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"ALUMINUM-TOXICITY EFFECT ON PHOSPHATE NUTRITION AND EXPRESSION OF PHOSPHATE TRANSPORTER GENES FROM RYEGRASS PLANTS"

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"ALUMINUM-TOXICITY EFFECT ON PHOSPHATE NUTRITION AND EXPRESSION OF PHOSPHATE TRANSPORTER GENES FROM RYEGRASS PLANTS"

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A mi hija Renata Ignacia

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Summary and thesis outline

The beef and dairy market in Southern Chile is based on grassland production and the main forage species is perennial ryegrass (*Lolium perenne* L). Large portions of soil used for this activity are acidic, with low phosphorus (P) availability and high levels of soluble aluminum (Al), affecting not only yield but also the quality and persistence of pastures. Previous studies have reported that P addition could alleviate Al toxicity in plants, but the influence of P nutrition on Al phytotoxicity is still controversial. Therefore, obtaining genotypes more P-efficient (uptake and distribution) in this species is likely to result in considerable economic and ecological benefits. Moreover, P efficient genotypes could ameliorate the effects of Al-toxicity. However, P uptake and transport mechanism underlying Al stress remain unknown in the pasture grass. In chapter I, we present a general introduction of this doctoral thesis, showing the hypothesis and aims of this study.

In chapter II we explore the general background of the response mechanisms of plants to phosphorus deficiency, focusing on phosphate (Pi) transporters PTH1s and their regulation in crops. This chapter aims to improve our knowledge on Pi uptake and transport by plants and how plants have developed strategies to cope with P deficiency mostly in acid soils.

In chapter III, we present functional characterization and expression patterns of two PHT1 members from ryegrass plants (*Lolium perenne*) to determine their roles in the specificity of Pi transport. Yeast mutant complementation assays showed that *LpPHT1;4* can complement the growth defect of the yeast mutant *Δpho84* under Pi deficient conditions, whereas the yeast mutant expressing *LpPHT1;1* was not able growth under Pi deficiency. These results suggest that LpPHT1;1 functions as a low-affinity Pi transporter, whereas

LpPHT1;4 acts as a high-affinity Pi transporter to maintain Pi homeostasis under stress conditions in ryegrass plants.

In chapter IV, we evaluated P-nutrition and Al-toxicity interaction on P uptake, antioxidant responses and the gene expression of phosphate transporters from ryegrass plants. Two ryegrass cultivars with different Al resistances, the Al-tolerant Nui cultivar and the Al-sensitive Expo cultivar were hydroponically grown under low (16 μM) and optimal (100 μM) P doses for 16 days. After P treatments, plants were exposed to Al doses (0 and 200 μM) under acidic conditions (pH 4.8) for 24 h. Al and P accumulation were higher in the roots of Nui than in those of Expo. Moreover, lower Al accumulation was found in shoots of Nui independently of the P supply. Oxidative stress induced by Al-toxicity and P-deficiency was more severe in the Al-sensitive Expo. Expression levels of the L. perenne phosphate transporters were higher in Nui than in Expo. While *LpPHT1* expression was up-regulated by P deficiency and Al toxicity in both cultivars, *LpPHT4* expression only increased in the Al-tolerant cultivar. These data suggest that the Al-tolerance of Nui is a consequence of Al immobilization by P mediated by the high expression of phosphate transporters.

All these results are discussed in the final chapter (V). In addition, studies about an Al transporter from ryegrass also were discussed and shown in annex 2. We conclude that: (1) Ryegrass Pi transporters show highly conserved characteristics among Pi transporters from Poaceae family (2) The functional analysis suggests that LpPHT1;1 (low-affinity Pi transporter) and LpPHT1;4 (high-affinity Pi transporter) play important roles in Pi uptake and translocation in ryegrass (3) Al-tolerance in ryegrass is mainly due to its high

efficiency to transport P from growth solution and to its greater antioxidant activity allowing an increased alleviation of Al-toxicity.

TABLE OF CONTENTS

Acknowledgements viii

Summary and Thesis Outline x

Table of contents xiii

CHAPTER I. ***General Introduction*** 1

1.1 Introduction 2

1.2 Hypothesis and research objectives 4

1.2.1 Hypothesis 4

1.2.2 Research objectives 4

1.2.2.1 General objective 4

1.2.2.2 Specific objectives 4

CHAPTER II. ***Plant response mechanisms to phosphorus deficiency in acid soils*** 5

Abstract 6

2.1 Introduction 7

2.2 Plant responses to low phosphate 8

2.3 Phosphorus uptake and transport 10

2.4 Phosphate transporters in plants 11

2.4.1 Structure and affinity of Pi transporters 14

2.4.2 Localization and expression patterns of Pi transporters 16

2.5 Phosphate uptake and aluminum toxicity in acid soils 18

2.6 Conclusions and perspectives 19

CHAPTER III. ***Functional properties and expression profiling of two phosphate transporters in response to phosphorus starvation in ryegrass (Lolium perenne L.)*** 21

Abstract 23

3.1 Introduction 24

3.2 Materials and Methods 27

3.2.1 Plant material and growth conditions 27

3.2.2 Phosphate transporters gene expression analyses 27

3.2.3 Phosphorus concentration analysis 29

3.2.4 Plasmid construction for yeast complementation assays 29

3.2.5 Yeast complementation assays 29

3.2.6 Bioinformatic analysis 31

3.2.7 Statistical analysis 31

3.3 Results 31

3.3.1 Ryegrass growth, P status and relative expression levels of *PHT1s* 31

3.3.2 Heterologous expression of *PHT1s* in yeast 34

3.3.3 Bioinformatic analysis of *PH*T1 genes 36

3.4 Discussion 40

Acknowledgments 43

Author contributions 43

CHAPTER IV. *Aluminium toxicity and phosphate deficiency activates antioxidant systems and up-regulates expression of phosphate transporters gene in ryegrass (Lolium perenne L.)* 44

