N-use efficiency; reduced sugars; tobacco plants.

Abbreviations: CKs, cytokinins; IPT, isopentenyltransferase; N, nitrogen; NUE, N-use efficiency; NO_3^-, nitrate; NUpE, nitrogen-uptake efficiency; NUtE, nitrogen-utilization efficiency; TNA, total nitrogen accumulation; TNC, total nitrogen content.

INTRODUCTION

N availability is considered one of the main limiting factors in crop production (Glass, 2003). This is particularly true for tobacco plants, where the decline in foliar biomass is directly correlated to N deficiency (Balachandran et al., 1997), since the tobacco cultivation requires high quantities of nitrate (NO_3^-) for maximum yield (Ruiz et al., 2006). Since only 30-40% of the N applied to the soil is used by the plant, greater NUE could improve crop yield and quality, reducing economic costs as well as decreasing environmental degradation caused by N-fertilizer application (Baligar et al., 2001). Therefore, the selection of cultivars with high NUE becomes critical, especially in crops such as tobacco, in which N is essential to reach harvest. Thus, crops that have high NUE offer greater yield under conditions of limited N supply or require lower N quantities to reach the same yield as crops with lower NUE (Ruiz et al., 2006; Svecnjak and Rengel, 2006).

NUE is defined as biomass production per unit of N available in the soil (Moll et al., 1982). This can be divided into two fundamental processes: i) the ability of the plant to take up N from the soil; and, ii) the efficient use of the N taken up, i.e. the capacity of the plant to transfer and utilize this element in plant organs (Ruiz et al., 2006). Genetic variability in NUE has been demonstrated in several species such as rice, alfalfa, and maize (Harrison et al., 2004; Lawlor, 2002). Improved NUE together with the evaluation of external factors such as agricultural practices or soil types have today become essential for maintaining

agricultural output, particularly when N is limited in the environment (Kant and Rothstein, 2011; Baligar et al., 2001).

The quality of tobacco is a complex combination of visual, physical, and chemical characteristics that are strongly influenced by N fertilization (Smeeton, 1987). The NO_3^- levels in tobacco leaves have a marked effect on their chemical composition, as it stimulates the formation of compounds that are harmful for human health, such as nitric oxide, volatile carcinogenic compounds, or specific nitrosamines that are directly related to the quantity of this anion in the leaf (Ruiz et al., 2006). Furthermore, alterations in the availability of NO_3^- in tobacco plants could provoke changes in the leaf nicotine content, and a close relationship has been reported between N metabolism and alkaloid synthesis (Hashimoto and Yamada, 1994). In addition, another parameter used to evaluate the tobacco quality are sugars, which are closely related to the quantity of NO_3^-, given that an increase in the quantity of NO_3^- in tobacco leaves lowers the sugar quantity and thus quality (Weybrew et al., 1983).

CKs are phytohormones that control the plant developmental programme. In addition, a relationship between CKs and macronutrient acquisition has been postulated (Franco-Zorrilla et al., 2002). Recent studies have indicated that CKs act as long-distance messengers signalling the N status of the plant (Takei et al., 2002), thus regulating the nutrient-uptake systems (Sakakibara et al., 2006). Previous work indicated that plants overexpressing IPT, an enzyme that catalyses the limiting step in CKs

synthesis, do not display biomass reduction caused by N deficiency (Rubio-Wilhelmi et al., 2011), and a CK-dependent NUE was suggested. Here, we aimed to examine the response of NUE and leaf quality of P_{SARK}*::IPT* tobacco plants submitted to N deficiency.

MATERIAL AND METHODS

Plant material, growth conditions and plant growth

Seeds of WT (*Nicotiana tabaccum* cv.SR1) and transgenic plants expressing P_{SARK}*::IPT* were germinated and grown as described before (Rubio-Wilhelmi et al., 2011). Growth conditions and N-treatments was as described elsewhere (Rubio-Wilhelmi et al., 2011). The N treatments began 60 days after sowing (DAS) and were maintained for 30 days. The treatments were 10 mM (Control), 7 mM and 1 mM $NaNO_3$. The experimental design was a randomized complete block with six treatments, arranged in individual pots with six plants per treatment, and three replicates. The experiment was repeated three times under the same conditions (n = 9).

Plant analysis

All plants were at the late vegetative stage when harvested. Middle leaves (positions 7^{th} and 8^{th}) were harvested, frozen immediately in liquid N_2, and kept at -80℃ until used. Plant material was lyophilised and used to determine N forms, nicotine and soluble sugar and starch. To determine the leaves:roots

ratio, leaves and roots from three plants per line were sampled at 90 DAS. The leaves and roots were dried in a forced-air oven at 70℃ for 24 h, and the dry weight (DW) was recorded.

NUE parameters

NO_3^- was analyzed from an aqueous extraction of 0.2 g of dried leaves and roots material in 10 mL of Millipore-filtered water (Cataldo et al., 1975). Reduced N concentrations were analysed after digestion of 0.2 g dry and milled leaves and roots material with H_2SO_4 (5 mL at 98%) and H_2O_2 (30%) (Sigma-Aldrich, Madrid, Spain) (Baethgen and Alley, 1989). **TNC** (Total Nitrogen Content) represents the sum of reduced N and NO_3^- and was expressed as mg/g DW. **TNA** (Total Nitrogen Accumulation) was calculated as TNC divided by leaves tissue DW values (Sorgona et al., 2006), the result being expressed as mg N. **NUtE** (Nitrogen-Utilization Efficiency) was calculated as leaves tissue DW divided by TNC (Siddiqi and Glass et al., 1981). The results were expressed as g DW/mg N. **NUpE** (Nitrogen-Uptake Efficiency) was calculated as TNA divided by roots tissue DW values (Elliot and Laüchli, 1985), the results were expressed as mg N g/DW root. Over the period under study, determination of N uptake fluxes were calculated from the relative growth rate (RGR), the fresh weight (FW), the nutrient total concentrations, and NO_3^- concentration contents of leaves and roots (Kruse et al., 2007).

Nicotine analysis

Half a leaf without midvein (100 mg) was harvested and frozen in liquid N_2. Leaf material was ground frozen in a 2 mL microcentrifuge tube. The samples were extracted by shaking the tubes vigorously for 2 h with 1.5 mL of 40% aqueous MeOH, containing 0.5% acetic acid. The resulting extract was centrifuged (13000 rpm, 12 min) and filtered through a 0.45µm PVDF membrane. Chromatographic analyses were carried out on a Phenomenex reverse-phase column (250 x 4.6 mm, Luna 5um C18 (2) 100A) (Keinänen et al., 2001). Nicotine standard were from Sigma-Aldrich. The mobile phase consisted of two solvents: (A) H_3PO_4 in water (pH 2.2) and (B) acetonitrile. HPLC gradient, 0-6 min, 12% B; 6-10 min, 18% B; and reaching 58% B in 30 min. The flow rate was 1 mL/min, the injection volume was 20 µL, and the column oven was set at 24 ºC. The eluent was monitored at 210, 254, 320, and 365 nm. The HPLC/UV analyses were carried out with an Agilent HPLC 1100 series.

Reduced sugar and starch analysis

Frozen Leaves homogenized for sugar analysis using mortar in 5 mL of ethanol at 96%. After centrifugation, the insoluble fraction of the extract was washed with 5 mL of ethanol at 70%. The extract was centrifuged at 5500 rpm for 10 min and the supernatant was stored at 4 ℃ for the determination of reduced sugars such as sucrose, glucose and fructose (Irigoyen et al., 1992), while the residue remaining from the centrifugation was dried for 48 hours at 40 ℃ and

used in the analysis of starch concentration (Irigoyen et al., 1992). The analysis of starch and reduced sugars were made with the anthrone reagent (Sigma-Aldrich, Madrid, Spain) at an absorbance of 650 nm against a standard curve of sucrose. The starch, reduced sugar concentrations were expressed as mg/g DW.

Statistical analysis

The data compiled were submitted to an analysis of variance (ANOVA) and the differences between the means were compared by Duncan's multiple-range test ($P > 0.05$).

RESULTS

Leaves:roots ratio and NUE parameters

The leaves:roots ratio was measured in WT and *P_{SARK}::IPT* tobacco plants. In WT plants leaves:roots ratio remained unchanged when the plants were exposed to 7 mM of N, whereas a significant decline was observed when the WT tobacco plants were exposed to a severe N deficiency (1mM) (Table 1). Nitrogen deficiency did not induced changes in the leaves:roots ratio in the transgenic plants (Table 1).

Table 1: Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on Total leave/Root ratio in two tobacco lines: 'WT' and 'IPT'.

	Total leaves:root ratio	
NO_3^- Treat.	**WT**	**IPT**
Control	9.41± 0.88 a	18.80± 0.41
Def 7 mM	9.83± 0.73 a	24.71± 1.10
Def 1 mM	6.98± 0.30 b	19.75± 3.24
P-value	*	NS
$LSD_{0.05}$	2.378	6.894

Values are means ± SE (n = 9) and differences between means were compared using LSD (P=0.05). Means followed by the same letter in the same column do not differ significantly. Levels of significance are represented by *P < 0.05, **P < 0.01, ***P < 0.001 and NS (not significant) P > 0.05.

Both wild-type and transgenic *P_{SARK}::IPT* tobacco lines showed a significant decrease in TNC under severe N deficiency (1 mM), but no changes were seen in the 7 mM of N treatments (Table 2). Treatments of 1 mM of N lowered the TNA in WT plants, while in *P_{SARK}::IPT* plants the TNA was reduced both under the 7 mM application as well as under 1 mM of N (Table 2). With respect to the two NUE components, NUtE increased in both tobacco lines under the severe N-deficiency treatment (1 mM), (Table 2). Both wild type and *P_{SARK}::IPT* plants showed a significant reduction of NUpE under the 1 mM treatment of N with respect to control (Table 2). Similar results were found in the N uptake flux that diminished significantly with the deficient N applications in both tobacco-plant lines (Fig. 1).

Table 2: Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on TNC (total nitrogen content), TNA (total nitrogen accumulation), NUtE (nitrogen-utilization efficiency) and NUpE (nitrogen-uptake efficiency) in two tobacco lines: 'WT' and 'IPT'.

Lines/NO_3^- Treatment	**TCN** (mg/g DW)	**TNA** (mg N)	**NUtE** (g DW/mg N)	**NUpE** (mg N g/DW root)
WT				
Control	55.61±4.02 a	376±27.21a	0.122±0.008 b	311±22.49 a
Def 7 mM	59.27±1.29 a (+6.5%)	320±6.98 a (-14.8%)	0.091±0.002 c (-25.4%)	325±7.09 a (+4.6%)
Def 1 mM	23.18±1.18 b (-42%)	105±5.35 b (-72%)	0.196±0.009 a (+60.5%)	86±4.40 b (-72.2%)
P-value	***	***	***	***
$LSD_{0.05}$	8.760	57.14	0.026	47.93
IPT				
Control	59.86±2.08 a	548±19.05 a	0.153±0.005 b	876±30.48 b
Def 7 mM	53.16±2.81 a (-11%)	419±22.22 b (-23.5%)	0.149±0.007 b (-2.6%)	1103±58.48 a (+25.7%)
Def 1 mM	28.48±1.16 b (-47%)	234±9.58 c (-57.2%)	0.289±0.012 a (+88.8%)	446±18.24 c (-49%)
P-value	***	***	***	***
$LSD_{0.05}$	7.376	61.53	0.030	136.71

Values are means ± SE (n = 9) and differences between means were compared using LSD (P=0.05). Means followed by the same letter in the same column do not differ significantly. Levels of significance are represented by *P < 0.05, **P < 0.01, ***P < 0.001 and NS (not significant) P > 0.05.

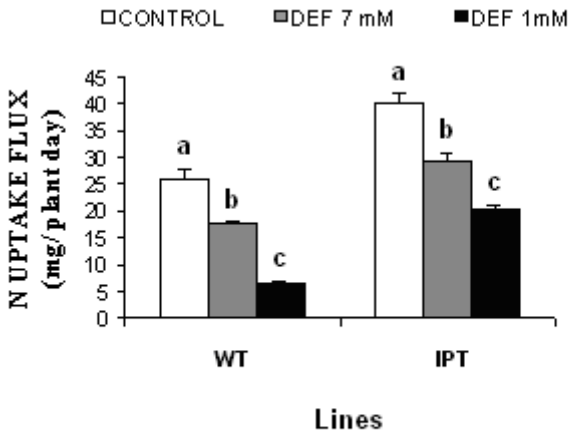

Fig. 1 Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on N uptake flux of two tobacco lines: 'WT' and 'IPT'.

Nicotine, reduced sugar, and starch

In the WT plants, N deficiency provoked a significant increase in the nicotine amounts both under the 7 mM application as well as under severe deficiency (1mM) (Fig. 2). A reverse situation was seen in the *P_{SARK}::IPT* tobacco plants, since the N deficiency treatment significantly lowered the amounts of nicotine (Fig. 2).

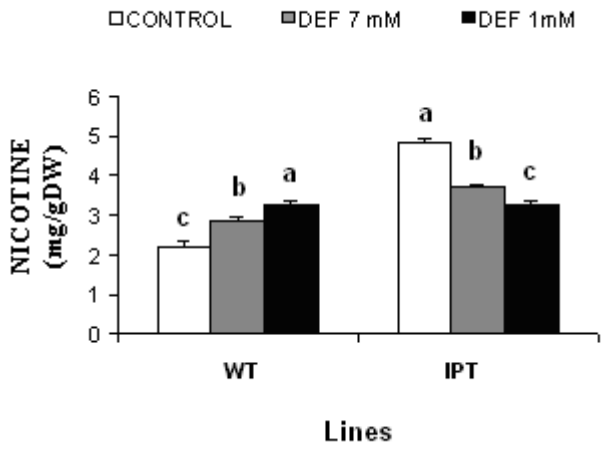

Fig. 2 Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on Nicotine concentration of two tobacco lines: 'WT' and 'IPT'.

The WT tobacco plants showed a significant increase in reducing sugars contents under N-deficiency conditions (Fig. 3A), and *P_{SARK}::IPT* plants (Fig. 3A). A similar trend was found in the quantity of starch in tobacco leaves of both lines, which showed a significant increase with N deficiency (Fig. 3B).

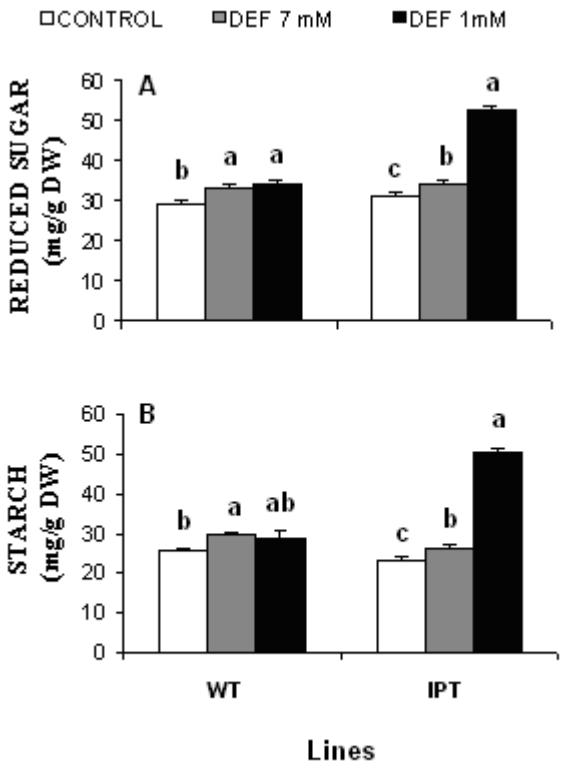

Fig. 3 Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on (A) sugar reduced concentration, (B) starch concentration of two tobacco lines: 'WT' and 'IPT'.

DISCUSSION

We have shown previously that the growth of WT tobacco plants under N-deficiency resulted in reduced foliar biomass and lower relative growth rates (Rubio-Wilhelmi el al., 2011). Similar results have been reported in different plant species (Tewari et al., 2007; Kováčik and Backor, 2007), confirming that plants submitted to limiting conditions of N redirect the photoassimilates towards the root zone to increase growth and intensify N uptake (Scheible et al., 2004). Our results appear to support this notion, as shown by a reduction of the leaves:roots ratio in WT tobacco plants subjected to N deficiency (Table 1). On

the other hand, the transgenic plants expressing *P_{SARK}::IPT* maintained active growth and biomass under severe N deficiency (Rubio-Wilhelmi et al., 2011). This sustained biomass, accompanied by maintenance of the leaves:roots ratio under N deficit (Table 1) would indicate that the CKs might impede the rerouting of the photoassimilates towards the root zone, which would otherwise limit N availability, favouring shoot growth.

The increase in nutrient-use efficiency has been described as a useful tool to improve agricultural systems (Fageria et al., 2008). Thus, because N is a determining element in tobacco yield, the selection of genotypes with high NUE is critical for the improvement of this crop. The TNC and TNA are determining parameters in the nutritional state of the plants, given that they show a direct relationship between yield and leaf content in N (Ruiz et al., 2008). In our work, tobacco plants of both lines submitted to severe N deficiency (1 mM) reduced the TNC and TNA (Table 2). However, the WT plants lowered TNA (72%) by a higher proportion than the transgenic plants, with respect to control (57.2%) (Table 2). This fact is correlated with the parameters related to NUE, since both plant lines responded similarly to the N deficiency. However, in the transgenic plants the NUtE increased to 88% under severe N deficiency (1 mM), while in WT plants the increase was only 60% (Table 2). This would indicate that under severe N deficiency (1 mM), the transgenic plants utilize N more efficiently. Furthermore, the transgenic plants also displayed more efficient N uptake than WT plants when both lines were under severe N limitation, the NupE decreasing by 72.2% in WT plants, whereas in the transgenic plants the decrease was only 49% (Table 2). This finding is again demonstrated by comparing the uptake flow

of N of both plant lines (Fig. 1). The results would explain previous observations that showed the maintenance of the biomass and foliar RGR in *P_{SARK}::IPT* tobacco plants under adverse growth conditions (Rubio-Wilhelmi et al., 2011). Similar results have been shown in varieties of *Brassica* under N deficiency, showing that varieties with greater NUE presented greater biomass (Ye et al., 2010).