Abstract 46

4.1 Introduction 47

4.2 Materials and Methods 49

4.2.1 Plant material and growth conditions 49

4.2.2 Plant Al and P analyses 51

4.2.3 Radical scavenging activity 51

4.2.4 Lipid peroxidation measurements 52

4.2.5 Superoxide dismutase and peroxidase activity 52

4.2.6 Protoplasts isolation 53

4.2.7 Detection of hydrogen peroxide (H_2O_2) production in shoot protoplasts by confocal microscopy 53

4.2.8 Detection of hydrogen peroxide (H_2O_2) production in root tissues 54

4.2.9 De novo assembly of ryegrass transcriptomes and search for phosphate transporter genes 55

4.2.10 Phosphate transporters gene expression analyses 55

4.2.11 Statistical analysis 56

4.3. Results 57

4.3.1 Phosphorus and aluminium concentrations 57

4.3.2 Lipid peroxidation and plant antioxidant responses 60

4.3.3 Production of H_2O_2 in ryegrass shoot protoplasts and roots 63

4.3.4 Regulation of phosphate transporters gene expression 65

4.4 Discussion 68

Author contributions 72

Acknowledgments 72

CHAPTER V. *General discussion, concluding remarks and future directions* 73

5.1 General discussion 74

5.2 Concluding remarks and future directions 78

References 80

Annexes 105

CHAPTER I

General Introduction

1.1 General Introduction

Phosphorus (P) is an essential macronutrient for plant growth, development and high crop productivity (Vance *et al.*, 2003; Richardson *et al.*, 2009). Plants acquire phosphorus as phosphate (Pi) anions from the soil solution, but its bioavailability is strongly affected by chemical and biological factors in the rhizosphere (Schachtman *et al.*, 1998; López-Arredondo *et al.*, 2014). Low P availability to plants is due to its substantial fixation by soil minerals and its low diffusion rate (10^{-12} to 10^{-15} m^2/s) (Shen *et al.*, 2011). Thus, to cope with low P availability plants have developed a number of morphological, biochemical and molecular strategies that increase Pi uptake and/or improve internal Pi use efficiency (Vance *et al.*, 2003). These strategies involve secretion of organic acids and phosphatases, changes in root system architecture and enhanced expression of phosphate transporters (PHTs) (Nussaume, 2011; Peret *et al.*, 2014; Chien *et al.*, 2018).

Plant PHTs are integral membrane proteins of similar size (approximately 518-587 amino acids), responsible for acquiring Pi from the rhizosphere and/or transferring it to different tissues and organs (Nussaume, 2011; Baker *et al.*, 2015; Gu *et al.*, 2016). These proteins belong to the major facilitator superfamily (MFS), which is the largest superfamily of active transporters, and they are generally symporters or antiporters driven by proton or sodium gradients (Smith *et al.*, 2003; Raghothama and Karthikeyan, 2005). PHTs identified in plants have been classified into four families; *PHT1*, *PHT2*, *PHT3* and *PHT4*, which are responsible for Pi transport across the membranes of cells, chloroplasts, mitochondria and Golgi bodies, respectively (Jain *et al.*, 2012).

In this context, it is well documented that an efficient regulation of the abundance of PHTs is essential for the plant to maintain Pi homeostasis (Wang *et al.*, 2010; Huang *et al.*, 2011; Gu *et al.*, 2016). In fact, a high P utilization efficiency in crops has been mainly attributed to an efficient P redistribution mediated by Pi transporters (Wang et al., 2010; Huang et al., 2011).

Members of the plant *PHT1* gene family, have been reported in many plant species, with their expression being regulated in response to different P status (López-Arredondo *et al.*, 2014; Teng *et al.*, 2017; Wang *et al.*, 2017*a*). Functional analyses have shown that some of the PHT1 members, are high-affinity Pi transporters while others are low-affinity Pi transporters, operating at micromolar and milimolar Pi concentration ranges, respectively (Ai *et al.*, 2009; Jain *et al.*, 2012; Wang *et al.*, 2017*b*). Although most *PHT1* genes are transcriptionally induced by Pi deficiency in both roots and shoots, the influence of *PHT1* gene expression on P acquisition efficiency (PAE) and/or P utilization efficiency (PUE) in crops is poorly understood.

Beef and dairy market in Southern Chile is based on grazing systems, including perennial ryegrass (*Lolium perenne* L.). Large portions of soil used for this activity are acidic, with low P availability and high levels of soluble aluminium (Al), affecting yield, quality and persistence of pastures (Mora *et al.*, 2006). Several reports have shown that P efficient genotypes have an enhanced ability to cope with Al toxicity (Kochian *et al.*, 2004; Magalhaes *et al.*, 2018). However, up to now little is known about P deficiency and Al toxicity interaction in pasture grass. Besides, the functions of many Pi transporters under other mineral stresses such Al toxicity, still remains elusive.

1.2 Hypothesis and research objectives

Based on the previous background, we addressed the following hypothesis:

1.2.1 Hypothesis

The expression of phosphate transporter genes will be up-regulated as a response to toxic levels of Al in ryegrass cultivars as a mechanism of acclimation in acid soils, enhancing P uptake efficiency and Al-tolerance

1.2.2 Research Objectives

1.2.2.1 General Objective

- To study the interaction of Al-toxicity and different P supplies on P uptake and antioxidant properties in ryegrass cultivars, as well as to analyze change effects involving gene expression of phosphate transporters

1.2.2.2 Specific Objectives

1. To identify sequences encoding phosphate transporters in ryegrass plants (*Lolium perenne* L.).

2. To analyze the gene expression and functional characterization of phosphate transporters in ryegrass cultivars subjected to P deficiency and Al toxicity.

3. To determine the effect of the interaction of P deficiency and Al-toxicity on the antioxidant systems in two ryegrass cultivars growing under acid conditions.

CHAPTER II

Plant response mechanisms to phosphorus deficiency in acid soils

Plant response mechanisms to phosphorus deficiency in acid soils: a focus on phosphate transporters

Abstract

Phosphorus (P) is one of the essential mineral nutrients required for all developmental processes and reproduction in plants. After nitrogen (N), P is the second major constituent of the fertilizers required in agriculture. The main source of phosphorus for plants is inorganic phosphate (Pi), which is characterized by its poor availability and low mobility. Thus, plants exposed to P deficiency activate a range of mechanisms that either result in an increased acquisition of P from P-deficient soil or in more efficient use of internal Pi. These include changes of root morphology and architecture, remodeling of different metabolic pathways, secretion of organic acids and phosphatases and improved P uptake and utilization efficiency through the expression of specific phosphate transporters. Genes encoding phosphate transporters involved in acquisition and mobility of Pi have been isolated and characterized in many plant species. Improving our knowledge of Pi acquisition and use by plants has a positive effect on reducing the dependency on fertilizer supply for crop production. The purpose of this review is to summarise current understanding of the role of P as an essential macronutrient for higher plants, recent knowledgement of plant Pi acquisition and transport mechanisms and how plants have developed strategy responses to the low availability of this element, mostly focusing on phosphate transport systems.

Keywords: Phosphorus utilization efficiency; phosphate uptake; phosphate transporter

2.1 Introduction

Phosphorus (P) is an essential macronutrient for growth, development and high productivity of crops. It serves various essential biological functions as a structural element in nucleic acids and phospholipids, in energy metabolism, in the activation of metabolic intermediates, as a component in signal transduction cascades and in regulation of enzymes (López-Arredondo et al., 2014). Despite its abundance in the environment (ranked as the 11th most abundant element), P is poorly available to plants due to inorganic fixation with cations such as iron (Fe), aluminum (Al) or calcium (Ca) and to the formation of P organic complexes (Hasan et al., 2016; Raghothama and Karthikeyan, 2005). Moreover, the movement of phosphate (Pi) ions is a relatively slow process that results in the concentration of Pi in the solution being depleted around plant roots leading to least available (Smith et al., 2003; Vance et al., 2003). To alleviate this problem, around 30 million tons of P fertilizer are extensively applied in agricultural systems (Koppelaar and Weikard, 2013). Rock phosphate is crucial for the production of P fertilizers, but rock phosphate is a non-renewable resource, which represent the only significant global reserve of Pi. Some estimates have shown that Pi reserves could be exhausted in the next 50–200 years (Cordell et al., 2009; López-Arredondo et al., 2017). Thus, Pi fertilizers will inevitably become a scarce, and consequently a costly input with severe effects on agricultural production and food security (Cordell et al., 2009).

As a consequence of low Pi availability, plants have developed a range of mechanisms that increase Pi acquisition from the soil and/or improve the internal efficiency of Pi within the plants (Ramaekers et al., 2010; Smith et al., 2003). The P acquisition efficiency (PAE) is the ability of plants to take up more P from the soil under P-limiting condition, whereas

utilization efficiency (PUE) is the capacity to produce a large amount of biomass per unit of P absorbed due to an efficient remobilization and translocation of P (Vance et al., 2003; Wang et al., 2010). Adaptive strategies to increase PAE and PUE include secretion of phosphatases and organic acids, modification of root architecture, formation of mycorrhizal symbiosis and induction of the expression of high-affinity phosphate transporter (PHTs) (Baker et al., 2015; Campos et al., 2018; Heuer et al., 2017; Peret et al., 2014). At a global scale, P deficiency and Al toxicity often coexist in acid soils and cannot be considered as independent factors, since both interact strongly through chemical and biochemical reactions (Kochian et al., 2004). Thus, improving PAE and PUE in crops have to relate to other major stresses, such as Al toxicity. The purpose of this review is to illustrate a current understanding of how plants have developed strategy responses to low P availability and Al toxicity, mostly focusing on the mechanism of Pi transport.

2.2 Plant responses to low phosphate

Plants exposed to P deficiency activate a range of mechanisms that either result in an increased acquisition of P from the P-limited soil (PAE) or in a more efficient use of internal P (PUE) (Vance et al., 2003; Wang et al., 2010). In general, some species or genotypes express one or many of these mechanisms, depending on the genotype, stage of development, and the degree and duration of P deficiency (López-Bucio et al., 2003; Ramaekers et al., 2010).

Changes of root morphology and architecture, increased root exudation of organic acid anions (OAs) and induction of Pi transporters are local responses to improve PAE in plants (Veneklaas et al., 2012). Likewise, changes in many metabolic pathways such as remobilization of Pi from older to young source tissues, remodeling of sugar and lipid

metabolism and the use of alternative ATP-independent pathways are adaptive plant responses to improve internal PUE (Plaxton and Tran, 2011).

Under P deficiency, modifications in root system architecture (RSA) result in a shallow root system bearing more and longer lateral roots to increase exploration capacity as well as denser root hairs, which increases root surface area (López-Bucio et al., 2003; Magalhaes et al., 2017). Root architecture responses to Pi availability vary significantly among and within species due to genetic determinants and environmental factors (Lynch and Brown, 2001). Moreover, alterations in RSA are associated with alternative strategies to cope with Pi deficiency such as establishment of symbiotic associations with fungi (Smith et al., 2011). Complementing an optimized root architecture, plants increase root exudation of organic acid anions (OAs) to enhance Pi acquisition (Ryan et al., 2001; Wang et al., 2010). The release of organic anions such as malate, citrate and oxalate into the rhizosphere increases P availability trough mobilizing sparingly soluble mineral P and organic P sources (Kochian et al., 2004; Richardson et al., 2009). Then, the subsequent induction of Pi transporters acts in efficient Pi uptake from rhizosphere. Thus, a higher PAE is also attributed to efficient P uptake from the soil by phosphate (Pi) transporters (Baker et al., 2015; Nussaume et al., 2011; Ramaekers et al., 2010). These proteins also play a crucial role in remobilization of Pi within the plant, which indicates their participation in the long-distance Pi deficiency response.

Pi-starved plants also show metabolic responses to improve P utilization efficiency (PUE). The efficient remobilization of internal Pi from vacuolar stores and redistribution of Pi from older to young source tissues are crucial to maintaining cytosolic Pi homeostasis under P deficiency conditions (Baker et al., 2015). Likewise, in order to reduce the demand

of P in lipid metabolism, plants conserve Pi by replacing their membrane phospholipids with dihexosylglycerolipid, sulfolipids and galactolipids (Plaxton, 2004; Byrne et al., 2011; Nakamura, 2013). Another strategy to reduce and recycle P is the use of alternative glycolysis pathway by activation of phosphoenolpyruvate carboxylase (PEPC)-malate (Kondracka and Rychter, 1997; Plaxton and Tran, 2011). Although the enhancement of PAE and PUE through breeding approaches is a powerful strategy that increases P efficiency in crops, the gene regulation networks controlling Pi homeostasis in crops are still poorly understood.

2.3 Phosphorus uptake and transport

Plants can only take up and metabolize P as inorganic phosphate (Pi), either as $H_2PO_4^-$ or HPO_4^{2-}, depending on the pH of the growing medium. Pi is the most limited nutrient due to its low diffusion rate (from 10−12 to 10−15 m^2 s^{-1}) and highly fixation by soil minerals (Menezes-Blackburn et al., 2018; Richardson et al., 2009; Smith et al., 2003). Thus, Pi uptake by the plant is a complex process, which needs to excessive application of Pi-fertilizer to maintain crop growth and yield in modern agriculture. However, only about 10–30% of the applied P-fertilizers is used by plants (Raghothama and Karthikeyan, 2005), being the rest lost due to fixation in the soil and conversion by microorganism into organic forms (López-Arredondo et al., 2014). Therefore, improved plant nutrition to maximize phosphate acquisition efficiency (PAE) and phosphate utilization efficiency (PUE) it's become necessary to sustain agricultural productivity.