Tobacco quality is a complex combination of physical, chemical, and visual characteristics that are strongly influenced by N fertilization and the foliar concentration of NO_3^- (Smeeton, 1987). Previous work have shown that the N deficiency in both tobacco lines diminished the quantity of NO_3^- (Rubio-Wilhelmi et al., 2011). This reduction noticably affects the improvement in quality, as it reduces the quantity of nitric oxide and volatile carcinogenic compounds that are related to the quantity of this anion in the leaf. In addition to the NO_3^- concentration, another parameter that determines the quality of the tobacco leaves is the nicotine. Tropano alkaloids such as nicotine are defined as secondary metabolites that contain atomic N in their molecules and their precursors are two amino acids such as ornithine and arginine (Hasimoto and Yamada, 1994). The N deficiency provoked an increase in the quantity of nicotine in WT plants (Fig. 2). This has been reported earlier in tobacco plants subjected to N deficiency that show typical symptoms of N deficiency (Xi et al., 2008). Nicotine as a secondary metabolite could change the chemical composition of the leaf, palliating the damage caused by pathogen attack or abiotic stress (Roda et al., 2003). The increase in nicotine amounts involves a loss in tobacco quality (Ruiz et al., 2006) since nicotine is the main harmful

component in cigarettes, playing an essential role in the development of cardiovascular diseases and cancer (Yildiz, 2004). However, the reduction in the quantity of nicotine shown by transgenic plants under N deficiency (Fig. 2), representing better leaf quality. Finally, the increase in reducing sugars and starch in transgenic plants under severe N deficiency (1 mM) (Fig. 3) would also notably favour the quality of tobacco leaf, since in tobacco, reducing sugars have the most favourable influence on the aroma and taste during smoking and thus is one of the important constituents for the evaluation of tobacco quality (Tariq et al., 2010). Finally, it should be pointed out that the increase of up to 69% of the soluble sugars and the starch found in the leaves of P_{SARK}::*IPT* tobacco submitted to severe N deficiency (1 mM) (Figs. 3A and B) would demonstrate that the photoassimilates remain in the shoot, favouring its growth while avoiding a reduction of foliar biomass as a result of N deficiency.

In short, our results appear to indicate that the overexpression of the CKs under N deficiency could be useful as strategy to improve the tobacco leaf quality reducing costs (fertilizers, wastes, environmental burden, etc.)

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REFERENCES

Baethgen WE, Alley MM. 1989. A manual colorimetric procedure for measuring ammonium nitrogen in soil and plant. Communications in Soil Science and Plant Analysis, 20, 961–969.

Balachandran S, Hull RJ, Martins RA, Vaadia Y, Lucas WF. 1997. Influence of environmental stress on biomass partitioning in transgenic tobacco plants expressing the movement protein of tobacco mosaic virus. Plant Physiology, 114, 475-481.

Baligar VC, Fageria NK, He ZL. 2001. Nutrient use efficiency in plants. Communications in Soil Science and Plant Analysis, 32, 921-950.

Cataldo DA, Haroon M, Schrader LE, Young VL. 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Communications in Soil Science and Plant Analysis, 6, 71-80.

Elliot GC, Laüchli A. 1985. Phosphorous efficiency and phosphate-iron interaction in maize. Agronomy Journal, 77, 399-403.

Fageria NK, Baligar VC, Li YC. 2008. The role of nutrient efficient plants in improving crop yields in the twenty first century. Journal of Plant Nutrition, 31, 1121-1157.

Franco-Zorrilla JM, Cubas P, Jarrillo JA, Fernández-Calvín B, Salinas J, Martinez-Zapater JM. 2002. Mutations at CRE1 impair cytokinin-induced repression of phosphate starvation responses in *Arabidopsis*. Plant Journal, 32, 353-360.

Glass ADM. 2003. Nitrogen use efficiency of crop plants: physiological constrains upon nitrogen absorption. Critical Review of Plant Science, 22, 453-470.

Harrison J, Hirel B, Limani AM. 2004. Variation in nitrate uptake and assimilation between two ecotypes of *Lotus japonicus* and their recombinant inbred lines. Physiologia Plantarum, 120, 124-131.

Hashimoto T, Yamada Y. 1994. Alkaloid biogenesis: molecular aspects. Annual Review of Plant Physiology and Plant Molecular Biology, 45, 257-285.

Irigoyen JJ, Emerich DW, Sánchez-Díaz M. 1992. Water stress induced changes in the concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. Physiologia Plantarum, 8, 455-460.

Kant S, Bi YM, Rothstein SJ. 2011. Understanding plant response to nitrogen limitation for the improvement of crop nitrogen use efficiency. Journal of Experimental Botany, 4, 1499-1509.

Keinänen M, Oldham NJ, Baldwin IT. 2001. Rapid HPLC Screening of Jasmonate-Induced Increases in Tobacco Alkaloids, Phenolics, and Diterpene Glycosides in *Nicotiana attenuate*. Journal of the Science of Food and Agriculture, 49, 3553-3558.

Kováčik J, Backor M. 2007. Changes of phenolic metabolism and oxidative status in nitrogen-deficient *Matricaria chamomilla* plants. Plant and soil, 297, 255-265.

Kruse J, Kopriva S, Hänsch R, Krauss GJ, Mendel RR, Rennenberg H. 2007. Interaction of sulfur and nitrogen nutrition in tobacco (*Nicotiana*

tabacum) plants; significance of nitrogen source and root nitrate reductase. Plant Biology, 9, 638–646.

Lawlor DW. 2002. Carbon and nitrogen assimilation in relation to yield: mechanisms are the key to understanding production systems. Journal of Experimental Botany, 53, 773-787.

Moll RH, Kamprath EJ, Jackson WA. 1982. Analysis and interpretation of factors which contribute to efficiency of nitrogen utilization. Agronomy Journal, 74, 562-564.

Roda AL, Oldham NJ, Svatos A, Baldwin IT. 2003. Allometric analysis of the induced flavonols on the leaf surface of wild tobacco (*Nicotiana attenuata*). Phytochemistry, 62, 527–536.

Rubio-Wilhelmi MM, Sanchez-Rodriguez E, Rosales MA, Blasco B, Rios JJ, Romero L, Blumwald E, Ruiz JM. 2011. Effect of cytokinins on oxidative stress in tobacco plants under nitrogen deficiency. Environmental and Experimental Botany, 62,167-173.

Ruiz JM, Rivero RM, Cervilla LM, Castellano R, Romero L. 2006. Grafting to improve nitrogen-use efficiency traits in tobacco plants. Journal of the Science of Food and Agriculture, 86, 1014-1021.

Sakakibara H, Takei K, Hirose N. 2006. Interactions between nitrogen and cytokinin in the regulation of metabolism and development. Trends in Plant Science, 11, 440-448.

Scheible WR, Morcuende R, Czechowski T, Fritz C, Osuna D, Palacios-Rojas N, Schindelasch D, Thimm, O, Uvardi MK, Stitt M. 2004. Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory

infrastructure of Arabidopsis in response to nitrogen. Plant Physiology, 136, 2483-2499.

Siddiqi MY, Glass AD. 1981. Utilization index: a modified phosphorous nutrition of eight forms of two clover species: *Trifolium ambiguum* and *T. repens*. Journal of Plant Nutrition, 4, 289–302.

Smeeton BW. 1987. Genetic control of tobacco quality. Recent Advances in Tobacco Science, 13, 3-26.

Sorgona A, Abenavoli MA, Gringeri PG, Cacco G. 2006. A comparison of nitrogen use efficiency definitions in citrus rootstocks. Scientia Horticulturae, 109, 389–393.

Svecnjak Z, Rengel Z. 2006. Nitrogen utilization efficiency in canola cultivars at grain harvest. Plant and Soil, 283, 299-307.

Takei K, Takahashi T, Sugiyama T, Yamaya T, Sakakibara H. 2002. Multiple routes communicating nitrogen availability from roots to shoots: a signal transduction pathway mediated by cytokinin. Journal of Experimental Botany, 53, 971-977.

Tariq M, Akbarb A, Haqb L, Khan A. 2010. Comparing Application Methods for Boron Fertilizer on the Yield and Quality of Tobacco (*Nicotiana tabacum* L.). Communications in Soil Science and Plant Analysis, 41(13), 1535-1537.

Tewari RK, Kumar P, Sharma PN. 2007. Oxidative stress and antioxidant responses in young leaves of mulberry plants grown under nitrogen, phosphorus or potassium deficiency. Journal Integrative Plant Biology, 49, 313-322.

Weybrew JA, Wan Ismail WA, Long RC. 1983. The cultural Management of flue-cured tobacco quality. Tobacco Science, 27, 56-61.

Xi X, Li C, Zhang F. 2008. Tobacco plants can use nitrogen taken up before mechanical wounding to synthesize nicotine afterwards. Plant Signalling and Behavior, 3, 87-90.

Ye X, Hong J, Shi L, Xu F. 2010. Adaptability mechanism of nitrogen-efficient germplasm of natural variation to low nitrogen stress in *Brassica napus*. Journal of Plant Nutrition, 33, 2028–2040.

Yildiz D. 2004. Nicotine, its metabolism and an overview of it biological effects. Toxicon, 43, 619-632.

Capítulo 6

Response of Carbon and Nitrogen-rich metabolites to nitrogen deficiency in P_{SARK}::IPT tobacco plants

ABSTRACT

Wild type (WT) and transgenic tobacco plants expressing isopentenyltransferase (IPT), a gene coding the rate-limiting step in cytokinin (CKs) synthesis, were grown under limited nitrogen (N) conditions. Here, we analyse the possible effect of N deficiency on C-rich compounds such as phenolic compounds, as well as on N-rich compounds such as polyamines (PAs) and proline (Pro), examining the pathways involved in their synthesis and degradation. N deficiency was found to stimulate phenolic metabolism and increase these compounds both in P_{SARK}::IPT as well as in WT tobacco plants. This suggests that nitrate (NO_3^-) tissue concentration may act as a signal triggering phenolic compound accumulation in N deficiency plants. In addition, we found the maintenance of PAs in the WT plants would be correlated with the higher stress response to N deficiency. On the contrary, the reduction of free PAs and Pro found in the *P_{SARK}::IPT* plants subjected to N deficiency would indicate the operation of a N-recycling mechanism that could stimulate a more efficient N utilization in *P_{SARK}::IPT* plants.

Keywords: Cytokinins; Nitrogen deficiency; Reactive oxidative species; Phenolic compounds; Polyamines; Proline.

Abbreviations: N, nitrogen; aa, amino acids; PAs, polyamines; ROS, reactive oxidative species; H_2O_2, hydrogen peroxide; Put, putrescine; Spm, spermine; Spd, spermidine; Pro; proline; Orn, ornithine; Arg, arginine, Glu, glutamate; CKs, cytokinins; IPT, isopentenyltransferase.

INTRODUCTION

Plant growth and development depends on an adequate supply of N in order to synthesize the amino acids (aa), proteins and nucleic acids (Sanchez et al., 2004). In addition, it is of great importance in the biochemistry of compounds such as enzymes, pigments, secondary metabolites, and PAs (Maathuis, 2009). N deficiency leads to wide reprogramming of primary and secondary metabolism (Scheible et al., 2004), and low N has an extensive impact on the overall plant metabolism, inducing a shift from N-based to C-based compounds. For example, a typical sign of N deficiency is the depletion in aa or other N-rich compound as PAs (Van Arendonk et al., 1998; Rubio-Wilhelmi et al., 2012) and the accumulation of phenolic metabolites (Kováčik et al., 2011; Fritz et al., 2006).

Phenolic compounds are C-rich metabolites that represent the largest group of plant secondary metabolites (Giorgi et al., 2009). For example phenolic compounds are important antioxidant and may play an important role as scavengers of free radicals and other oxidative species (Grace and Logan, 2000). Phenolic compounds are generally synthesised through the shikimate pathway (Dixon and Paiva, 1995). From the metabolism of carbohydrates and glycolysis, the 3-deoxy-D-arabino-heptulosonate 7-phosphate is biosynthesised by its corresponding synthase (DAHPS, EC 4.1.2.15), a key enzyme controlling the C flow towards phenolic metabolism. The pathway continues, producing the aa aromatic phenylalanine, which is afterwards deaminated by the enzyme phenylalanine ammonium lyase (PAL, EC 4.3.1.5), the key enzyme in phenolic

biosynthesis. PAL catalyses the non-oxidative deamination of L-phenylalanine to form cinnamic trans-acid. Phenolic compounds are oxidatively degraded primarily by polyphenol oxidase (PPO, EC 1.10.3.2) and also by peroxidases (POX, EC 1.11.1.7). The increase in phenolic compound under low N is mainly attributed to the enhanced PAL activity (Kováčik et al., 2007). It has been postulated that the appearance of reactive oxygen species (ROS), especially hydrogen peroxide (H_2O_2), under N deficiency could act as a signal activating PAL activity and the synthesis of phenols (Kováčik et al., 2009). Others assumed a carbon-nutrient balance hypothesis, according to which secondary metabolism is directed towards C-rich metabolites, in N limited plants. Thus, this type of secondary compounds may accumulate forming a pool of C in plants subjected to N deficiency due to coordinated regulation the C and N metabolism (Fritz et al., 2006; Blaschke et al., 2002).

PAs are known as a group of natural compounds with aliphatic N structure, that play important roles in many physiological processes, such as cell growth and development and the response to environmental stresses (Singh Gill and Tuteja; 2001). Spermidine (Spd) and spermine (Spm) and their obligate precursor putrescine (Put), are the most commonly found PAs in higher plants and could be present as free, conjugated and bound forms (Singh Gill and Tuteja, 2001). They are formed in the oxoglutaric acid pathway of aa biosynthesis. The first step in PAs biosynthesis in higher plants is Put synthesis from decarboxylation of either ornithine (Orn) or arginine (Arg) , in reactions catalysed by the enzymes ornithine decarboxylase (ODC; EC 4.1.1.17) and arginnine decarboxylase (ADC; EC 4.1.1.19) via agmatine (Agm). Spd and Spm

are formed by the subsequent addition of an aminopropil moiety onto Put and Spd, respectively. Intracellular free PAs pool, depend also on several processes including degradation and conjugation. PAs are oxidatively deaminated by the action of amines oxidases, include the copper diamine oxidases (DAO; EC 1.4.3.6) and polyamine oxidases (PAO; EC 1.5.3.3). DAO reaction product from Put is γ-aminobutiric (GABA) that is formed via pyrroline. PAO yields pyrroline, diaminepropane and H_2O_2 (Bouchereau et al., 1999). PAs and Pro pathways are metabolically linked in their biosynthesis through the common precursor glutamate (Glu), and consequently, to the substrates Arg and Orn (Balestrasse et al., 2005). Glu is conversed into Pro by two reactions, catalysed by glutamate dehydrogenase and Δ^1-pyrroline-5-carboxylate synthetase (P5CS, E.C. 2.7.2.11/1.2.1.41). Another precursor of Pro synthesis is Orn, which is transaminated by ornithine-δ-aminotransferase (OAT, E.C. 2.6.1.13). The most dramatic changes in PAs and Pro metabolism and content are those brought about by nutrient starvation (Szabados and Savouré, 2009; Flores, 1991). K^+ starvation in barley and *Arabidopsis* led to the accumulation of Put via ADC activation, also other mineral deficiencies were found to be associated with a stimulation of PAs oxidation (Watson and Malmberg, 1996; Richards and Coleman, 1952). N deficiency led to a depletion of the free PAs and Pro levels in different plant species as *Poa* or *Phaseolus* and possibly the use of degraded PAs and Pro as N source (Sanchez et al., 2004; Van Arendonk et al., 1998).

CKs are phytohormones that control the plant developmental programme and a relationship between CKs and macronutrient acquisition has been postulated (Brenner et al., 2005). Recent studies have indicated that CKs could

act as long-distance messengers signalling the N status of the plant (Takei et al., 2002), thus regulating the nutrient-uptake systems (Sakakibara et al., 2006). Previous work indicated that tobacco plants expressing *P_{SARK}::IPT*, an enzyme that catalyses the limiting step in CKs synthesis, increase the amount of CKs, inhibiting the formation of ROS and preventing the oxidative stress caused by N deficiency (Rubio-Wilhelmi et al., 2011; Rivero et al., 2007). Numerous studies have indicated that compounds such as phenols, PAs or Pro and its synthesis with oxidative stress such compounds being described classically as a ROS scavenger (Groppa and Benavides, 2008; Matysik et al., 2002; Grace and Logan, 2000). Here, we aim to determine the effects of N-deficiency on the synthesis and degradation of phenolic compounds, PAs and Pro in WT and *P_{SARK}::IPT* tobacco plants.

MATERIALS AND METHODS

Plant material, growth conditions and plant growth

Seeds of WT (*Nicotiana tabaccum* cv.SR1) and transgenic plants expressing *P_{SARK}::IPT* were germinated and grown in soil for 30 days (d) in a tray with wells (each well 3 cm x 3 cm x 10 cm). During this time, no differences in germination or plant development between WT and the transgenic plants were observed. Afterwards, the seedlings were transferred to a growth chamber under controlled conditions with relative humidity of 50±10%, at 28ºC/20ºC (day/night), and a 16h/8h photoperiod with a PPFD (photosynthetic photon-flux density) of 350 µmol $m^{-2}s^{-1}$ (measured with an SB quantum 190 sensor, LI – COR Inc.,

Lincoln, NE, USA). Under these conditions, plants were grown in individual pots (25 cm upper diameter, 17 cm lower diameter, and 25 cm high) of 8 L in volume and filled with a 1:1 perlite:vermiculite mixture. During 30 d, the plants were grown in a complete nutrient solution containing: 10 mM $NaNO_3$, 2 mM NaH_2PO_4, 5 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 2 mM Na_2SO_4, 2 µM $MnCl_2$, 0.75 µM $ZnCl_2$, 0.25 µM $CuCl_2$, 0.1 µM $(NH_4)_6Mo_7O_{24}$, 5 µM Fe-EDDHA, and 50 µM H_3BO_3, pH 5.8. The nutrient solution was renewed every 3 d and the soil was rinsed with distilled water to avoid nutrient accumulation. The N treatments began 60 d after sowing (DAS) and was maintained for 30 d. The treatments were 10 mM (Control), 7 mM and 1 mM $NaNO_3$. The experimental design was a randomized complete block with six treatments, arranged in individual pots with six plants per treatment, and three replicates. The experiment was repeated three times under the same conditions (n = 9). All plants were at the late vegetative stage when harvested. Middle leaves (positions 7^{th} and 8^{th}) were harvested, frozen immediately in liquid N_2, and kept at -80ºC until used.

Phenolic compound and PAs analysis by HPLC/UV

Phenolic compounds were assayed in accordance with the method of Sanchez-Rodriguez et al. (2011). The HPLC/UV analyses were carried out with an Agilent HPLC 1100 series.

For the identification of PAs, 3 g of fresh leaves was homogenized in 4 mL of 6% (v/v) cold perchloric acid (PCA), kept on ice for 1 h, and then

centrifuged at 21000 *g* for 30 min. The pellet was extracted twice with 2 mL of 5% PCA and recentrifuged. The three supernatants were pooled and used to determine the levels of free PAs. The supernatant was benzoylated in accordance with the method of Aziz and Larher (1995). The HPLC/UV analyses were carried out with an Agilent HPLC 1100 series.

Pro concentration

For the determination of the free-Pro concentration, leaves were homogenized in 5 mL of ethanol at 96%. The insoluble fraction of the extract was washed with 5 mL of ethanol at 70%. The extract was centrifuged at 3500 *g* for 10 min and the supernatant was preserved 4ºC for the Pro determination (Irigoyen et al., 1992).

Enzyme extractions and assays

For determination of DAHPS (DS-Mn, DS-Co) and phenylalanine PAL activities, 0.2 g whole fresh leaf was homogenized in 100 mM potassium-phosphate buffer (pH 8.0) containing 1.4 mM 2-mercaptoethanol. The homogenate was centrifugated at 15,000 *g* for 15 min at 4ºC. The supernatant was passed throught a Sephadex G-25 column (24 x 100 mm) previously equilibrated with the same buffer. DAHPS activity was assayed using a modified method of Morris et al. (1989). PAL activity was measured by a method of Tanaka et al. (1974).

For determination of SKDH and PPO activities, whole fresh leaf was homogenized in 50 mM potassium phosphate buffer (pH 7.0). Homogenates were centrifuged at 15,000 *g* for 15 min at 4ºC. Shikimate dehydrogenase (SKDH, EC1.1.1.25) activity was determined according to Ali et al. (2006). PPO assay was performed in mixture containing 2.85 mL of 50 mM potassium phosphate buffer (pH 7.0), 50 µL of 60 mM catechol and 0.1 mL of supernatant. Increase in absorbance was read over 3 min at 390 nm (Aquino-Bolaños and Mercado-Silva, 2004). For determination of cinnamate 4-hydroxylase (C4H, EC 1.14.13.11), fresh sample of leaf was homogenized in 200 mM potassium phosphate buffer (pH 7.5) containing 2 mM of 2-mercaptoethanol. Homogenates were centrifuged at 10,000 *g* for 15 min at 4ºC. C4H activity was assayed by using the method described by Lamb and Rubery (1975). For 4-coumarate coenzyme A ligase (4CL, EC 6.2.1.12) activity was performed. The extract buffer was 0.05 M Tris-HCl (pH 8.8) containing 14 mM mercaptoethanol and 30% glycerol. The activity was determined with the spectrophotometric method, using caffeic acid as the preferred phenolic substrate (Knoblock and Hahlbrock; 1975). For determination of guaicol peroxidasa (GPOX, EC 1.11.1.7) the extract buffer was 50 mM Tris-HCl (pH 7.5) containing 5 mM mercaptoethanol, 2 mM DTT (dithiothreitol), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM EDTA-Na. Homogenates were centrifuged at 16,500 *g* for 30 min at 4ºC. GPOX activity was determined by monitoring guaiacol oxidation at 485 nm (Ghanati et al., 2005).

ADC and ODC activities were determined according to Xu et al., (2011) with some modifications. Plant material (1.5 g) was homogenized in 50 mM

potassium phosphate buffer (pH 6.3) containing 5 mM EDTA, 0.1 mM PMSF, 40 µM pyridoxal phosphate (PLP), 5 mM DTT, 20 mM ascorbic acid, and 0.1% polyvinylpyrrolidone. The homogenate was centrifuged at 12000 *g* for 40 min at 4℃ and the supernatant was dialyzed at 4℃, agains t 3 mL of 10 mM potassium phosphate buffer (pH 6.3) containing 0.05 mM PLP, 1 mM DTT, 0.1 mM EDTA for 24 h in darkness. The dialyzed extract was used for enzyme assay. DAO and PAO activities were determined by measuring the generation of H_2O_2, a product of the oxidation of PAs, as described by Xu et al. (2011), with some modifications. Plant material (0.5 g) was homogenized in 100 mM potassium phosphate buffer (pH 6.5). The homogenate was centrifuged at 10000 *g* for 20 min at 4℃. The supernatant was used for en zyme assays.

P5CS, extraction was carried out according to Sumithra et al. (2006). Leaves were homogenized with extraction buffer containing 100 mM Tris-HCl (pH 7.5), 10 mM β-mercaptoethanol, 10 mM $MgCl_2$ and 1 mM PMSF and then centrifugated at 10 000 *g* for 15 min. The supernatant was used for enzyme assays. For OAT and proline deshidrogenase (PDH, E.C. 1.5.99.8) extraction, leaves were homogenized in 100 mM K-phosphate buffer (pH 7.8). The homogenate was filtered and centrifuged at 12 000 *g* for 20 min (4 ºC) (Charest and Phan, 1990). OAT was assayed according to Charest and Phan (1990) in 0.2 M Tris-KOH buffer (pH 8.0) containing 5 mM ornithine, 10 mM α-ketoglutarate and 0.25 mM NADH. The decrease in absorbance of NADH was monitored at 340 nm for 1 min after initiating the reaction with the addition the enzyme extract. PDH activity was assayed by the reduction of NAD^+ at 340 nm.

Statistical analysis

The data compiled were submitted to an analysis of variance (ANOVA) and the differences between the means were compared by Duncan's multiple-range test ($P > 0.05$).

RESULT

Phenolic compounds, PAs and Pro

The concentration of soluble phenolic compounds was measured in WT and transgenic tobacco plants expressing P_{SARK}*::IPT* (Table 1). The plants were grown under normal conditions (10 mM N) and under two N-deficient treatments (i.e. 7 and 1 mM N). The hydroxycinnamic acid content showed a significant increase under 7 and 1mM N treatments in WT tobacco plants, while in transgenic plants this significant increase was detected only under severe N deficiency, i.e. the 1mM treatment (Table 1). With respect to flavonoids and glycosides, the control treatment showed the lowest concentrations of these compounds both in the case of the WT plants as well as in the transgenic plants, showing a significant increase in these compounds with the N deficiency (Table 1). Therefore, both lines of tobacco plants, WT and P_{SARK}*::IPT*, registered a higher total phenol concentration in leaf, this being significant for both lines in the case of the severe N deficiency treatment (1 mM). Finally, it bears highlighting that under any of the treatments, the WT plants reached higher total phenol concentrations than did transgenic plants (Table 1).

Table 1: Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on Phenolic compounds in two tobacco lines: 'WT' and 'IPT'

Lines/NO_3^- Treatment	**Hydroxycinnamic acids and derivatives** ($mg.g^{-1}DW$)	**Flavonoids and glycosides** ($mg.g^{-1}DW$)	**Others** ($mg.g^{-1}DW$)	**Total** ($mg.g^{-1}DW$)
WT				
Control	5.31±0.19 c	0.52±0.00 c	2.65±0.06 b	8.49±0.14 b
Def 7 mM	6.12±0.26 b	0.59±0.00 b	3.01±0.27 b	9.73±0.51 b
Def 1 mM	10.91±0.18 a	0.92±0.02 a	5.58±0.45 a	17.42±0.36 a
P-value	***	***	**	***
$LSD_{0.05}$	0.761	0.044	1.078	1.287
IPT				
Control	2.59±0.22 b	0.42±0.00 c	1.12±0.16 b	4.14±0.21 b
Def 7 mM	2.89±0.12 b	0.37±0.01 b	1.06±0.07 b	4.34±0.12 b
Def 1 mM	5.76±0.27 a	0.56±0.00 a	2.34±0.06 a	8.67±0.20 a
P-value	***	***	***	***
$LSD_{0.05}$	0.764	0.038	0.383	0.652

Values are means ± SE (n = 9) and differences between means were compared using LSD (P=0.05). Means followed by the same letter in the same column do not differ significantly. Levels of significance are represented by *P < 0.05, **P < 0.01, ***P < 0.001 and NS (not significant).

PAs are a group of aliphatic amine compounds that are ubiquitous in all plant cells. We found that in WT tobacco plants, the N deficiency did not cause significant changes in the concentration of Spd and free Agm, and no detectable amounts of free Put or Spm were found under any of the treatments. On the other hand, in the transgenic P_{SARK}*::IPT* plants, a significant decrease in the free PAs, Put, and Spd was seen under N deficiency (Table 2) and Spm was not detected. Both WT and P_{SARK}*::IPT* tobacco plants showed a decrease in Pro concentration under N-deficient conditions (Fig. 1).

Table 2: Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on free polyamines in two tobacco lines: 'WT' and 'IPT'

Lines/ NO_3^- Treatment	Free polyamines (mg g^{-1}FW)				
WT	PUT	SPD	SPM	AGM	TOTAL
Control	n.d	0.10±0.03	n.d	3.78±0.15	3.88±0.12
Def 7mM	n.d	0.05±0.00	n.d	4.20±0.51	4.26±0.51
Def 1mM	n.d	0.08±0.00	n.d	3.05±1.02	3.13±1.02
P- value		NS		NS	NS
$LSD_{0.05}$		0.06		2.30	2.29
IPT					
Control	0.35±0.00a	0.23±0.01a	n.d	3.95±0.62a	4.54±0.60a
Def 7mM	0.26±0.03b	0.07±0.00c	n.d	3.72±0.37a	4.06±0.41a
Def 1mM	0.01±0.00c	0.14±0.03b	n.d	1.35±0.37b	1.50±0.35b
P- value	**	**		*	**
$LSD_{0.05}$	0.06	0.06		1.63	1.62

Values are means ± SE (n = 9) and differences between means were compared using LSD (P=0.05). Means followed by the same letter in the same column do not differ significantly. Levels of significance are represented by *P < 0.05, **P < 0.01, ***P < 0.001 and NS (not significant).

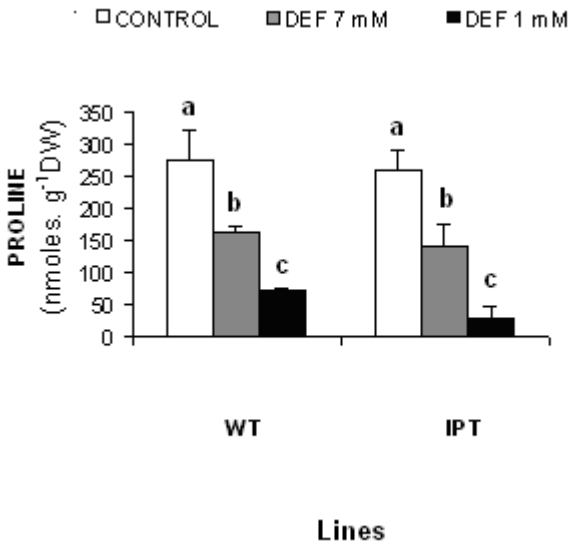

Fig. 1 Effect of 10 mM NO_3^- (control) and NO_3^- deficiency (7 and 1 mM) on proline content in leaves of two tobacco lines: 'WT' and 'IPT' under N deficiency. Values are the Means+S.E (n=9).

Phenolic compound metabolism

The activity of some of the enzymes controling the synthesis of phenolic compounds was measured in the leaves of WT and P_{SARK}*::IPT* tobacco plants (Fig. 2). Under the severe N-deficiency treatment (1 mM), the WT plants showed a significant increase in the activity of these enzymes, with the exception of DAHP DS-Co (Fig. 2B). The 7mM N application only induced the reduction of DAHP DS-Mn activity with respect to the controls (Fig. 2A). In the case of the transgenic P_{SARK}*::IPT* plants, the enzymatic activities increased when grown under 1 mM N, with the exception of DAHP-Co and SKDH which remain unchanged (Figs. 2B and 2C). When grown under 7 mM N treatments, the activity of the enzymes did not significantly differ with respect to controls, except for the enzyme C4H that showed increased activity with respect to the N control (Fig. 2F). On the other hand, the activities of PPO and GPOX (Figs. 3A and 3B), enzymes involved in the degradation of phenol compounds, were lower under N deficiency treatments, this trend being significant only under the severe deficiency dosage of 1 mM in the case of the P_{SARK}*::IPT* tobacco plants (Figs. 3A and 3B).

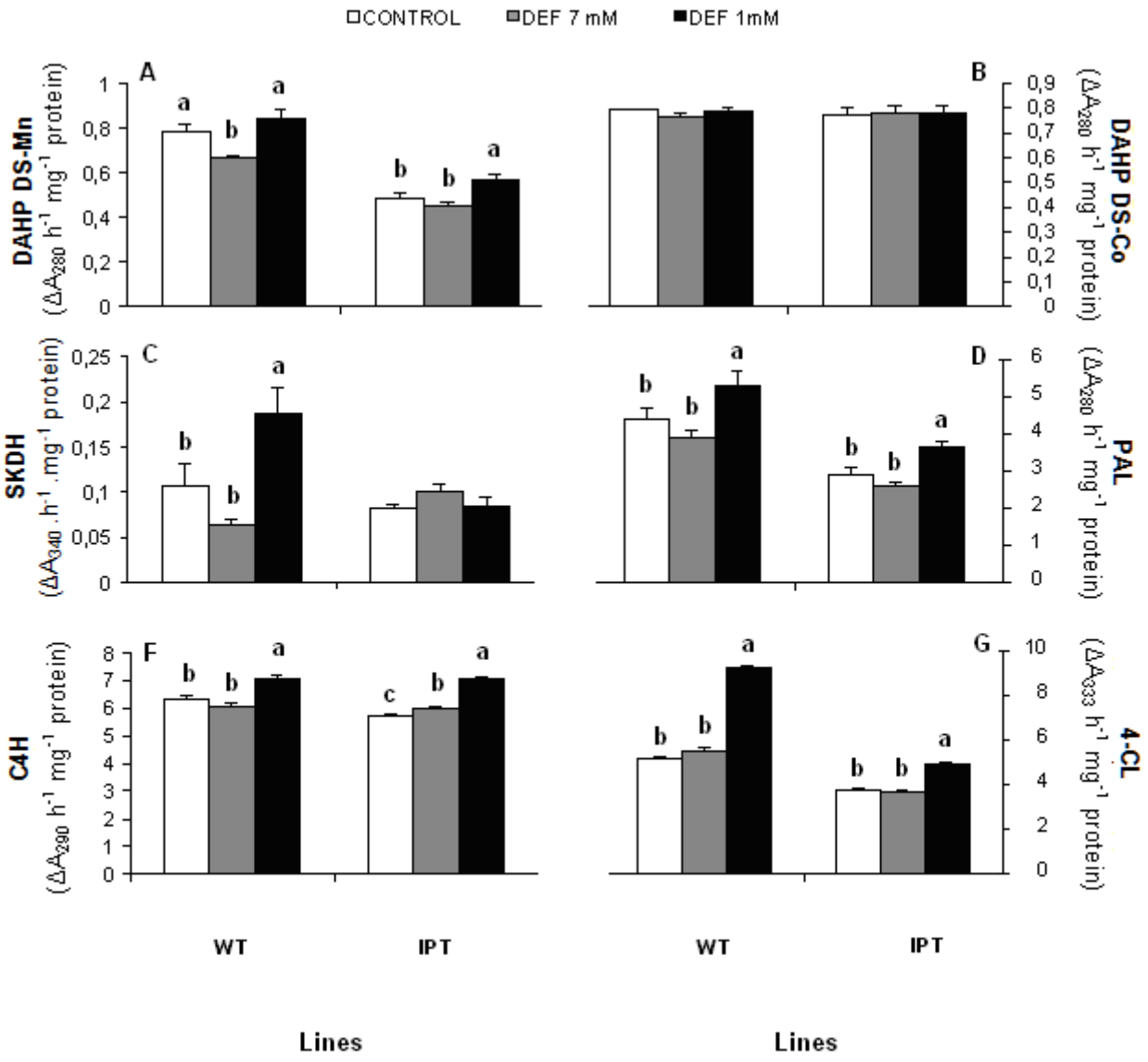

Fig. 2 Response of flavonoid and phenylpropanoid synthesis-related enzymes activity in leaves of two tobacco lines: 'WT' and 'IPT' under N deficiency. Values are the Mean+S.E (n=9).