Transport of ions across the plasmalemma (the boundary between the apoplasm and the symplasm) is a critical step that mediates and regulates the uptake of nutrients by plants (Schachtman et al., 1998; Shen et al., 2011; Baker et al., 2015). Early studies of Epstein in

the 1950s (Epstein, 1953) demonstrated that kinetics of ion absorption in barley roots were in close agreement with classical enzyme kinetics and elucidated that the ion uptake by plants could be described through two phases (i) a high-affinity system operating at low external nutrient concentrations and (ii) a low-affinity system operating at higher external concentration, being both phases mediated by proteins embedded in the plasmalemma.

Pi concentration in plant tissues has been measured as 5–20 mM, whereas the available level of Pi in soils is typically less than 10 μM (Shen et al., 2011), resulting in Pi uptake against a steep concentration gradient. An energy mediated cotransport process, driven by a proton gradient generated by plasma membrane H^+-ATPase has been proposed for Pi uptake in plants (Muchhal and Raghothama, 1999; Smith et al., 2003). Pi absorption is accompanied by H^+ influx, with a stoichiometry of 2 to $4H^+$ per $H_2PO_4^-$ transported (Raghothama and Karthikeyan, 2005; Schachtman et al., 1998).

2.4 Phosphate transporters in plants

Efficient Pi transport systems are essential for plants to mediate uptake from the soil and to maintain adequate levels of cellular Pi (Raghothama and Karthikeyan, 2005; Smith et al., 2003; Versaw and Garcia, 2017). The Phosphate transporters (PHTs) are integral membrane proteins responsible for Pi uptake, translocation and remobilization within the plants (Guo et al., 2011; Nussaume et al., 2011). The identified plant PHTs can be classified into four families: PHT1, PHT2, PHT3 and PHT4. These PHTs are responsible for phosphate transport across the membranes of cells, chloroplasts, mitochondria and Golgi-compartment, respectively (Rausch and Bucher, 2002). Members of the PHT1 family not only play a key role in Pi uptake from the soil solution but also in internal root-to-shoot distribution (López-Arredondo et al., 2014; Ayadi et al., 2015).

The PHT1 transporters in plants were initially identified based on their similarity to the Pi transporter from the yeast *Saccharomyces cerevisiae* (PHO84) (Bun-Ya et al., 1991) and fungal H^+/Pi symporters from *Neurospora crassa* (Versaw, 1995) and *Glomus versiforme* (Harrison and Van Buuren, 1995). Two separate approaches were used for their identification in *Arabidopsis thaliana*, either by the heterologous complementation of the yeast PHO84 mutant by plant cDNA libraries (Muchhal et al., 1996) or by the identification of *Arabidopsis* ESTs closely related to these proteins (Mitsukawa et al., 1997; Smith et al., 1997). Their function as Pi transporters was confirmed in the plant by the analysis of *Arabidopsis thaliana* mutants through reverse genetic approaches (Misson et al., 2004; Shin et al., 2004). After the first PHT1 genes (*AtPTH1* and *AtPTH2*) were cloned from *Arabidopsis*, many PHT1 homologs have been identified and characterized in several crop species (Table 1). In this context, 9 and 13 PHT1s were identified in the *Arabidopsis* and rice genomes and named as AtPTH1-9 and OsPTH1-13, respectively (Mudge et al., 2002; Paszkowski et al., 2002; Ai et al., 2008; Ye et al., 2015).

Table 1. Phosphate transporter members from PHT1 family in important crops

Plant species	***PHT1* gene**	Localization and expression pattern	Reference
Glycine max	*GmPHT1* to *GmPHT12*	Expressed in roots and shoots under low and normal P conditions. Responsible for direct Pi uptake from soil and Pi translocation within the plant	Wu et al., 2011 Fan et al., 2013
Oryza sativa	*OsPHT1, OsPHT2, OsPHT3, OsPHT6, OsPHT9, OsPHT10*	Expressed in roots and shoots under low and normal P conditions. Responsible for direct Pi uptake from soil and Pi translocation within the plant	Paszkowski et al., 2002 Ai et al., 2008 Sun et al., 2012
Oryza sativa	*OsPHT11*	Expressed in mycorrhizal roots. Linked exclusively to mycorrhizal colonization	Paszkowski et al., 2002
Oryza sativa	*OsPHT8*	Expressed in various tissues and organs from roots to seeds under low and normal Pi conditions. Responsible for direct Pi uptake from soil and Pi distribution from source to sink organs	Jia et al., 2011 Li et al., 2015
Oryza sativa	*OsPHT4*	Expressed in various tissues and organs from roots to seeds under low and normal Pi conditions. Responsible for direct Pi uptake from soil, in embryo development and seed germination	Zhang et al., 2015 Ye et al., 2015
Triticum aestivum	*TaPHT1, TaPHT2, TaPHT4, TaPHT6, TaPHT7*	Expressed in roots and shoots under low and normal P conditions. Responsible for direct Pi uptake from soil and Pi translocation within the plant.	Miao et al., 2009 Guo et al., 2014 Liu et al., 2013 Teng et al., 2017
Hordeum vulgare	*HvPHT6*	Expressed in roots and shoots under low and normal P conditions. Responsible in remobilization of stored Pi out of old leaves into younger organs	Rae et al., 2003 Huang et al., 2011
Hordeum vulgare	*HvPHT1, HvPHT2, HvPHT3*	Expressed in roots under low P conditions. Responsible for direct Pi uptake from soil	Smith et al., 1999 Preuss et al., 2011
Zea mays	*ZmPHT1, ZmPHT2, ZmPHT3, ZmPHT6*	Expressed in roots and shoots under low and normal P conditions. Responsible for Pi uptake from soil, Pi translocation in shoots and Pi uptake during pollen tube growth	Nagy et al., 2006 Liu et al., 2016

2.4.1 Structure and affinity of Pi transporters

Plant Pi transporters are proteins of similar size (approximately 518-587 amino acids) and show several conserved phosphorylation and N-glycosylation sites implicated in their post-transcriptional control (Karthikeyan, 2002; Jain et al., 2012). All members of the plant PHT1 family and the above-mentioned fungal Pi transporters exhibit high sequence similarity with each other (Muchhal and Raghothama, 1999; Zhang et al., 2014; Gu et al., 2016). The polypeptides of plant Pi transporters generally contain 12 membrane-spanning domains (MSD) separated into two groups of six by a large charged hydrophilic region (Fig.1) (Smith, 2002; Vance et al., 2003 Guo et al., 2011). These Pi transporters belong to the major facilitator superfamily (MFS), which are generally symporters or antiporters driven by proton or sodium gradients (Raghothama and Karthikeyan, 2005; Baker et al., 2015).