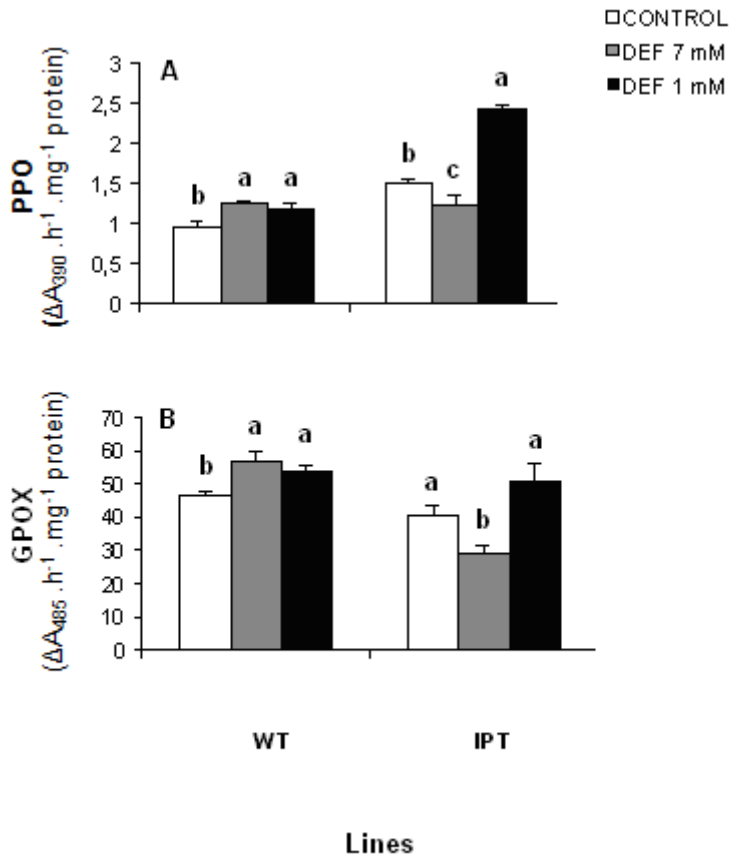

Fig. 3 Response of flavonoid and phenylpropanoid degradation-related enzymes activity in leaves of two tobacco lines: 'WT' and 'IPT' under N deficiency. Values are the Mean+S.E (n=9).

PAs and Pro metabolism

The enzymes involved in the PAs synthesis are shown in Fig. 4. The activity of ADC, the enzyme mediating Agm synthesis, increased in WT tobacco plants grown under 1 mM of N as compared to controls and no significant differences were seen in the transgenic P_{SARK}*::IPT* plants grown at the N deficiency rates (Fig. 4A). A greater ODC activity was found in the WT plants grown under N deficiency, although significant differences were only seen in the 7 mM treatment. A different response was seen in the P_{SARK}*::IPT* plants, where ODC activity was higher under the control N treatment (Fig. 4B). The enzymes

involved in the degradation of PAs, PAO and DAO followed a similar trend, with higher activities under severe N deficiency treatment 1mM (Figs. 4C and 4D).

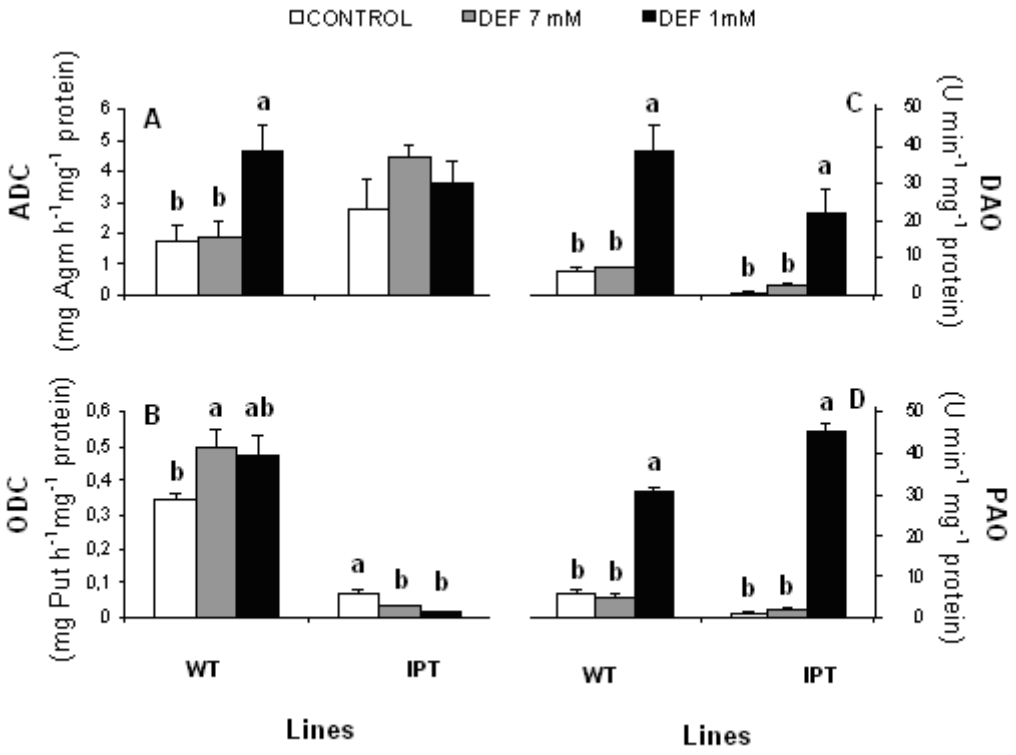

Fig. 4 Effect of 10 mM NO_3^- (control) and NO_3^- deficiency (7 and 1 mM) on arginnine decarboxylase (ADC) (A), ornithine decarboxylase (ODC) (B), diamine oxidases (DAO) (C) and polyamine oxidases (PAO) (D) in leaves of two tobacco lines: 'WT' and 'IPT'. Values are the Mean+S.E (n=9).

Table 3 shows the enzymatic activities responsible for the synthesis and degradation of Pro. Pro synthesis by OAT, was not appreciably affected by N deficiency. On the other hand, P5CS activity decreased with N deficiency and this decrease was significant under the 1mM deficiency treatment (Table 3). However, in the transgenic P_{SARK}*::IPT* plants, the P5CS activity was significantly lower both at the 7mM and the 1mM rates of N deficiency (Table 3). The

degradation of Pro by the enzyme PDH was significantly reduced with N deficiency in both lines of tobacco plants (Table 3).

Table 3: Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on proline metabolism in two tobacco lines: 'WT' and 'IPT'

Lines/NO_3^- Treatment	**OAT** ($\Delta Abs_{340} h^{-1} mg\ prot^{-1}$)	**P5CS** ($\Delta Abs_{340} h^{-1} mg\ prot^{-1}$)	**PDH** ($\Delta Abs_{340} h^{-1} mg\ prot^{-1}$)
WT			
Control	0.187±0.018	0.678±0.095 a	2.982±0.125 a
7 mM	0.134±0.020	0.357±0.030 a	2.119±0.112 b
1 mM	0.184±0.023	0.245±0.029 b	0.122±0.032 c
P-value	NS	**	***
$LSD_{0.05}$	0.060	0.176	0.289
IPT			
Control	0.213±0.047	0.433±0.012 a	3.895±0.525 a
7 mM	0.268±0.030	0.297±0.026 b	2.192±0.263 b
1 mM	0.214±0.027	0.150±0.034 c	0.924±0.068 c
P-value	NS	***	***
$LSD_{0.05}$	0.105	0.075	0.998

Values are means ± SE (n = 9) and differences between means were compared using LSD (P=0.05). Means followed by the same letter in the same column do not differ significantly. Levels of significance are represented by *P < 0.05, **P < 0.01, ***P < 0.001 and NS (not significant).

DISCUSSION

N deficiency increased the concentration of phenols as well as the enzymes involved in their synthesis and degradation in WT and transgenic *P_{SARK}::IPT* plants (Table 1; Fig. 2). These compounds have been shown to inhibit of oxidative damage (Rice-Evans et al., 1996), and the increased activity of enzymes associated with phenol synthesis, PAL in particular, and tissue phenol concentrations have been reported in plants grown under diverse abiotic stress, including N deficit (Sanchez-Rodriguez, 2011; Kováčik 2007; Dixon and Paiva, 1995). Furthermore, it has been suggested that ROS are molecules involved in

signalling between the perception of stress and the expression of the enzyme PAL, and therefore an increase in ROS would act as a signal, increasing phenolic compounds synthesis. Previous results by our group have shown that under N deficiency, only WT tobacco plants increased in ROS (Rubio-Wilhelmi et al., 2011), and therefore, the greater PAL activity and the higher quantity of phenols in both plant lines did not correlate with the increment in ROS. Recently, the role of N in the regulation of the synthesis of phenol compounds, and the role of nitrate (NO_3^-) in the regulation phenylpropanoid metabolism has been proposed (Fritz et al., 2006). Thus, several genes associated with phenolic metabolism are induced by N deficiency, including members of the gene families encoding enzymes such as PAL, 4CL, and C4H (Scheible et al., 2004; Wang et al., 2003). Therefore, the increase in the activity of the enzymes involved in the phenolic compounds metabolism shown in this work, as well as the increase in the quantity of phenols observed in both WT and P_{SARK}*::IPT* plants submitted to N deficiency, appears to supports the notion of N as a possible regulator of phenolic metabolism, and that changes in the N concentrations could trigger variations in phenolic metabolism. The low concentrations of NO_3^- and total reduced N found previously both in the WT and in P_{SARK}*::IPT* lines subjected to N deficiency (Rubio-Wilhelmi et al., 2011) would support the hypothesis that changes in NO_3^- tissue concentrations could act as a signal, triggering phenolic accumulation in N deficient plants (Frizt et al., 2006) and therefore phenolic compounds could be synthesised as a C pool in plants as a response to low N concentrations.

PAs and Pro metabolism is associated with the tolerance of plants to adverse environmental conditions (Szabados and Savouré, 2009; Singh Gill and Tuteja, 2001). Our results show that in response to a severe N deficiency, the synthesis and degradation of PAs are stimulated and the amounts of free PAs are maintained in the WT plants (Fig. 4; Table 2). On the other hand, N deficiency induced a reduction in free PAs amounts in the transgenic P_{SARK}*::IPT* plants, and this reduction was associated with the increased activities of DAO and PAO (Table 2; Figs. 4C and 4D). In the WT plants, the maintenance of PAs was associated with the improvement of ADC activity (Fig. 4A), which has been shown to play predominant roles in the accumulation of PAs under stress conditions (Wang et al., 2011). It has been postulated that PAs may function along the signalling processes associated with the plant responses to different stress signals (Liu et al., 2007). Furthermore, their antioxidant effect due a combination of their anionic and cationic-binding properties in radical scavenging has been reported (Wimalasekera et al., 2011). Therefore, the previously noted increase in ROS in WT plants, caused by the N deficiency (Rubio-Wilhelmi et al., 2011), could be involved in the maintenance of these compounds despite their high N content. However, the increased DAO and PAO activities (Figs. 4C and 4D) could be considered a N-recycling mechanism, given that GABA, a product of these enzymes, is subsequently transaminated and oxidised to succinic acid, which is incorporated into the TCA cycle. Thus, this pathway ensures the recycling of C and N from Put (Bouchereau et al., 1999).

The accumulation of Pro has been associated with the response of plants to environmental stress (Yang et al., 2009; Choudhary et al., 2005). Nevertheless, the reduction in Pro concentrations have been also reported during N deficiency (Kovačik et al., 2011; Lemaître et al., 2008). WT and transgenic *P_{SARK}::IPT* plants displayed a reduction in Pro (Table 3, Fig. 1), that appears to be associated with the inhibition of P5CS, and would indicate a possible redirection of N towards the synthesis of other essential aa.

In conclusion, N deficiency induced changes in the primary and secondary metabolism. Our work indicates that N deficiency treatments stimulated phenolic metabolism and increased phenol contents in both WT and transgenic *P_{SARK}::IPT* plants. Therefore, phenolic compounds could be accumulated, indicating NO_3^- tissue concentration may act as a signal triggering phenolic compound accumulation in N deficiency plants. The maintenance of PAs in the WT plants would be correlated with the higher stress response to N deficiency. On the other hand, the reduction of free PAs and Pro found in the *P_{SARK}::IPT* plants subjected to N deficiency would indicate the operation of a N-recycling mechanism that could stimulate a more efficient N utilization as seen by the previously reported enhanced growth at otherwise inimical low N fertilization regimes.

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REFERENCES

Ali MB, Singh N, Shohael AM, Hahn EJ, Paek KJ. 2006. Phenolics metabolism and lignin synthesis in root suspension cultures of Panax ginseng in response to copper stress. Plant Science, 171, 147–154.

Aquino-Bolaños E, Mercado-Silva E. 2004. Effects of polyphenol oxidase and peroxidase activity, phenolics and lignin content on the browning of cut jicama. Postharvest Biology and Technology, 33, 257-283.

Aziz A, Larher F. 1995. Changes in polyamine titers associated with the proline response and osmotic adjustment of rape leaf discs submitted to osmotic stress. Plant Science, 112, 175–186.

Balestrasse KB, Gallego SM, Benavides MP, Tomaro ML. 2005. Polyamines and proline are affected by cadmium stress in nodules and roots of soybean plants. Plant and Soil, 270, 343-353.

Blaschke L, Forstreuter M, Sheppard LJ, Leith IK, Murray MB, Polle A. 2002. Lignification in beech (*Fagus sylvatica*) grown al elevated CO_2 concentrations: interaction with nutrient availability and leaf maturation. Tree Physiology, 22, 469-477.

Bouchereau A, Aziz A, Larher F, Martin-Tanguy J. 1999. Polyamines and environmental challenges: recent development. Plant Science,140, 103-125.

Brenner WG, Romanov GA, Kölmer I, Brürkle L, Schmülling T. 2005. Immedite-early and delayed cytokinin response genes of *Arabidopsis thaliana* identificied by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascade. Plant Journal, 44, 314-333.

Charest C, Phan CT. 1990. Cold acclimation on wheat: properties of enzymes involved in proline metabolism. Physiologia Plantarum, 80, 159-168.

Choudhary NL, Sairam RK, Tyagi A. 2005. Expression of delta1-pyrroline-5-carboxilate synthetase gene during drought in rice (*Oryza sativa* L.). Indian Journal of Biochemistry and Biophysics, 42,366-370.

Dixon RA, Paiva NL. 1995. Stress-induced phenylpropanoid metabolism. Plant Cell, 7, 1085–1097.

Flores HE. 1991. Changes in polyamines metabolism in response to abiotic stress, In: R. Slocum, HE, Flores (Eds.) The biochemistry and physiology of polyamines in plants, CRC Press, Boca Raton, FL, pp. 214-225.

Fritz C, Palacio-Rojas N, Feil R, Stitt M. 2006. Regulation of secondary metabolism by the carbon-nitrogen status in tobacco: nitrate inhibits large sectors of phenilpropanoid metabolism, Plant Journal, 46,533-548.

Ghanati F, Morita A, Yokota H. 2005. Deposition of suberin in roots of soybean induced by excess boron. Plant Science, 168, 397–405.

Giorgi A, Mingozzi M, Madeo M, Speranza G, Cocucci, M. 2009. Effect of nitrogen starvation on the phenolic metabolism and antioxidant properties

of Yarrow (*Achillea collina* Becker ex Rchb.). Food Chemistry,114, 204-211.

Grace CS, Logan BA. 2000. Energy dissipation and radical scavenging by the plant phenilpropanoid pathway. Philosophical Transaction of the Royal Society London B, 355, 1499-1510.

Groppa MD, Benavides MP. 2008. Polyamines and abiotic stress: recent advances, Amino Acids, 34, 35-45.

Irigoyen JJ, Emerich DW, Sánchez-Diaz M. 1992. Water stress induced changes in the concentrations of proline and total soluble sugars in nodulated alfafa (*Medicago sativa*) plants. Physiologia Plantarum, 84, 55-60.

Knoblock KH, Hahlbrock K. 1975. Isoenzymes of p-coumarate: CoA ligase from cell suspension cultures of *Glycine max*. European Journal of Biochemistry, 52, 311-320.

Kováčik J, Klejdus B, Bačkor M, Repčák, M. 2007. Phenilalanine ammonia-lyase activity and phenolic compounds accumulation in nitrogen-deficient *Matricaria chamomilla* leaf rosettes. Plant Science, 172, 393-399.

Kováčik J, Klejdus B, Bačkor M. 2009. Nitric oxide signals ROS scavenger-mediated enhancement of PAL activity in nitrogen-deficient *Matricaria chamomilla* roots: side effects of scavengers. Free Radical Biology and Medicine, 46, 1686-1693.