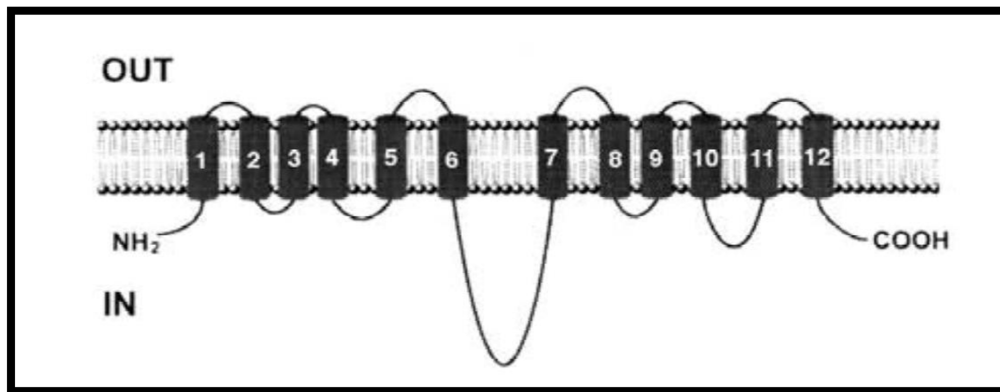

Figure 1. Topology of a typical plant transporter protein responsible for the movement of phosphate from the external soil solution across the plasmalemma and into the cytoplasm of root cells. They harbor 12 membrane spanning domains arranged in a ’6+6’ configuration (Smith, 2002).

Kinetic characteristics (K_m value) are important factors for understanding the regulatory functions of PHTs members in Pi uptake and/or Pi translocation across tissues (Guo et al., 2014). The affinities of plant PHT1s have been established by heterologous expression in the *pho84* mutant of *S. cerevisiae* and/or *Xenopus oocytes* (Baker et al., 2015). The plant PHT1 transporters show a range of measured affinities for Pi and are generally divided into high- and low-affinity transporters (Smith et al., 2003; Nussaume et al., 2011). The high-affinity phosphate transporters are usually expressed at low Pi concentrations and have a K_m ranging from 3 to 10 µM, whereas the low-affinity system function at high Pi availability having a K_m ranging from 50 to 300 µM or in mM range (Lopez-Arredondo et al., 2014). Interestingly, the low-affinity transport system appears to be expressed constitutively in plants, whereas the high-affinity uptake system is strongly enhanced during Pi deficiency (Furihata et al., 1992; Park et al., 2007; Sun et al., 2012). However, recent studies suggest that Pi transporters might operate with a dual-affinity system (Ayadi et al., 2015). The existence of dual affinities of plant nutrient transporters have been determined for *Arabidopsis* K+ transporter and the nitrate NRT1;1 transporter (Fu and Luan, 1998; Sun et al., 2014). Changes in the affinities of the PHT1s could occur due to post-translational modifications, such as phosphorylation and dephosphorylation (Ceasar et al., 2016). Further studies on the structure and function of PHT1 transporters are required to determine the natural regulatory process *in planta*.

2.4.2 Localization and expression patterns of Pi transporters

Different members of the PHT1 gene family show varying patterns of expression with respect to tissue and concentration of Pi (Table 1) (Gu et al., 2016; Liu et al., 2017). The Pi transporters encoded by members of the *Pht1* gene family are predominantly expressed in

root epidermal cells and root hairs and usually act as high-affinity Pi transporters under P deficient conditions (Kavka and Polle, 2016; Versaw and Garcia, 2017). However, *Pht1* transcripts are also detected in leaves, stems, cotyledons, flowers and seeds (Table 2), suggesting their involvement not only in Pi uptake by roots, but also in internal root-to-shoot distribution and translocation within the plant (Ai et al., 2009; Chen et al., 2014; Sun et al., 2012; Li et al., 2015). Thus, Pi transporters could act independent of the P status to maintain Pi homeostasis (Huang et al., 2011; Liang et al., 2014).

Several *Pht1* genes have been identified that are specifically induced and expressed in cortical root cells harboring arbuscular mycorrhizal (AM) (Yang et al., 2012; Ceasar et al., 2014; Liu et al., 2016; Ferrol et al., 2019). In this case, plants take up P from the soil through the “mycorrhizal pathway”. The AM fungus first acquires P from the soil solution by a PHT1 transporters that is expressed in the extraradical mycelium, Pi is then translocated from the extraradical to the intraradical mycelium and finally transferred to the plant via AM-inducible plant PHT1 transporters (Javot et al., 2007; Walder et al., 2015). The AM-induced *PHT1* genes have been identified in several crop species, including potato (Rausch et al., 2001), maize (Liu et al., 2016), barley (Christophersen et al., 2009) rice (Glassop et al., 2007; Yang et al., 2012; Chen et al., 2013) and wheat (Teng et al., 2017). These specific Pi transporters are generally present at much higher levels in AM roots than other Pi transporters (Javot et al., 2007). In the plant, the direct P uptake pathway and mycorrhizal pathway contribute positively to improve P uptake under P deficient conditions.

Table 2. Expression patterns of the nine PHT1 transporters from *Arabidopsis thaliana*, based on a compilation of histological and transcriptomics data, combining information for both +P and –P conditions. (Adapted from Nussaume et al., 2011).