Kováčik J, Klejdus B, Štork F, Hedbavny J. 2011. Nitrate deficiency reduces cadmium and nickel accumulation in Chamomile plants. Journal Agriculture and Food Chemistry, 59, 5139-5149.

Lamb C, Rubery PH. 1975. A spectrophotometric assay for trans-cinnamic acid 4-hydroxylase activity. Analitycal Biochemistry, 68, 554–561.

Lemaître T, Gaufichon L, Boutet-Mercey S, Christ A, Masclaux-Daubresse C. 2008. Enzimatic and metabolic diagnostic of nitrogen deficiency in *Arabidopsis thaliana* Wassileskija accession. Plant Cell Physiology, 49, 1056-1065.

Liu J-H, Kitashiba H, Wang J, Ban Y, Moriguchi T. 2007. Polyamines and their ability to provide environmental stress tolerance to plants. Plant Biotechnology, 24,117-126.

Maathuis FJM. 2009. Physiological funcions of mineral macronutrients. Current Opinion in Plant Biology, 12, 250-25.

Matysik J, Alia-Bhalu B, Mohanty P. 2002. Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. Current Science, 82, 525-532.

Morris PF, Doong RL, Jensen RA. 1989. Evidence from *Solanum tuberosum* in support of the dual-pathway hypothesis of aromatic biosynthesis. Plant Physiology, 89, 10–14.

Rice-Evans CA, Miller NJ, Paganga G. 1996. Structure-antioxidant activity relationships flavonoids and phenolic acids. Free Radical Biology and Medicine, 20, 933-953.

Richards FJ, Coleman EG. 1952. Ocurrence of putrescine in potassium deficient barley. Nature, 170, 160-461.

Rivero RM, Kojima M, Gepstein A, Sakakibara H, Mittler R, Gepstein S, Blumwald E. 2007. Delayed leaf senescence induces extreme drought

tolerance in a flowering plant. Proceeding of the National Academy of Science USA, 104, 19631–19636.

Rubio-Wilhelmi MM, E. Sanchez-Rodriguez E, M.A. Rosales MA, B. Blasco B, J.J. Rios JJ, L. Romero L, E. Blumwald E, J.M. Ruiz JM. 2011. Effect of cytokinins on oxidative stress in tobacco plants under nitrogen deficiency. Environmental and Experimental Botany, 62, 167-173.

Rubio-Wilhelmi MM, Sanchez-Rodriguez E, Rosales MA, Blasco B, Rios JJ, Romero L, Blumwald E, Ruiz JM. 2012. Ammonium formation and assimilation in P_{SARK}::IPT tobacco transgenic plants under low N. Journal of Plant Physiology, 169,157-162.

Sakakibara H, Takei K, Hirose N. 2006. Interactions between nitrogen and cytokinin in the regulation of metabolism and development. Trends in Plant Science, 11, 440-448.

Sanchez E, Rivero RM, Ruiz JM, Romero L. 2004. Changes in biomass, enzymatic activity and protein concentration in roots and leaves of green bean plants (*Phaseolus vulgaris* L. cv. Strike) under high NH_4NO_3 application rates. Scientia Horticulturae, 99, 237-248.

Sánchez-Rodríguez E, Moreno DA, Ferreres F, Rubio-Wilhelmi MM, Ruiz JM. 2011. Differential responses of five cherry tomato varieties to water stress: Changes on phenolic metabolites and related enzymes, Phytochemistry, 72, 723-729.

Scheible W-R, Morcuende R, Czechowski T, Frizt C, Osuna D, Palacios-Rojas N, Schindelash D, Thimm O, Udvardi MK, Stitt M. 2004. Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory

infrastructure of *Arabidopsis* in response to nitrogen. Plant Physiology, 136, 2483-2499.

Singh Gill S, Tuteja N. 2001. Polyamines and abiotic stress tolerante in plants. Plant Signaling and Behavior, 5, 26-33.

Sumithra K, Jutur PP, Carmel BD, Reddy AR. 2006. Salinity-induced changes in two cultivars of *Vigna radiate*: responses of antioxidative and proline metabolism. Plant Growth Regulation, 50, 11-22.

Szabados L, Savouré A. 2009. Proline: a multifunctional amino acid. Trends in Plant Science, 15, 89-97.

Takei K, Takahashi T, Sugiyama T, Yamaya T, Sakakibara H. 2002. Multiple routes communicating nitrogen availability from roots to shoots: a signal transduction pathway mediated by cytokinins. Journal of Experimental Botany, 53, 971-977.

Tanaka Y, Kojima M, Uritani I. 1974. Properties, development and cellular localization of cinnamic acid 4-hydrolase in cul-injured sweet potato. Plant Cell Physiology 15, 843–854.

Van Arendonk JJCM, Karanov E, Alexiera V, Lambers H. 1998. Polyamine concentrations in four Poa species differing in their maximum relative growth rate, Brown with free access to nitrate and limiting nitrate supply. Plant Growth Regulation, 24, 77-89.

Wang B-Q, Zhang Q-F, Liu J-H, Li G-H. 2011. Overxpression of PtADC confers enhanced dehydrated and drought tolerance in transgenic tobacco and tomato: Effect on ROS elimination. Biochemical and Biophysical Research Communications, 413, 10-16.

Wang R, Okamoto M, Xing X, Crawford NM. 2003. Microarray analysis of the nitrate response in Arabidopsis roots shoots reveals over 1,000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and sulphate metabolism. Plant Physiology, 132, 556-567.

Watson MB, Malmberg RL. 1996. Regulation of *Arabidopsis thaliana* (L.) Heynh arginine decarboxilase by potassium deficiency stress. Plant Physiology, 111, 1077-1083.

Wimalasekera R, Tebartz F, Scherer GFE. 2011. Polyamines, polyamine oxidases and nitric oxide in development, abiotic and biotic stresses. Plant Science, 181, 593-603.

Xu Y, Shi GX, Ding CX, Xu XY. 2011. Polyamine metabolism and physiological responses of *Potamogeton crispus* leaves under lead stress. Russian Journal of Plant Physiology, 58, 460-466.

Yang SL, Lang SS, Gong M. 2009. Hydrogen peroxide-induced proline and metabolic pathway of its accumulation in maize seedlings. Journal of Plant Physiology, 166, 1694-1699.

Capítulo 7

P_{SARK}::IPT expression causes protection of photosynthesis in tobacco plants during N deficiency

ABSTRACT

Wild type (WT) and transgenic tobacco plants expressing isopentenyltransferase (IPT), a gene coding the rate-limiting step in cytokinins (CKs) synthesis, were grown under limited nitrogen (N) conditions. Our results indicated that while WT tobacco plants showed a drastic reduction in photosynthetic rate (*A*) and the maximum carboxylation rate of Rubisco (Vcmax), the maximum rate of the electron transport (Jmax) and the use of triose-P (TPU) parameters, the expression an *IPT* gene driven by P_{SARK}, a stress-and maturation-induced promoter, leads the maintenance of photosynthesis as well as VCmax, Jmax and TPU parameters and leaf biomass related with the maintenance of sm-Rubisco transcripts levels under N deficiency. Limited increase in sugar concentration as well as the maintenance of leaf biomass observed in transgenic plants would indicate CKs may play a role in the sink limitation caused by deficiency of N, enhancing the strength of the sink, such as young leaves, enhancing foliar biomass under severe N deficiency.

Keywords: Cytokinins; nitrogen deficiency; photosynthetic rates; sugar metabolism; tobacco plants.

Abbreviations: *A*, photosynthetic rate; CKs, cytokinins; IPT, isopentenyltransferase; Fru, fructose; Glu, glucose; Glu-6P, glucose-6P; Jmax, maximum rate of the electron transport; Man, mannose; N, nitrogen; Rubisco,

Ribulose 1,5-bisphosphate carboxilase/oxygenase; Suc, Sucrose; TPU, use of triose-P; Vcmax, maximum carboxylation rate of Rubisco.

INTRODUCTION

N is a mineral element that plants require in greatest amount and it is often the growth limiting nutrient (Antal et al., 2010). The role of N in agricultural production is intimately connected with photosynthesis because more than half of the total leaf N is allocated to the photosynthesis apparatus (Makino and Osmond, 1991; Lawlor, 2002). Two days of N deprivation led to coordinate repression of the majority of genes assigned to photosynthesis, chlorophyll synthesis, and plastid proteins synthesis (Scheible et al., 2004). Thus, N shortage results in a marked decrease in plant photosynthesis, decreasing in both *A* and the quantum yield of photosynthesis (Cruz et al., 2003; Boussadia et al., 2010). Limited N supply, causes diminution in the content of chloroplastidic pigments and a reduction in the synthesis of several enzymes involved in the Calvin cycle, particularly in Ribulose 1,5-bisphosphate carboxilase/oxygenase (Rubisco, EC 4.1.1.39) (Terashima and Evans, 1988). Overall leads, sink limitation due to decreased in shoot growth, results in accumulation of carbohydrates in matures leaves, higher levels of carbon allocated to the roots and an increase in root-to-shoot biomass ratio (Paul and Foyer, 2001; Hirari et al., 2004; Remans et al., 2006). Therefore N deficiency affects, primary photosynthesis, sugar metabolism and carbohydrate partitioning between source and sink tissues (de Groot et al., 2003; Scheible et al., 2004).

CKs are phytohormones involved in numerous important biological processes associated with plant growth and development, including the response of plants to abiotic stress (Haberer and Kieber, 2002; Rivero et al., 2007). An unfavourable environmental was found to have an impact on CKs content in plants and changes in endogenous CKs levels were reported to alter the stress tolerance in plants (Werner et al., 2010; Nishiyama et al., 2011). Thus, CKs, selectively could affect the expression of certain genes, which is of special importance for stress protection (Chernyad`ev 2009). It has been proposed the protective action of CKs under stress conditions, preserving the structure and function of the photosynthetic machinery inducing the synthesis of proteins of the electron-transport chain, photosynthetic chlorophyll-protein complexes and Rubisco (Chernyad`ev 2009). Thus, Rivero et al. (2010) demostred the prevention of the degradation of the photosynthetic protein complexes, and the maintenance of photosynthesis during water stress in the plants expressing an *IPT* gene driven by P_{SARK}, a stress-and maturation-induced promoter. Furthermore it has been suggested that CKs may also be involved in the regulation of assimilate partitioning affecting source-sink relationships (Roitsch and Ehneβ, 2000). Peleg et al. (2010) demonstrated that the expression of P_{SARK}*::IPT* in rice affected plant hormone homeostasis and altered the source-sink balance showing that CKs could maintain the strength of this sink increasing the amount of starch and sucrose (Suc) during water-deficit. Moreover, it has been demostrated that CKs supplied at physiological concentration could change the direction of assimilate export from individual leaves, thus completely inverting the source-sink relationship in favour of the shoot.

Furthermore, the availability of N can alter the levels of CKs. It was proposed that CKs may be root-derived signal which controls uptake and utilization of assimilates and biomass distribution in response to N (Beck, 1999; Sakakibara et al., 2006). Therefore, N deficiency would lower CKs endogenous levels while an application of N would raise CKs levels in the shoot (Argueso et al., 2009). Thus, CKs can act as a signal communicating to the shoot if the N application of the root is adequate, thereby regulating the biomass distribution and nutrient uptake systems (Sakakibara et al., 2006). Previous work indicated that tobacco plants overexpressing *P_{SARK}::IPT* increase the amount of CKs, inhibiting the formation of ROS and preventing biomass reduction caused by N deficiency (Rivero et al., 2007; Rubio-Wilhelmi et al., 2011). There are numerous evidence linking N deficiency with CKs and photosynthesis, therefore, the aim of the present work was to evaluate the effect of CKs on photosynthetic parameters and sugar metabolism in WT and *P_{SARK}::IPT* tobacco plants under N deficiency.

MATERIAL AND METHODS

Plant material and growth conditions

Seeds of WT tobacco (Nicotiana tabacum 'SR1') and transgenic line expressing *P_{SARK}::IPT* were sown in soil (Metro-Mix 200; Sun Gro) in a growth chamber (500 µmol photons m^{-2} s^{-1}, 16-h photoperiod, 25ºC) for 15 d until the appearance of the first two true leaves. During this time, no differences in germination time and in plant development between the WT and *P_{SARK}::IPT*

lines were observed. After, plants were transferred and transplanted (10-L pots filled with vermiculite) to a greenhouse (UC-Davis, CA, US), where they were grown for 1 week to allow acclimation of the plants to the new conditions (1,000 µmol photons m^{-2} s^{-1}, 16-h photoperiod, 28ºC–30ºC/23ºC–25ºC day/night). During 20 d, the plants were grown in a complete nutrient solution containing: 10 mM $NaNO_3$, 2 mM NaH_2PO_4, 5 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 2 mM Na_2SO_4, 2 µM $MnCl_2$, 0.75 µM $ZnCl_2$, 0.25 µM $CuCl_2$, 0.1 µM $(NH_4)_6Mo_7O_{24}$, 5 µM Fe-EDDHA, and 50 µM H_3BO_3, pH 5.8. The nutrient solution was renewed every 3 d and the soil was rinsed with distilled water to avoid nutrient accumulation. The N treatments began 40 d after sowing (DAS) and was maintained for 30 d. The treatments were 10 mM (Control), 7 mM and 1 mM $NaNO_3$. The experimental design was a randomized complete block with six treatments, arranged in individual pots with six plants per treatment, and three replicates. All plants were at the reproductive stage when harvested. Middle leaves (positions 7^{th} and 8^{th}) were harvested, frozen immediately in liquid N_2, and kept at -80ºC until used. Leaves, roots and flowers were dried in a forced-air oven at 70ºC for 24 h for dry biomass determination.

A/CO_i curves

Gas-exchange measurements were conducted in the eighth fully expanded leaf in each genotype (wild type, *P_{SARK}::IPT*) with a gas exchange system (LI-6400; Li-Cor). Leaves were first equilibrated at a photon density flux of 1,000 mmol m^{-2} s^{-1} for at least 20 min. After this, photosynthesis was induced with 1,000 mmol photons m^{-2} s^{-1} and 400 mmol mol^{-1} CO_2 surrounding the leaf (Ca). Leaf

temperature was maintained at 25ºC, and the leaf-to-air vapor pressure deficit was kept between 1 and 1.3 kPa. These conditions were kept constant for the determination of A. CO_2 response curves were performed at steady state at least 30 min after clamping the leaf. CO_2 response curves, corresponding to eighth fully expanded leaves of 6 different plants, were obtained per each plant genotype (the wild type and P_{SARK}*::IPT* lines) and were repeated every 5 d. *A* and rates of CO_2 assimilation under varying intercellular CO_2 concentrations (Ci) were first measured at 400 mmol mol^{-1} Ca. Then, Ca was increased stepwise up to 1,800 mmol mol^{-1} and returned to its original value, followed by a stepwise decrease down to 0 mmol mol^{-1} Ca. *A* and Ci were measured at 12 different Ca values for each curve. From the *A*/Ci curves, the following photosynthetic parameters were calculated according to Long and Bernacchi (2003): Vcmax, Jmax that is equivalent to the ribulose-1,5-bisP (RuBP) regeneration rate, as well as TPU. In order to avoid miscalculation of *A* and Ci due to leakage into the gasket of the gas analyzer, we performed CO_2 response curves using an empty chamber. The values obtained for *A* and Ci in the empty chamber were compared with those of the chamber filled with a tobacco leaf and subtracted from the values obtained with the empty chamber. The relation between *A* and Ci was fitted with the software Photosyn Assistant (Dundee Scientific). The program uses the model proposed by Farquhar et al. (1980), as subsequently modified by von Caemmerer and Farquhar (1981), Sharkey et al. (1985), Harley and Sharkey (1991), and Harley et al. (1992).

qPCR

cDNAs were obtained from two independent RNAs corresponding to the same sample using the SuperScript VILO synthesis kit (Invitrogen). This procedure was done with tissues from WT plants and *P_{SARK}::IPT* plants growing under control conditions (10 Mm) and deficient conditions (7Mm, 1Mm) so that every sample was represented by two independent cDNAs.

From each cDNA, three replicates were placed on a 96-well plate, so that every sample was represented by six replicates. For all targets analyzed, the primers were designed using ABI Primer Express software. For IPT expression, the primers used were IPT-forward (5´-CCAAGGCCAGAGTTAAGCAG-3´) and IPT-reverse (5´-TTTGCGTCAAGCTGCAATAG-3´). For Rubisco small subunit (smRubisco), the primers used were smRubisco-forward (5´-AGTGCGGCAACGGTAATATC-3´) and smRubisco-reverse (5´-TCAACAAAGTCCGGAGAACC-3´). An internal control Elongation factor 1 *α* (EF-1*α*) which expression did not change over the amplification in the different samples, were processed in parallel. The primers used were as follows: EF-1*α*-forward (5´-TGAGATGCACCACGAAGCTC-3´) EF-1*α*-reverse (5´-CCAACATTGTCACCAGGAAGTG-3´). The amplification was performed in a total reaction volume of 20 mL. Reactions included 2 mL of template, 10 mL of Fast SYBR Green Master Mix, 0.9 mL of reverse primer, 0.9 mL of forward primer, and sterile molecular biology-grade water to a total volume of 20 mL. All PCRs were performed with the exact reaction cycling conditions as follows: 95ºC for 10 min followed by 40 cycles of 95ºC for 3 s and 60ºC for 30 s. A melt

curve for every target analyzed was included with the following conditions: 95ºC for 15 s, 60ºC for 1 min, and 95ºC for 15 s. Amplification and data analysis were carried out on an ABI StepOne Plus real-time PCR system (Applied Biosystems) taking as internal control EF-1*α* and as a sample control WT plants growing under control conditions (10 mM). All template and primer concentrations were optimized for the reactions.