Spatial distribution	Phosphate transporter	Reference
Silique abscission zone	*PHT4*[a]	Karthikeyan et al., 2002-2009
Axillary buds	*PHT2*[a] *PHT4*[a]	Mudge et al., 2002
Hydathodes Leaf Vascular tissues	*PHT1*[a] *PHT3*[ab] *PHT4*[ab]* *PHT5*[ab]	Karthikeyan et al., 2002-2009 Mudge et al., 2002 Misson et al., 2004
Old primary root	*PHT2*[a] *PHT3*[a] *PHT4*[a]	Karthikeyan et al., 2002-2009 Mudge et al., 2002 Misson et al., 2004
Laretal root	*PHT1*[abc]* *PHT2*[abc]* *PHT3*[abc], *PHT4*[abc]*	Karthikeyan et al., 2002-2009 Mudge et al., 2002 Misson et al., 2004
Root cap /Root tip	*PHT1*[ac] *PHT3*[ab] *PHT4*[abc]*	Karthikeyan et al., 2002-2009 Mudge et al., 2002 Misson et al., 2004
Root hair zone	*PHT1*[abc]* *PHT2*[abc]* *PHT3*[abc] *PHT4*[abc]* *PHT5*[ab] *PHT7*[ab]* *PHT8*[ab] *PHT9*[ab]	Karthikeyan et al., 2002-2009 Mudge et al., 2002 Misson et al., 2004

Senescing leaves	*PHT4*[b]* *PHT5*[abc]	Nagarajan et al., 2011 Mudge et al., 2002
Pollen/anthers	*PHT3*[b] *PHT4*[ab]* *PHT6*[abc] *PHT7*[abc]*	Karthikeyan et al. 2002-2009 Mudge et al., 2002
Flower abscission zone	*PHT4*[ab]*	Karthikeyan et al. 2002-2009 Mudge et al., 2002
Young floral buds	*PHT5*[ab] *PHT6*[ab]	Mudge et al., 2002

[a] Histological studies (promoter::GFP/GUS fusions or *in situ* hybridization).
[b] Transcriptomics studies corresponding to a summary of several data sets presented on thee FP server (Winter et al., 2007).
[c] Due to the design of most transcriptomic chips,it is not possible to distinguish the expression patterns of *PHT1/PHT2* as well as *PHT4/PHT7.*
* When a *PHT1* transporter is predominantly (but not exclusively) expressed in one or several tissues.

2.5 Phosphate uptake and aluminum toxicity in acid soils

Al toxicity and P deficiency very often coexist in soils and cannot be considered as independent factors, since both interact strongly through chemical and biochemical reactions (Kochian et al., 2004; Pariasca-Tanaka et al., 2009). The solubility of Al in neutral and alkaline soils is too low to be toxic to plants, but under acidic conditions, it becomes soluble to the toxic monomeric form Al^{3+}. Al toxicity (Al^{3+}) causes the inhibition and reduction in root growth and reacts with available P and converts it into insoluble aluminum-phosphate complex, which is not available to plants (Kochian et al., 2004).

Consequently, several studies have been carried out to investigate Al and P interactions in plant nutrition. However, the influence of P nutrition on Al phytotoxicity is still controversial. In this context, previous studies have reported that P addition could alleviate

Al toxicity in plants (Liao et al., 2006). This alleviation effect is commonly based on two possible mechanisms: (i) direct interactions through the formation of Al–P complexes in the soil solution and/or in the root surface, decreasing accumulation of Al in leaves (Gaume et al., 2001; Nakagawa et al., 2003) and (ii) indirect effects, through improved root growth and nutrient uptake and increasing immobilization of Al in roots through enhancing secretion of organic acid anions (Yang et al., 2011; Chen et al., 2012).

Moreover, molecular approaches have shown that genes involved in Al resistance are also associated with PAE and PUE in crops (Kochian et al., 2004; Sun et al., 2008; Ruíz-Herrera and López-Bucio, 2013). The firts genes indentified in Al resistance were those that encode malate (ALMT) and citrate (MATE) efflux transportes (Kochian et al., 2015). Greater exudation of organic acids into the rhizosphere help to P mobilize from the soil, favoring P uptake (Delhaize et al., 2009). In fact, P deficiency up-regulated the ALMT1 expression in *Arabidopsis* (Müller et al., 2015). Thus, an efficient defence mechanism against Al toxicity will also serve as an efficient mechanism of P acquisition in plants (Yang et al., 2011). Therefore, Al-tolerant plant genotypes could use P more effectively than the Al-sensitive genotypes.

Improved P efficiency can only be achieved if plants are tolerant of Al toxicity (Heuer et al., 2017). In fact, recent studies have speculated a pleiotropic mechanism between Al resistance genes and P deficiency responses (Magalhaes et al., 2018; Bernardino et al., 2019). Further work should focus on explore Al resistance genes to improve crop P efficiency crops on acidic soils.

2.6 Conclusions and perspectives

In recent years, knowledge of molecular and biochemical mechanisms of Pi uptake and transport in plants has greatly increased. However, a higher number of studies are required to obtain efficient genotypes in P uptake and utilization to improve P-efficiency in crops and reduce dependence on nonrenewable inorganic P resources. Understanding the mechanism involved in sensing of P deficiency could facilitate selection and breeding to increase crop production in P-stressed soils. Although several Pi transporters have been identified in plants, it is necessary to increase knowledge on regulation of Pi transporters under other stresses, such as Al toxicity. Finally, the post-transcriptional regulation of Pi transporters, including the control of protein translation, cell targeting, transport activity and proteolysis offers great potential for future research.

CHAPTER III

Functional properties and expression profiling of two phosphate transporters in response to phosphorus starvation in ryegrass (Lolium perenne L.)

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Functional properties and expression profiling of two phosphate transporters in response to phosphorus starvation in ryegrass (*Lolium perenne* L.)

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Abstract

Members of the phosphate transporter family 1 (PHT1) are integral membrane proteins that operate in phosphate (Pi) uptake, distribution and remobilization within plants. In this study, we report on the functional characterization and expression of two PHT1 members from ryegrass plants (*Lolium perenne*) and determine their roles in the specificity of Pi transport. The expression level of *LpPHT1;4* was strongly influenced by phosphorus (P) status, being higher under P-starvation condition. In contrast, the expression level of *LpPHT1;1* was not correlated with P supply. Yeast mutant complementation assays showed that *LpPHT1;4* can complement the growth defect of the yeast mutant *Δpho84* under Pi deficient conditions, whereas the yeast mutant expressing *LpPHT1;1* was not able to restore growth. Phylogenetic and molecular analyses indicated high sequence similarity to previously identified PHT1s from other species in the Poaceae. These results suggest that LpPHT1;1 functions as a low-affinity Pi transporter, whereas LpPHT1;4 acts as a high-affinity Pi transporter to maintain Pi homeostasis under stress conditions in ryegrass plants. This study will form the basis for the long term goal of improving the phosphate use efficiency of ryegrass plants.

Keywords: *Lolium perenne* L, phosphate starvation; phosphate transporter genes; heterologous expression.