Sugar determination

Leaves glucose-6P (Glu-6P), Suc, glucose (Glu), mannose (Man), fructose (Fru) were quantified as described (Smith and Zeeman, 2006) with some modifications. Middle leaves were sampled from WT and P_{SARK}*::IPT* tobacco plants and immediately frozen in liquid-N. Then 0.25 g was incubated in ethanol (80%) for 3 min at 95 ºC and centrifuged 3000 g 10 min, keeping supernatant (soluble carbohydrate fraction). For sugars quantification, the soluble carbohydrate fraction dissolved in ethanol (80%) was evaporated using a rotary evaporator. The samples were dissolved in ddH_2O and subsequently analysed by high performance liquid chromatography (HPLC) using Aminex HPX-87C column.

Enzymes assay

Enzymes were extracted from 0.1 g of leaves by homogenizing in 50 mM Hepes/NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 0.005% Triton X-100, 1 % insoluble PVP, and 5 mM DTT. The homogenate was centrifuged at 20 000 *g*

for 20 min. Supernatant was used to measure suc-P synthase (SPS, EC 2.4.1.14; SPS), suc synthase (SS, EC 2. 4. 1. 13), vacuolar and citosolic invertases (EC 3.2.1.26) activities. Resuspended pellet was used for measured of cell wall invertase (EC 3.2.1.26).

SS and SPS assays were performed in a manner similar to that previously described (Hubbard et al., 1989; Miron and Schaffer, 1991; Klann et al., 1993). SS and SPS reactions and controls contained 40 µL of extract in a total volume of 70 µL. Suc synthase assays contained 50 mM Hepes/NaOH (pH 7.5), 15 mM $MgCl_2$, 25 mM Fru, and 25 mM UDP-Glu. Suc-P synthase assays contained 50 mM Hepes/NaOH (pH 7.5), 15 mM $MgCl_2$, 25 mM Fru-6-P, 25 mM Glu-6-P, and 25 mM UDP-Glu. Controls were boiled for 10 min immediately after the addition of the enzyme extract. The reactions were incubated for 30 min at 37ºC and then stopped by transfer to a boiling water bath. Reactions and controls were stored at -20ºC until the Suc was assayed using the anthrone assay (Van Hamdel, 1968). To the 70µL sample, 70 µL of 30% KOH was added, boiled for 10 min, and cooled. Anthrone reagent was then added, and the tubes incubated for 20 min at 40ºC. Absorbance was measured at 650 or 595 nm and compared to Suc standards.

Vacuolar, citosolic and cell wall invertases assays contained either 100µL of extract in a total volume of 300 µL. The final assay contained 500 mM sodium acetate/NaOH pH 4.5 for vacuolar and cell wall invertase or pH 7.5 for citosolic invertase and 120 mM Suc. Both reactions and controls were neutralized with the addition of 30 µL of 2.5 M Tris base before boiling to prevent acid hydrolysis

of the Suc. Controls were boiled immediately, and the reactions were incubated at 37ºC for 30 min before boiling. Reaction and controls were stored at -20ºC until the reducing sugar assay was performed. Reducing sugars in the invertases assays were measured using the Somogyi modification of Nelson's reducing sugar assay (Nelson, 1944; Somogyi, 1952).

RESULTS

Effects of N deficiency on plant biomass

Table 1 shows foliar, root and flower biomass in WT and P_{SARK}*::IPT* tobacco plants under N deficiency. In WT plants, a significant decrease in foliar biomass was seen under severe N deficiency (1mM). However, transgenic line did not show significant differences in foliar biomass under any of the treatments (Table 1). Besides, root biomass was not affected by the N deficiency in WT plants; on the contrary, P_{SARK}*::IPT* plants shown significant decreased in this parameter under 1mM treatment (Table 1). Finally, in WT plants, 7 mM dosage of N deficiency produced a significant increase in flower biomass compared to control (10 mM); while transgenic plant shown a reduction in flower biomass under N severe deficiency (1 mM), however this reduction was not significant (Table 1).

Table 1: Foliar, root and flower biomass in WT and transgenic tobacco plants subjected to N deficit.

	Foliar Biomass (g DW)		Root Biomass (g DW)		Flower Biomass (g DW)	
NO_3^- treat.	**WT**	**IPT**	**WT**	**IPT**	**WT**	**IPT**
Control	9.89±0.53a	12.66±0.89	8.44±1.15	5.77±0.23a	6.83±0.58b	7.31±0.80
7 mM	9.80±0.29a	12.03±0.67	8.24±1.37	6.70±0.90a	9.14±0.27a	7.16±0.36
1 mM	6.61±0.34b	11.15±1.01	7.42±0.20	4.02±0.50b	5.92±0.41b	5.57±0.66
P-value	***	NS	NS	*	**	NS
$LSD_{0.05}$	1.214	2.631	3.597	1.009	1.338	1.916

Values are means ± SE (n = 9) and differences between means were compared using LSD (P = 0.05). Means followed by the same letter in the same column do not differ significantly. Levels of significance are represented by *P < 0.05, **P < 0.01, ***P < 0.001 and ns (not significant) P > 0.05.

Effects of N deficiency on *IPT* and sm-Rubisco expression analysis

Relative expression *IPT* gene driven by P_{SARK}, a stress-and maturation-induced promoter levels during control and under N deficient in *P_{SARK}::IPT* transgenic plants shown in Fig 1. When the plants grown under 7 mM N treatment did not show differences in *P_{SARK}::IPT* expression levels, however severe N deficiency (1 mM) increased *P_{SARK}::IPT* expression levels compared to control (10 mM). On the other hand, sm-Rubisco relative expression levels in both lines of tobacco plants are shown in Fig. 2. WT plants registered a significant decrease in sm-Rubisco relative expression levels under N deficiency (Fig. 2). The lowest expression levels were shown under 1 mM N treatment (Fig. 2). However, in transgenic plants, sm-Rubisco relative expression levels remained unchanged under the different N dosages (7 mM and 1 mM) compared to control (10 mM; Fig. 2).

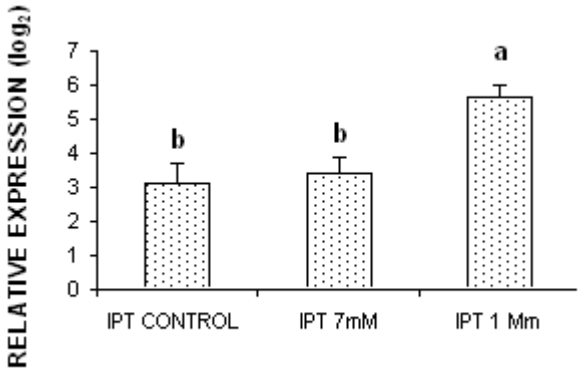

Fig 1: Relative expression IPT gene driven by P_{SARK}, a stress-and maturation-induced promoter levels during control and under N deficient in P_{SARK}::IPT transgenic plants. Bars represent means+s.e. (n=9); for each lines.

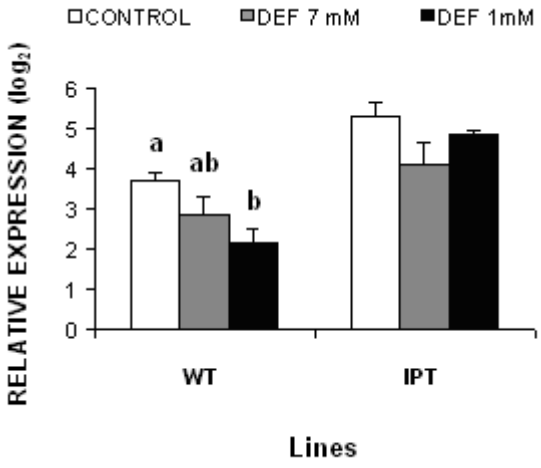

Fig 2: Relative expression sm-Rubisco subunit levels during control and under N deficient in WT and transgenic plants. Bars represent means+s.e. (n=9); for each lines.

Effects of N deficiency on A/C_i curves, Vcmax, Jmax and TPU

We measured rates of CO_2 assimilation under varying intercellular CO_2 concentrations (Ci) and produced *A*/Ci curves (Figs. 3A and B). A/Ci curves showed a significant decrease in both lines of tobacco plants under the 1 mM

dosage of N (Figs. 3A and B), being more pronounced in WT plants (Fig. 3A). A treatment of 7 mM of N had no significant effect in neither of tobacco lines (WT and *P_{SARK}::IPT*; Figs. 3A and B).

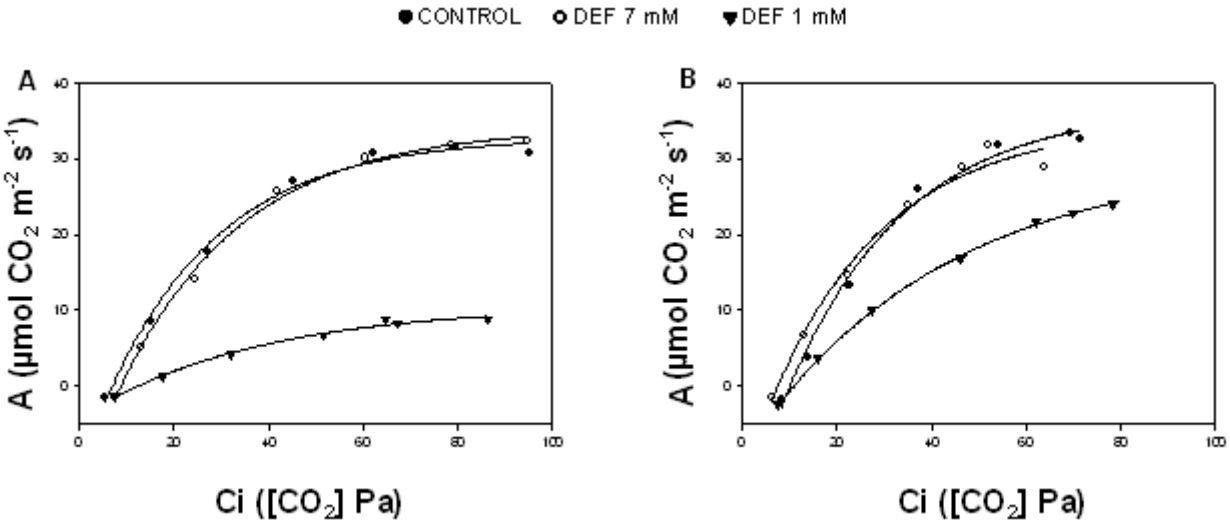

Fig 3: CO_2 assimilation rates at different intercellular CO_2 concentrations (A/Ci curves) of WT and transgenic tobacco plants under N deficiency.

From the curves, one can calculate biochemical factors such as the Vcmax, Jmax and TPU (Figs. 4A, B and C). The application of 1 mM N resulted in reductions of 72, 70 and 66% respectively compared to control (10 mM) in WT tobacco plants (Figs. 4A, B and C). Vcmax, Jmax and TPU did not show significant differences under 7mM N treatment and control condition (10 mM; Figs. 4A, B and C). In transgenic plants, *P_{SARK}::IPT*, a decrease in Vcmax and Jmax was observed under severe N deficiency (1 mM; 48 and 45% respectively) (Figs. 4A, B and C). However, TPU showed significant differences under both N rates (1 mM, 7 mM) and control conditions, resulted in reduction of 21% in the case of 1 mM dosage (Figs. 4A, B and C).

Effects of N deficiency on sugars metabolism

Table 3 shows Glu-6P, Suc, Glu, Man and Fru concentrations in WT and *P_{SARK}::IPT* tobacco plants under N deficiency. In WT plants, severe N deficiency (1 mM) resulted an increase Glu-6P, Glu, Man y Fru concentration, being the increase of 45, 89, 194 y 51% respectively compared to control conditions (10 mM; Table 3). When the WT plants grown under 7 mM N treatment, only Suc concentration showed a significant decrease respect to control (10 mM; Table 2). In the case of *P_{SARK}::IPT* plants under severe N deficiency dosage (1 mM), only Glu and Man concentrations presented an increase compared to control condictions (10 mM; 50 y 57% respectively). Fru concentration was not affected by the reduction in N. On the contrary, Glu-6P, Glu concentrations showed a reduction under severe deficiency treatment (1 mM; Table 2).

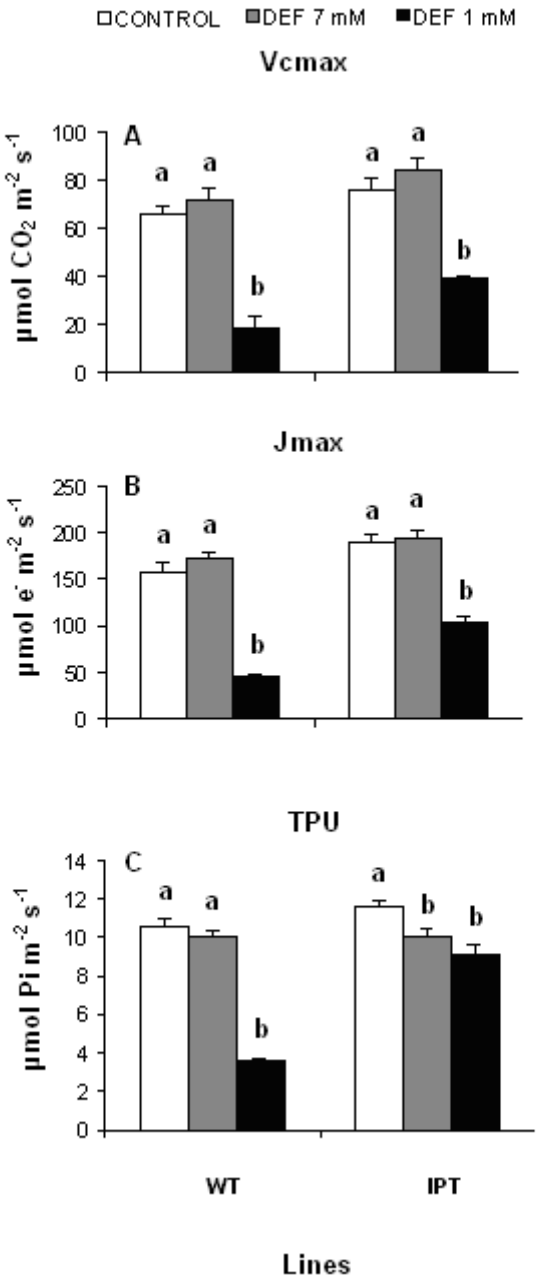

Fig 4: Vcmax (A), Jmax (B), TPU (C) in WT and transgenic tobacco plants under N deficiency. Bars represent means+s.e. (n=9); for each lines.

Table 2: Glu-6P, Suc, Glu, Man, Fru Concentrations in WT and transgenic tobacco plants subjected to N deficit.

Lines/NO_3^- Treatment	**Glucose 6P** (mg g^{-1}DW)	**Sucrose** (mg g^{-1}DW)	**Glucose** (mg g^{-1}DW)	**Mannose** (µg g^{-1}DW)	**Fructose** (mg g^{-1}DW)
WT					
Control	56.20±3.45 b	17.24±0.25 a	11.20±0.47 b	1.22±0.29 b	8.39±0.52 b
7 mM	78.98±3.50 a	14.97±0.63 b	9.15±0.79 b	0.81±0.25 b	9.32±0.71 b
1 mM	81.01±5.78 a	18.55±0.36 a	21.22±1.21 a	3.59±0.45 a	12.67±0.43 a
P-value	**	**	***	***	**
$LSD_{0.05}$	14.027	1.429	2.818	1.105	1.848
IPT					
Control	71.31±6.12 a	17.03±0.58 a	9.81±0.30 b	1.22±0.09 b	8.24±0.67
7 mM	62.90±0.59 ab	13.20±1.15 b	12.02±0.92 b	1.35±0.16 b	9.25±1.14
1 mM	52.19±1.12 b	14.63±0.24 ab	14.67±0.87a	1.92±0.31 a	9.49±0.04
P-value	*	*	**	***	NS
$LSD_{0.05}$	11.559	2.432	2.421	0.263	2.459

Values are means ± SE (n = 9) and differences between means were compared using LSD (P = 0.05). Means followed by the same letter in the same column do not differ significantly. Levels of significance are represented by *P < 0.05, **P < 0.01, ***P < 0.001 and ns (not significant) P > 0.05.

The enzymes envolved in Suc synthesis are shown in Fig 5. WT tobacco plants presented an increase in SPS and SS activities under both N deficiency treatments (7 mM, 1 mM). Activities were higher under 1 mM dosage (Fig. 5). In P_{SARK}*::IPT* plants, 7mM of N resulted in increase of SPS, while SS activity showed a significant increase when plant grown under severe N deficiency (1 mM; Fig. 5). Finally, in WT tobacco plants, the enzymes involved in Suc degradation, cell wall, vacuolar and citosolic invertases, followed a similar trend, with higher activities under the severe N deficiency treatment (1 mM; Table 3), while in P_{SARK}*::IPT* plants, vacuolar and citosolic invertases activities remain unchanged under N deficiency, only cell wall invertase activity showed significant decrease under 7mM treatment (Table 3).