3.1 Introduction

Low phosphorus (P) availability is considered the principal problem for agricultural production in many soils (Wang et al. 2010; Zhang et al. 2014). Phosphate (Pi) is central to plant metabolism, playing key roles in photosynthesis, energy metabolism and environmental stress response (Plaxton and Tran, 2011; Chien et al. 2018; Ham et al. 2018). Phosphate fertilizers used to mitigate P deficiency are extremely inefficient because only 10–30% of applied P is used by plants (Vance et al. 2003) the remainder is rendered unavailable by the formation of insoluble complexes of P with cations such as aluminium (Al), iron (Fe) or calcium (Ca), as well as P adsorption by mineral surfaces and organic matter, which decreases P availability for plants (Kochian et al. 2004; López-Arredondo et al. 2017). Moreover, rock phosphate reserves used to produce P-fertilizers are a non-renewable resource and limitation of supply could impact both agricultural productivity and food security (Cordell and White, 2014; Sattari et al. 2016).

Plants exposed to P deficiency activate a range of morphological, physiological, biochemical and molecular mechanisms that result in increased uptake of phosphate (Pi) from the soil solution as well as more efficient use of internal Pi leading to improved growth and yield (Plaxton and Tran, 2011; Chien et al. 2018). Mechanism underlying these responses include changes in root morphology and architecture (Peret et al. 2014), secretion of organic anions and phosphatases (Veneklaas et al. 2012; Chien et al. 2018) and enhanced expression of Pi transporters (Versaw and Garcia, 2017; Wang et al. 2017). Many of these adaptation mechanisms have been attributed to P-efficient genotypes, which are characterized by higher phosphorus acquisition efficiency (PAE) and phosphorus use efficiency (PUE) (Wang et al. 2010; Veneklaas et al 2012; Gu et al. 2016; Maharajan et al.

2018). PAE is referred to as the capacity of plant roots to acquire P from the soil and PUE is related to the amount of biomass (fruits, forage and grains) produced per unit P due to efficient remobilization and translocation of P within the plant (Wang et al. 2010; López-Arredondo et al. 2014). In this context, the efficient uptake and translocation of Pi as well as the re-use of stored P in plants are ascribed to a coordinated function of Pi transporters under P limitation (Huang et al. 2011; Gu et al. 2016; Wang et al. 2018). Although many Pi transporters have been identified in several species (Muchhal and Raghothama, 1999; Ye et al. 2015; Liu et al. 2016; Teng et al. 2017), there is a dearth of studies regarding the characterization and function of Pi transporters and the impact that these have on the PAE and PUE of forage plants.

Members of phosphate transporter family 1 (PHT1) are integral plasma membrane proton-coupled Pi-H^+-symporters that differ in affinity for Pi and are differentially expressed in response to environmental Pi availability and internal Pi status (Wang et al. 2017). Low-affinity Pi transporters have K_m values in the millimolar range, whereas high-affinity Pi transporters operate at the micromolar Pi concentration range (Smith et al. 2003; Raghothama and Karthikeyan, 2005). Both Pi transporter systems show a diversity of expression patterns in different plant tissues, indicating that *PHT1* family members play key roles in direct Pi uptake from the soil as well as in Pi distribution and remobilization within the plant (Nussaume et al. 2011; López-Arredondo et al. 2014; Baker et al. 2015). Additionally, their expression profiles are genotype-dependent and also related with the stage of development and the degree and duration of the P deficiency period (Ai et al. 2009; Sun et al. 2012; Wang et al. 2018).

Perennial ryegrass (*Lolium perenne* L.) is the most important forage plant grown in Chilean Andisols (Mora et al. 2006). These soils are characterized by low pH and low Pi availability, resulting in limited yield and quality of forage. Therefore, improvement of Pi uptake and P utilization efficiency in ryegrass is a central approach to reduce demand for P fertilizers for production in the region. However, the molecular mechanisms regarding the response of ryegrass to P deficiency have not been extensively explored (Byrne et al. 2011; Rasmussen et al. 2014). In our previous work, we found that phosphate transporters (*LpPHT1;1/4)* from ryegrass were up-regulated by long-term Pi deficiency treatments (Parra-Almuna et al. 2018). Further identification and functional analyses of ryegrass PHT1 family will elucidate the mechanisms underlying PAE and PUE in ryegrass and aid in the selection of high-producing genotypes for use in P-limiting soils. In this study, we performed for the first time the functional and expression analysis of the *LpPHT1;1* and *LpPHT1;4* genes to determine their roles in maintaining Pi homeostasis in perennial ryegrass.

3.2 Materials and Methods

3.2.1 Plant material and growth conditions

Seeds from ryegrass (*Lolium perenne* L.; cv. Nui) were rinsed with 2% v/v sodium hypochlorite for 15 min, washed several times with distilled water and then germinated on moist filter paper in a growth chamber at 21 ºC. After 10 days of germination, the seedlings were transferred into 7-L plastic pots filled with a continuously aerated basal nutrient solution proposed by Taylor and Foy (1985). After an acclimation period of 7 days, a time-course experiment at 6, 12, 24, 48 and 72 h was performed under P-optimal (100 µM) and P-starvation (0 µM) conditions. Potassium (K) was supplied as KCl to maintain equal concentrations of K in solution. During the experiment, the pH of the nutrient solution was adjusted to 6.0 and checked daily. Root and shoot samples were collected at each harvest time and quickly stored for later analyses.

3.2.2 Phosphate transporters gene expression analyses

The expression patterns of phosphate transporter genes (*LpPHT1;1* and *LpPHT1;4*) were evaluated in shoots and roots from ryegrass plants grown as above. Total RNA was extracted using the NucleoSpin® RNA Plant Kit (Macherey-Nagel GmbH and Co. KG, Düren, Germany). The concentration and purity of RNA were checked spectrophotometrically using a NanoDrop™ (Thermo Scientific, Wilmington, DE, USA). The RNA concentration was adjusted to 50 ng μL^{-1} for the synthesis of the first strand of cDNA using a High-Capacity cDNA Reverse Transcription Kit (Invitrogen, Carlsbad, CA, USA). The expression level of genes was analysed by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) by using a qPCR Step One Plus

(Applied Biossystems, Foster City, CA, USA) and the Brilliant II SYBR® Master Mix Kit (Agilent, Santa Clara, CA, USA). Specific primers to amplify *LpPHT1;1* (GeneBank accession MF966998) and *LpPHT1;4* (GeneBank accession MF966999) genes were obtained from (Parra-Almuna et al. 2018). Two housekeeping genes, eukaryotic elongation factor 1 alpha *eEF1α*(h) (GeneBank accession GO924753) and *eEF1α*(s) (GeneBank accession GO924801) were used as internal control (Table 1). Cycling conditions were 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s, 59 °C for 1 min, and 72 °C for 5 s. The normalized values were subjected to a $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All qRT-PCR reactions were determined on three biological replicates with three technical replicates.