Table 3: Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on invertases activity in WT and transgenic tobacco plants

Lines/NO_3^- Treatment	**Cell wall Invertase** (µmol Glu mg^{-1}prot h^{-1})	**Vacuolar Invertase** (µmol Glu mg^{-1}prot h^{-1})	**Citosolic Invertasa** (µmol Glu mg^{-1}prot h^{-1})
WT			
Control	1.65±0.08 b	4.33±0.37 b	1.09±0.11 b
7 mM	1.54±0.30 b	3.89±0.37 b	1.01±0.09 b
1 mM	2.88±0.30 a	6.26±0.70 a	1.58±0.13 a
P-value	**	*	**
$LSD_{0.05}$	0.776	1.527	0.356
IPT			
Control	1.88±0.16 ab	3.35±0.31	1.03±0.13
7 mM	1.53±0.07 b	3.25±0.11	1.17±0.14
1 mM	2.13±0.17 a	4.08±0.28	1.13±0.11
P-value	*	NS	NS
$LSD_{0.05}$	0.432	0.595	0.391

Values are means ± SE (n = 9) and differences between means were compared using LSD (P = 0.05). Means followed by the same letter in the same column do not differ significantly. Levels of significance are represented by *P < 0.05, **P < 0.01, ***P < 0.001 and ns (not significant) P > 0.05.

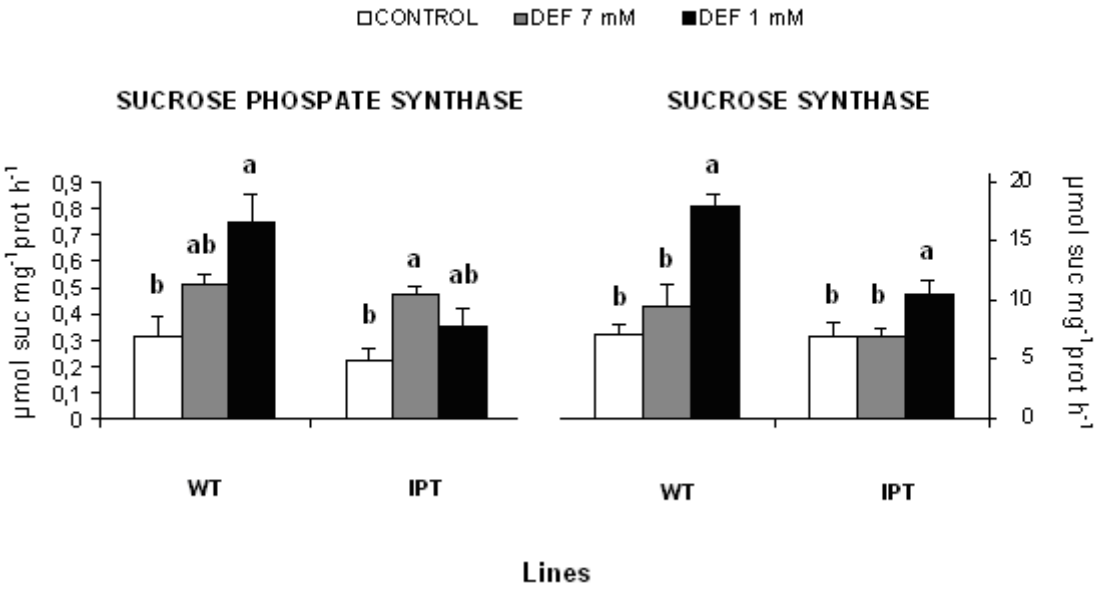

Fig 5: SPS and SS activity in WT and transgenic tobacco plants under N deficiency. Bars represent means+s.e. (n=9); for each lines.

DISCUSSION

The effect of low N availability on partitioning plant biomass, has already been widely studied (Nguyen et al., 2003; Scheible 2004). N content was negatively correlated with the proportion of C allocated to the root (de Groot et al., 2003). The reduction in foliar biomass as well as the root biomass maintenance observed in WT plants submitted to severe N deficiency would agree with Groot et al. (Table 1). On the contrary, P_{SARK} *:: IPT* plants under severe N deficiency (1 mM) reduced root biomass and maintained leaf biomass, which suggests that in these plants the translocation of sugars (carbon compounds) to the root is disrupted despite N deficiency. This response could be related to increased IPT gene expression levels under severe N dosage (1 mM; Fig. 1) which shows the response of promoter P_{SARK} to severe N deficiency. These results indicates that CKs may play a role in plant biomass partitioning under low availability of N. In this sense, it has been indicated that CKs supplied at physiological concentrations to *Urtica dioica* plans could change the direction of photoassimilates, thus completely inverting the source-sink relationship in favour of the shoot (Beck, 1999).

It has been demostrated the protective action of CKs under stress conditions preserved the structure and function of the photosynthetic machinery (Chernyad´ev, 2009), thus CKs could induce the synthesis of proteins of the electron-transport chain, photosynthetic chlorophyll-protein complexes and Rubisco (Chernyad´ev, 2009). Several authors show *A* maintenance under water stress in plants which expressing IPT gene driven by different promoters

(Rivero et al., 2009; Merewitz et al., 2011). In WT plants, severe N deficiency (1 mM) provoked drastic decrease in *A* (Fig. 3A) linked to drastic decrease in Vcmax, Jmax and TPU parameters (72, 70 and 66% respectively; Figs. 4A, B and C). Furthermore, in P_{SARK}*::IPT* plants under 1mM N dosage, reduction of *A* (Fig. 3B) and Vcmax; Jmax and TPU parameters (48, 45 and 21% respectively; Figs. 4A, B and C) was less pronounced than in WT tobacco plants. It suggest that CKs could protect biochemical processes such as carboxylation rate of Rubisco which may enhance *A*. In P_{SARK}*::IPT* tobacco plants under severe N deficiency, the maintenance of Jmax and TPU appears to supports a role of CKs in the protection of the electron transport, leading to the regeneration of RUBP and the capacity of the chloroplast reactions to use triose-P under low availability of N.

Sm-Rubisco are encoged by a family of nuclear genes. It has been proposed that this subunit may be involved in the regulation of photosynthetic ratios and thus the carboxylase / oxygenase Rubisco activity (Khrebtukova and Spreitzer, 1996). Our results show a decrease in sm-Rubisco transcripts in WT plants under N deficiency according to a decrease Vcmax. However P_{SARK}*::IPT* plants preserved sm-Rubisco transcripts levels under N deficiency compared to control conditions (10 mM). It suggets the CKs could be involved transcripcional regulation of this gene under N deficiency.

Regarding the enzymes involved in sugars metabolism, an increase in both synthesis and degradation of Suc was observed in the WT plants subjected to severe N deficiency (1 mM, Table 3, Fig. 6). P_{SARK} *::IPT* tobacco

plants showed a similar trend although both synthesis and degradation were increased less than in WT plants (Table 3, Fig. 6). It has been demostrated that N deficiency leads a sugar accumulation due to less carbon is consumed and exported from source leaves for N assimilation and growth (Paul and Driscoll, 1997, Scheible et al., 2004) the degradation of Suc by invertase, observed especially in WT plants (Table 3) could be directed to the formation of carbon skeletons. Thus, carbon skeletons may be aminated forming new amino acids, particularly affected during the N deficiency (Sanchez et al., 2004). In fact previous work by our group show that WT plants under N deficiency increased processes such as protein degradation or photorespiration which could lead an ammonia formation. This ammonia can be reassimilated by the cycle GS / GOGAT. Thus carbon skeletons, from the Suc degradation could enhance N remobilization process, which are key under low availability of N (Rubio-Wilhelmi et al., 2012). Besides, general increase in other sugars concentration in mature leaves (Table 2) and a reduction in leaf biomass (Table 1) found in WT plants subjected to severe N deficiency (1 mM) could be related with sink limitation within the whole plant due to decreased the growth under N deficiency (Paul and Foyer, 2001). In transgenic plants, limited increase in sugar concentration as well as the maintenance of leaf biomass observed would indicate that CKs may play a role in the sink limitation caused by deficiency of N. Overall could be agree with several authors that indicated CKs may be involved in the maintenance of sink strength in plants subjected to various types of stress such as saline or water (Peleg et al., 2011, Perez-Alfocea et al. 2010).

In conclusion, our work indicated that expression an IPT gene driven by P_{SARK}, a stress-and maturation-induced promoter, leads the maintenance of photosynthesis as well as VCmax, Jmax and TPU parameters and leaf biomass under N deficiency. Therefore, our results suggest that CKs could protect biochemical processes such as carboxylation rate of Rubisco, enhancing sm-Rubisco transcrits levels under low availability of N. Also, CKs could be involved in the protection of the electron transport, leading to the regeneration of RUBP and the capacity of the chloroplast reactions to use triose-P under N deficiency. Regarding sink-source relationships, CKs would act maintain the strength of the sink, such as young leaves, enhancing foliar biomass under severe N deficiency (1 mM).

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REFERENCES

Antal T, Mattila H, Hakala-Yatkin M, Tyystjärvi T, Tyystjärvi E. 2010. Acclimation of photosynthesis to nitrogen deficiency in *Phaseolus vulgaris*. Planta, 232, 887-898.

Argueso CT, Ferreira FJ, Kieber JJ. 2009. Environmental perception avenues: the interaction of cytokinin and enviromental response pathways. Plant Cell and Environment, 32, 1147-1160.

Beck E. 1999. Towards an understanding of plant growth regulation: cytokinins as major signals for biomass distribution. In: Strnad M, Pec P, Beck E, eds. Advances in regulation of plant growth and development. Prague: Peres Publishers, 97-110.

Chernyad´ev II. 2009. The protective action of cytokinins on the photosynthetic machinery and productivity of plants under stress. Applied Biochemistry and Microbiology, 45, 351-362.

Cruz JL, Mosquim PR, Pelacani CR, Araújo WL, DaMatta FM. 2003. Photosynthesis impairment in cassava leaves in response to nitrogen deficiency. Plant and Soil, 257, 417-423.

De Groot CC, Marcelis LFM, van der Boogaard R, Kaiser WM, Lambers H. 2003. Interaction of nitrogen and phosphorus nutrition in determining growth. Plant and Soil, 248, 257-268.

Farquhar GD, Caemmerer SV, Berry JA. 1980. A biochemical model of photosynthetic CO_2 assimilation in leaves C-3 species. Planta, 149, 78-90.

Haberer G, Kieber JJ. 2002. Cytokinins: new insights into a classic phytohormone. Plant Physiology, 128, 354-362.

Harley PC, Loreto F, Dimarco G, Sharkey TD. 1992. Theoretical considerations when estimating the mesophyll conductance to CO_2 flux by analysis of the response of photosynthesis to CO_2. Plant Physiology, 98, 1429-1436.

Harley PC, Sharkey TD. 1991. An improved model of C3 photosynthesis at high CO_2: reversed O_2 sensitivity explained by lack of glycerate reentry into the chloroplast. Photosynthesis Research, 27, 169-178.

Hirai MY, Yano M, Goodenowe DB, Kanaya S, Kimura T, Awazuhara M, Arita M, Fujiwara T, Saito K. 2004. Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in *Arabidopsis thaliana*. Procceding of the National Academy of Science USA, 101, 10205-10210.

Hubbard NL, Huber SC, Pharr DM. 1989. Sucrose phosphate synthase and acid invertase as determinants of sucrose concentration in developing muskmelon *(Cucumis melo* L.) fruits. Plant Physiology, 91, 1527-1534.

Khrebtukova I, Spreitzer RJ. 1996. Elimination of the chlamydomonas gene family that encodes the small subunit of ribulose-1,5-bisphosphate carboxylase oxygenase. Procceding of the National Academy of Science USA, 93, 13689-13693.

Klann E, Yelle S, Bennett AB. 1992. Tomato acid invertase complementary DNA. Plant Physiology, 99, 351-353.

Lawlor DW. 2002. Carbon and nitrogen assimilation in relation to yield: Mechanisms are the key to understanding production system. Journal of Experimental Botany, 53, 773-787.

Long SP, Bernacchi CJ. 2003. Gas exchange measurements, what can they tell us about the underlaying limitations to photosynthesis? Procedures and sources of error. Journal and Experimental Botany, 54, 2393-2401.

Makino A, Osmond B. 1991. Effects of nitrogen nutrition on nitrogen partitioning between chloroplast and mitochondria in pea and wheat. Plant Physiology, 96, 355-365.

Merewitz EB, Gianfagna T, Huang B. 2011. Photosynthesis, water use, and root viability under water stress as affected by expression of SAG12-ipt controlling cytokine synthesis in *Agrostis stolonifera*. Journal of Experimental Botany 62, 383-395.

Miron D, Schaffer AA. 1991. Sucrose phosphate synthase, sucrose synthase, and invertase activities in developing fruit of *Lycopersicon esculentum* Mill. and sucrose accumulating *Lycopersicon hirsutum* Humb. and Bonpl. Plant Physiology, 95, 623-627.

Nelson N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. Journal of Biochemistry, 153, 375-380.

Nguyen TTH, Ie Sung S, Katsuichiro K, Usui K. 2003. Accumulation of some nitrogen compounds in response to salt stress and their relationships with salt tolerance in rice (*Oryza sativa* L.) seedlings. Plant Growth Regulation 41, 159-164.

Nishiyama R, **Watanabe Y, Fujita Y, Le DT, Kojima M, Werner T, Vankova R, Yamaguchi-Shinozaki K, Shinozaki K, Kakimoto T, Sakakibara H, Schmülling T, Phan Tran L-S.** 2011. Analysis of cytokinin mutants and regulation of cytokinin metabolic genes reveals important regulatory roles of cytokinins in drought, salt and abscisic acid responses, and abscisic acid biosynthesis. Plant Cell, 23, 2169-2183.

Paul MJ, Driscoll SP. 1997. Sugar repression of photosynthesis: the role of carbohydrates in signalling nitrogen deficiency through source:sink imbalance. Plant Cell and Environment, 20, 110-116.

Paul MJ, Foyer CH. 2001. Sink regulation of photosynthesis. Journal of Experimental Botany, 52, 1383-1400.

Peleg Z, Reguera M, Tumimbang E, Walia H, Blumwald E. 2010. Cytokinin-mediated source/sink modifications improve drought tolerance and increase grain yield in rice under water-stress. Plant Biotechnology Journal, 9, 747-758.

Pérez-Alfocea F, Albacete A, Ghanem ME, Dodd IC. 2010. Hormonal regulation of source-sink relations to maintain crop productivity under salinity: a case study of root-to-shoot signalling in tomato. Functional Plant Biology, 37, 592-603.

Remans T, Nacry P, Pervent M, Girin T, Tillard P, Lepetit M, Gojon A. 2006. A central role of the nitrate transporter NRT2.1 in the integrated morphological and physiological responses of the root system to nitrogen limitation in *Arabidopsis.* Plant Physiology, 140, 909-921.

Rivero RM, Gimeno J, Van Deynze, Walia H, Blumwald E. 2010. Enhanced cytokinins synthesis in tobacco plants expressing P_{SARK}::IPT prevents the degradation of photosynthetic proteins complexes during drought. Plant Cell Physiology, 51, 1929-1941.

Rivero RM, Kojima M, Gepstein A, Sakakibara H, Mittler R, Gepstein S, Blumwald E. 2007. Delayed leaf senescence induces extreme drought tolerance in a flowering plant. Procceding of the National Academy of Science USA, 104, 19631–19636.

Roitsch T, Ehneβ R. 2000. Regulation of source/sink relations by cytokinins. Plant Growth Regulation, 32, 359-367.

Rubio-Wilhelmi MM, Sanchez-Rodriguez E, Rosales MA, Blasco B, Rios JJ, Romero L, Blumwald E, Ruiz JM. 2011. Effect of cytokinins on oxidative stress in tobacco plants under nitrogen deficiency. Environmental and Experimental Botany, 62, 167-173.

Sakakibara H, Takei K, Hirose N. 2006. Interactions between nitrogen and cytokinins in the regulation of metabolism and development. Trends in Plant Science, 11, 440-448.

Scheible W-R, Morcuende R, Czechowski T, Frizt C, Osuna D, Palacios-Rojas N, Schindelash D, Thimm O, Udvardi MK, Stitt M. 2004. Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of *Arabidopsis* in response to nitrogen. Plant Physiology, 136, 2483-2499.

Sharkey TD, Berry JA, Raschke K. 1985. Starch and sucrose synthesis in *Phaseolus vulgaris* as affected by light, CO_2, and abscisic acid. Plant Physiology, 77, 617-620.

Somogyi M. 1951. Notes on sugar determination. Journal of Biological Chemistry, 195, 19-23.

Terashima I, Evans JR. 1988. Effects of light and nitrogen nutrition on the organization of the photosynthetic apparatus in spinach. Plant and Cell Physiology, 29, 143-155.

Von Caemmerer S, Farquhar GD. 1981. Some relationships between the biochemistry of photosynthesis and the gas-exchange of leaves. Planta, 153, 376-387.

Werner T, Nehnevajova E, Köllmer I, Novák O, Stmad M, Krämer U, Schmüling T. 2010. Root-specific reduction of cytokinins causes enhanced root growth, drought tolerance, and leaf mineral enrichment in *Arabidopsis* and tobacco. Plant Cell, 22, 3905-3920.

Capítulo 8

Discusión general

Uno de los factores más limitantes del crecimiento de los cultivos es la disponibilidad de N, de tal manera que la aplicación de fertilizantes nitrogenados se ha convertido en una práctica agronómica muy extendida. De acuerdo con las últimas investigaciones el incremento del doble de la producción agrícola, que se ha producido en los pasados 40 años, se ha asociado con un aumento de siete veces en la fertilización nitrogenada. Pero la aplicación de estos fertilizantes es costosa económica y medioambientalmente, por lo que la utilización de plantas con menor requerimiento de N que no implique una disminución en la cantidad y la calidad de la cosecha, es la estrategia más lógica para mantener y desarrollar una agricultura sostenible, rentable económicamente y respetuosa con el medioambiente.

Por tanto, conocer las respuestas fisiológicas de las plantas a la deficiencia de N podría ayudar en la búsqueda de genotipos capaces de utilizar de forma más eficiente el N. Así, la consecuencia fundamental de la deficiencia de N en las plantas es la represión de genes de la fotosíntesis y de la síntesis de clorofilas que provoca la senescencia de las hojas y por tanto la reducción de la biomasa foliar que conlleva pérdidas en la producción de los cultivos. A pesar de que estas consecuencias se conocen de manera amplia, pocos son los estudios que engloban el estudio fisiológico y agronómico de la deficiencia de N orientados hacia la reducción de la aplicación de fertilizantes. Por ello, en esta tesis doctoral utilizamos plantas de tabaco WT y P_{SARK}*::IPT* que expresan el gen *IPT* asociado al promotor P_{SARK} que se induce en la madurez y con el estrés. Estas plantas mostraron distintas respuestas fisiológicas frente a la deficiencia de N.

Las plantas de tabaco WT sometidas a deficiencia severa de N (1 mM) presentaron una reducción de biomasa foliar acompañado de un mantenimiento de la biomasa radicular. Ésto ha sido descrito previamente por algunos autores en distintos tipos de plantas sometidas a deficiencia de N (Tewari et al., 2004, 2007; Kovacik and Backor, 2007) y corrobora que las plantas en situaciones limitantes de N redirigen los fotoasimilados hacia la parte radicular manteniendo el crecimiento de la raíz e intensificando la absorción de N (Scheible et al., 2004). Pero a pesar de esta redirección de fotoasimilados hacia la raíz, las plantas WT acumularon gran cantidad de ellos en las hojas maduras lo que confirma la perdida de fuerza del sumidero que suponen las hojas jóvenes y por tanto la reducción del crecimiento de la parte aérea observado en plantas WT bajo deficiencia de N.

El principal proceso alterado por la deficiencia de N es la fotosíntesis ya que normalmente existe una relación directa entre capacidad fotosintética y cantidad de N por unidad de área en la hoja (Walcroft et al., 1997). La deficiencia de N provocó en las plantas WT una reducción generalizada de parámetros fotosintéticos, especialmente relacionados con la Rubisco ya que esta enzima constituye un elevado porcentaje de las proteínas totales de la planta (Antal et al., 2010). Por tanto, la alteración de los parámetros de crecimiento observado en las plantas WT, parecen ser consecuencia de la reducción drástica de *A* asociado con la disminución del Vcmax y con la reducción en los niveles de expresión de sm-Rubisco observados en las plantas WT sometidas a deficiencia de N. Otros parámetros fotosintéticos como el TPU y el Jmax se vieron afectados por la baja disponibilidad de N. Una de

las consecuencias de la alteración de la cadena de transporte electrónico es el incremento en la formación de ROS, ampliamente conocidas por generar daño oxidativo y por tanto reducción del crecimiento (Jaleel et al., 2009; Arora et al., 2002). La deficiencia de N provocó en las plantas WT un incremento en la cantidad de ROS frente al cual estas plantas respondieron incrementado la actividad de algunas de las enzimas del metabolismo oxidativo como SOD, CAT y GPX o manteniendo compuestos como las PAs que aunque ricos en N se han descrito como antioxidantes. La reducción de la biomasa foliar demuestra que dicha respuesta no fue suficiente para evitar el daño oxidativo en las plantas WT posiblemente debido a que procesos tan importantes en la detoxificación ROS como el ciclo de Halliwell-Asada no fueron capaces evitar la acumulación de éstas.

Por otro lado la reducción de la biomasa foliar se asocia con la disminución de todas las formas de N medidas en las hojas de plantas WT bajo deficiencia severa de N (1 mM). El descenso de las formas de N en conlleva una activación de procesos de reciclaje o removilización de N, propios de situaciones de baja disponibilidad de N. Las plantas WT activaron procesos como la degradación de proteínas, reducción en la síntesis de Pro y la fotorrespiración, todos ellos son capaces de formar NH_4^+ que rápidamente será reasimilado ya que resulta muy tóxico para las plantas. Este NH_4^+ fue asimilado por el ciclo GS/GOGAT para la formación de Aas esenciales como el Glu y la Gln, donadores de grupos amino en la síntesis de otros Aas. Así mismo, el aumento en la síntesis y degradación de Suc observado bajo deficiencia severa de N (1mM) parece estar asociado a la generación de nuevos esqueletos

carbonados los cuales serán aminados para dicha síntesis de Aas. Por tanto, las plantas WT activan mecanismos de removilización de N, en un intento por parte de la planta de sintetizar nuevos Aas como respuesta a la deficiencia severa de N (1mM).

El incremento en la eficiencia del uso de los nutrientes se ha descrito como una herramienta útil para mejorar los sistemas agrícolas (Fageria et al., 2008). Se ha relacionado previamente un elevado NUE con un incremento en la biomasa (Ye et al., 2010). En nuestro trabajo los parámetros relacionados con el NUE, TCN, TNA y NUpE se redujeron en las plantas WT sometidas a deficiencia severa de N especialmente el NUpE asociado a una reducción acusada del flujo de absorción de N por parte de la raíz. A pesar de que observamos un incremento del NUtE bajo deficiencia de N, éste no fue suficiente para el mantenimiento de la biomasa foliar en plantas WT.

La implicación de las CKs en la señalización del estado nutricional ha sido ampliamente estudiada y se conoce que esta fitohormona influye en la homeostasis de algunos nutrientes como N, P, S o Fe. Pero especialmente la relación entre CKs y N ha sido la más estudiada y se sabe que actúan como mensajeros a larga distancia que controlan la asimilación de N y el estatus de éste en la planta (Sakakibara et al., 2006). Por tanto, las CKs podrían actuar como señal a larga y corta distancia comunicando a la parte aérea si la aplicación de N en la raíz es adecuada y regulando así los sistemas de absorción del N y de otros nutrientes del suelo (Sakakibara et al., 2006).

Las plantas de tabaco *P_{SARK}::IPT* expresan el gen *IPT* unido al promotor *P_{SARK}* el cual como a otros estreses, se muestra sensible a la deficiencia de N ya que incrementa su expresión relativa bajo condiciones de deficiencia severa de N (1 mM). Las plantas *P_{SARK}::IPT* muestran un mantenimiento de la biomasa foliar y una reducción de la biomasa radicular en condiciones de baja disponibilidad de N. Este fenómeno parece estar asociado a que las plantas transgénicas fueron capaces de mantener *A* bajo deficiencia severa de N (1mM) lo cual se relaciona de forma directa con la conservación de los niveles de expresión de sm-Rusbisco observados en situaciones de baja disponibilidad de N ya que esta subunidad ha sido relacionada con la regulación de la actividad carboxilasa/oxigenasa de la Rubisco (Khrebtukova and Spreitzer, 1996). Normalmente bajo situaciones de estrés se ha descrito un descenso de actividad de la Rubisco acompañado de un descenso del ratio de asimilación fotosintético de CO_2. En nuestro caso las CKs son capaces de reducir este descenso en la fotosíntesis mediante el mantenimiento de la expresión relativa de sm-Rubisco y por tanto de la fijación de CO_2. Así, un menor descenso de la fotosíntesis permite a la planta sostener el crecimiento foliar manteniéndose la fuerza del sumidero formado por las hojas jóvenes que explica una acumulación limitada de azúcares en las hojas intermedias de las plantas *P_{SARK}::IPT*. Sin embargo la biomasa radicular se redujo durante la deficiencia de N, hecho que induce a pensar que en estas plantas la translocación de azúcares hacia la raíz se encuentra interrumpida, lo que también favorecería la translocación de compuestos carbonados hacia las hojas jóvenes, ayudando al mantenimiento de la biomasa foliar durante la deficiencia de N.

Por otro lado, una menor afectación de la fotosíntesis implica que las plantas P_{SARK}*::IPT* no muestren un incremento en la síntesis de ROS por lo que estas plantas no activaron la respuesta antioxidante propia de situaciones de disponibilidad de N. Algunos compuestos no enzimáticos que se han relacionado de forma clásica con la respuesta antioxidante como los fenoles, presentaron un aumento en su concentración, pero éste parece estar más relacionado con una respuesta de la planta a bajas cantidades de NO_3^- y otras formas de N, encontradas en las hojas de las plantas P_{SARK}*::IPT* que con la respuesta antioxidante. La acumulación de otros compuestos como PAs y Pro y su metabolismo se ha asociado a la tolerancia de algunas plantas a condiciones adversas. Las plantas P_{SARK}*::IPT* mostraron un descenso significativo en ambos compuestos lo que podría explicarse ya que éstos poseen una elevada cantidad de N en su estructura lo que los hace especialmente sensibles a la deficiencia de dicho nutriente. Por ultimo, otros compuestos ricos en N como los Aas redujeron su concentración en las plantas transgénicas sometidas a deficiencia, pero a la luz de nuestros resultados, esto no parece comprometer la biomasa foliar.

La baja disponibilidad de N en el medio conlleva un inducción rápida de la senescencia foliar por disminución del nivel de CKs, lo que se traduce en una disminución del crecimiento y de la productividad de las plantas, y por lo tanto en una reducción drástica del NUE. La expresión del gen *IPT* en plantas de tabaco sometidas a deficiencia severa de N provoca un retraso en la senescencia y por tanto una mejora en la eficiencia en el uso de N así como de su absorción lo que ofrece un mantenimiento de la producción bajo condiciones

de baja disponibilidad de N. Esto, señala a las plantas P_{SARK}*::IPT* como una herramienta eficaz para la reducción del uso de fertilizantes nitrogenados, costosos económica y medio ambientalmente, sin que esto acarre una perdida de producción.

REFERENCES

Antal T, Mattila H, Hakala-Yatkin M, Tyystjärvi T, Tyystjärvi E. 2010. Acclimation of photosynthesis to nitrogen deficiency in *Phaseolus vulgaris*. Planta, 232, 887-898.

Fageria NK, Baligar VC, Li YC. 2008. The role of nutrient efficient plants in improving crop yields in the twenty first century. Journal of Plant Nutrition, 31, 1121-1157.

Khrebtukova I, Spreitzer RJ. 1996. Elimination of the Chlamydomonas gene family that encodesthe small subunit of ribulosa-1,5-bisphosphate carboxylase oxygenase. Procceding of the National Academy of Science USA, 93, 13689-13693.

Kováčik J, Backor M. 2007. Changes of phenolic metabolism and oxidative status in nitrogen-deficient *Matricaria chamomilla* plants. Plant and soil, 297, 255-265.

Sakakibara H, Takei K, Hirose N. 2006. Interactions between nitrogen and cytokinin in the regulation of metabolism and development. Trends in Plant Science, 11, 440-448.

Scheible W-R, Morcuende R, Czechowski T, Frizt C, Osuna D, Palacios-Rojas N, Schindelash D, Thimm O, Udvardi MK, Stitt M. 2004.

Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of *Arabidopsis* in response to nitrogen. Plant Physiology, 136, 2483-2499.

Tewari RK, Kumar P, Sharma PN. 2007. Oxidative stress and antioxidant responses in young leaves of mulberry plants grown under nitrogen, phosphorus or potassium deficiency. Journal of Integrative Plant Biology, 49, 313-322.

Tewari RK, Kumar P, Tewari N, Srivastava S, Sharma PN. 2004. Macronutrient deficiencies and differential antioxidant responses-influence on the activity and expression of superoxide dismutase in maize. Plant Science, 166, 687-694.

Walcroft AS, Whitehead D, Silvester WB, Kelliher FM. 1997. The response of photosynthetic model parameters to temperature and nitrogen concentration in *Pinus radiata*. Plant Cell Environment, 20, 1338-1348.

Ye X, Hong J, Shi L, Xu F. 2010. Adaptability mechanism of nitrogen-efficient germplasm of natural variation to low nitrogen stress in *Brassica napus*. Journal of Plant Nutrition, 33, 2028–2040.

Capítulo 9

Conclusiones

1. La deficiencia de N conlleva una reducción de la biomasa y de la tasa de crecimiento relativo foliar en plantas de tabaco WT, debido a un descenso de las diferentes formas de N y una inducción de especies reactivas de oxigeno (ROS) y por tanto del daño oxidativo. Por el contrario, en las plantas de tabaco *P_{SARK}::IPT* la deficiencia de N no afectó la producción de ROS manteniéndose la biomasa y la tasa de crecimiento relativo foliar a pesar de que también se reduce la concentración de las distintas formas de N. Esta repuesta diferencial nos sugiere que el aumento de CKs en plantas de tabaco *P_{SARK}::IPT* podría actuar como señal impidiéndose la aparición de las respuestas típicas de la deficiencia de N.

2. Ante una deficiencia de N severa las plantas WT activan los procesos de generación de NH_4^+ independientes de la reducción del NO_3^-, como son la fotorrespiración, la degradación de proteínas y el aumento de los niveles relativos de aas de transporte, todo ello con el fin de suministrar N a zonas de crecimiento. Por el contrario, las plantas transgénicas *P_{SARK}::IPT* no inducen estas respuestas típicas de la deficiencia de N lo que les permite en estas condiciones un mantenimiento de la biomasa foliar y una mejora en la calidad de las hojas de tabaco así como en la eficiencia del uso del N.

3. La deficiencia de N estimula el metabolismo fenólico incrementando la cantidad de fenoles, lo cual podría definirse como respuesta a la baja concentración NO_3^- existentes en los tejidos. El mantenimiento de la cantidad de PAs en plantas de tabaco WT bajo deficiencia de N podría estar relacionado con el mayor estrés oxidativo observado previamente en dichas plantas. Por el

contrario la reducción de PAs y Pro en las plantas transgénicas podría representar un mecanismo de reciclaje de N bajo situaciones de baja disponibilidad de dicho nutriente.

4. La expresión del gen *IPT* en plantas de tabaco sometidas a deficiencia de N conlleva un mantenimiento de la fotosíntesis de tal manera que las CKs podrían actuar protegiendo este proceso fisiológico lo que se demuestra por el mantenimiento de los niveles de transcrito de la sm-Rubisco, la cadena de transporte electrónico y la capacidad del cloroplasto para usar las triosas-P. En relación a la relación fuente-sumidero, la inducción en la síntesis de CKs podría ayudar a potenciar la fuerza del sumidero que representan las hojas jóvenes, mejorando la biomasa foliar bajo condiciones de deficiencia severa de N.

Anexo

Curriculum vitae

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2000-2006: Licenciada en Biología. Universidad de Granada. Facultad de Ciencias

2006-2007: Master en Biología agraria y Acuicultura. Universidad de Granada, Facultad de Ciencias. Departamento de fisiología vegetal. (Supervisor: Dr. Luis Romero)

2007-2008: Certificado de Aptitud Pedagogica (CAP). Universidad de Granada.

2008-2012: Estudiante predoctoral Universidad de Granada, Facultad de Ciencias, Departamento de fisiología vegetal.

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EXPERIENCIA PROFESIONAL

2006-presente: Estudiante predoctoral Universidad de Granada, Facultad de Ciencias, Departamento de fisiología vegetal.

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PUBLICACIONES MÁS RELEVANTES

Rubio-Wilhelmi MM, Sanchez-Rodriguez E, Rosales MA, Blasco B, Rios JJ, Romero L, Blumwald E, Ruiz JM. 2011. Effect of cytokinins on oxidative stress in tobacco plants under nitrogen deficiency. Environmental and Experimental Botany, 72, 167-173.

(Correspondiente al capítulo 3)

Rubio-Wilhelmi MM, Sanchez-Rodriguez E, Rosales MA, Blasco B, Rios JJ, Romero L, Blumwald E, Ruiz JM. 2011. Cytokinin-Dependent

Improvement in Transgenic P_{SARK}*::IPT* Tobacco 2 under Nitrogen Deficiency. Journal Agriculture and Food Chemistry, 59, 10491-10495.
(Correspondiente al capítulo 4)

Rubio-Wilhelmi MM, Sanchez-Rodriguez E, Rosales MA, Blasco B, Rios JJ, Romero L, Blumwald E, Ruiz JM. 2012. Ammonium formation and assimilation in P_{SARK}*::IPT* tobacco transgenic plants under low N. Journal of Plant Physiology, 169, 157-162.
(Correspondiente al capítulo 5)

Rubio-Wilhelmi MM, Sanchez-Rodriguez E, Leyva R, Blasco B, Romero L, Blumwald E, Ruiz JM. 2012. Response of Carbon and Nitrogen-rich metabolites to nitrogen deficiency in P_{SARK}*::IPT* tobacco plants. Plant Physiology and biochemistry (En revision)
(Correspondiente al capítulo 6)

Rubio-Wilhelmi MM, Reguera M, Sanchez-Rodriguez E, Romero L, Blumwald E, Ruiz JM. 2012. P_{SARK}*::IPT* expression causes protection of photosynthesis in tobacco plants during N deficiency.
(Correspondiente al capítulo 7)

PARTICIPACIÓN EN CONGRESOS

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Poster: Implicaciones del metabolismo de la prolina en la deficiencia de nitrógeno: Papel de las citoquininas
XIX Reunión de la sociedad Española de Fisiología vegetal y XII Congreso Hispano-Luso de fisiología vegetal.
Castellón de la Plana (Spain) 2011