Table 1. Primer sequences used for relative expression analysis and TOPO® Cloning procedure of phosphate transporter genes from *Lolium perenne* L.

Gene name*	Forward primer (5´- 3´)	Reverse primer (5´- 3´)	Utilization
LpPHT1;1	CCTGGGATTGCTTTCTCAC	TGGTTGCGTCATCGTCATAG	qRT-PCR
LpPHT1;4	AACCAGCGTACCAGGACAAC	GAGGATGATGCGCCAGAC	qRT-PCR
eEF1α(h)	ATGTCTGTTGAGCAGCCTTC	GCGGAGTATATAAAGGGGTAGC	qRT-PCR
eEF1α(s)	CCGTTTTGTCGAGTTTGGT	AGCAACTGTAACCGAACATAGC	qRT-PCR
LpPHT1;1	CACCATGGCGGGTGAACAGCTCAA	CTAGGCACCAGCAGGGGTAA	pENTR™/D-TOPO® vector
LpPHT1;4	CACCATGGCGCGGTCGGAGCAGCA	CTACGCCATCGACGGCTGC	pENTR™/D-TOPO® vector

*: *LpPHT1;1*; Phosphate transporter 1, *LpPHT1;4*; Phosphate transporter 4; eEF1α; Eukaryotic elongation factor 1 alpha.

3.2.3 Phosphorus concentration analysis

Shoot and root samples (0.25 g) were ashed in a muffle at 500 ºC for 10 h and afterward digested with 2 M HCl and filtered to determine P concentrations. The P concentration was measured by the molybdovanadate method (Sadzawka et al. 2004). A molybdovanadate solution was prepared by mixing equal parts 8 mM NH_4VO_3, 1.5 mM $(NH_4)_6Mo_7O_{24}•4H_2O$ and 1.5 M HNO_3. Filtered digestion solutions were mixed with 4 ml of the molybdovanadate solution, maintained for 1 h at room temperature and then spectrophotometrically analysed at 466 nm. Two reference samples with known P concentration were included for each analytical run as internal controls.

3.2.4 Plasmid construction for yeast complementation assays

The open reading frame (ORF) of phosphate transporters (*LpPHT1;1/4*) were obtained by PCR amplifications of ryegrass root cDNA using gene specific primers based on the sequences described by Parra-Almuna et al. (2018), (Table 1). PCR products were visualized on an agarose gel to verify amplicon and then purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel GmbH and Co. KG, Düren, Germany). The purified PCR-products were cloned into pENTR™/D-TOPO® (Invitrogen) gateway entry vector following the manufacturer´s instructions. Constructs were confirmed by sequencing, and then recombined into gateway destination vectors pDRf1-GW (Addgene plasmid #36026) via LR clonase enzyme mix (Invitrogen).

3.2.5 Yeast complementation assays

The yeast wild type (WT) *Saccharomyces cerevisiae* BY4741 strain (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) and the yeast Pi uptake-defective mutant *Δpho84* (*MATa;*

his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YML123c:: kanMX4) strains were transformed with the constructs pDRf1-*LpPHT1;1* and pDRf1-*LpPHT1;4* according to Gietz and Schiestl (2007). Transformed yeasts were selected on synthetic defined medium without uracil (SD-ura) by autotrophy to uracil. Plates were incubated at 28 ºC for 2 days.

The pDRf1-*LpPHT1;1* and pDRf1-*LpPHT1;4* constructs were tested for their ability to complement the growth defect of the yeast Pi transport-deficient strain *Δpho84*. The WT strain and the yeast mutant strain *Δpho84* carrying the empty vector were used as positive and negative controls, respectively. Transformants and control cells were precultured in liquid SD-ura without phosphate medium (Formedium, Norfolk UK), supplemented with a basal Pi concentration from 1mM (KH_2PO_4) overnight at 28 ºC in an orbital shaker. Precultured yeasts were transferred to fresh medium, adjusted to an OD_{600} value of 0.2–0.3 and grown to exponential phase (OD_{600}~0.8). Cells were harvested by centrifugation at 4000 rpm for 5 min and washed three times with sterile deionized water and then resuspended in the SD-ura without phosphate medium and adjusted to a final OD_{600} of 1. Then, 5 µL of serial 1:10 dilutions were spotted on SD-ura without phosphate medium agar plates containing two different Pi concentrations (10 and 0.1 mM as KH_2PO_4). Plates were maintained at 28 °C for 3 days.

A time-course experiment for growth and Pi transport activity of the pDRf1-*LpPHT1;1* and pDRf1-*LpPHT1;4* expressing cells was carried out in selective media containing 60 µM of Pi at 4, 8, 12, 24 and 32 hours. At each time point, yeast growth (OD_{600}) and Pi concentration in the medium were determined. The malachite green phosphate assay was used to determine the Pi concentration in the medium according to Ohno and Zibilske (1991). For each experiment, three independent cultures were used.

3.2.6 Bioinformatic analysis

Multiple sequence alignment of phosphate transporters was carried out using ClustalW (Thompson et al. 1994) and evolutionary tree was built using MEGA software version 6.0 (Tamura et al. 2013). The evolutionary distances were calculated for amino acid sequences using the Poisson correction method (Zuckerkandl and Pauling, 1965), then a phylogenetic tree was constructed using Neighbor-Joining method (Saitou and Nei, 1987). Tree robustness was evaluated by using 1000 Bootstrap replications (Felsenstein, 1985).

An evolutionary conservation analysis using multiple aligned phosphate transporters and a three dimensional structure of *Lolium perenne* PHTs proteins obtained from Swiss-Model server (Waterhouse et al. 2018), were used to calculate position-specific conservation scores by the empirical Bayesian algorithms (Mayrose et al. 2004) in ConSurf (Ashkenazy et al. 2010).

3.2.7 Statistical analysis

The experimental data were evaluated by analyses of variance (ANOVA) using the software SigmaPlot (v12.0) (Jandel Scientific Software, San Rafael, CA, USA). Comparisons of means were performed using Tukey's test at $p \leq 0.05$. The relationship between P status and molecular parameters were tested by Pearson correlation analysis.

3.3 Results

3.3.1 Ryegrass growth, P status and relative expression levels of PHT1s

During the time-course, plants grown under Pi-starvation had significantly lower ($p \leq 0.05$) shoot P concentrations by 6 h of starvation compared with plants grown in the presence of

normal Pi-supply. Similarly, after 6 h of Pi-starvation, the root P concentration declined significantly compared with plants grown under normal Pi-supply ($p \leq 0.05$; Fig. 1).

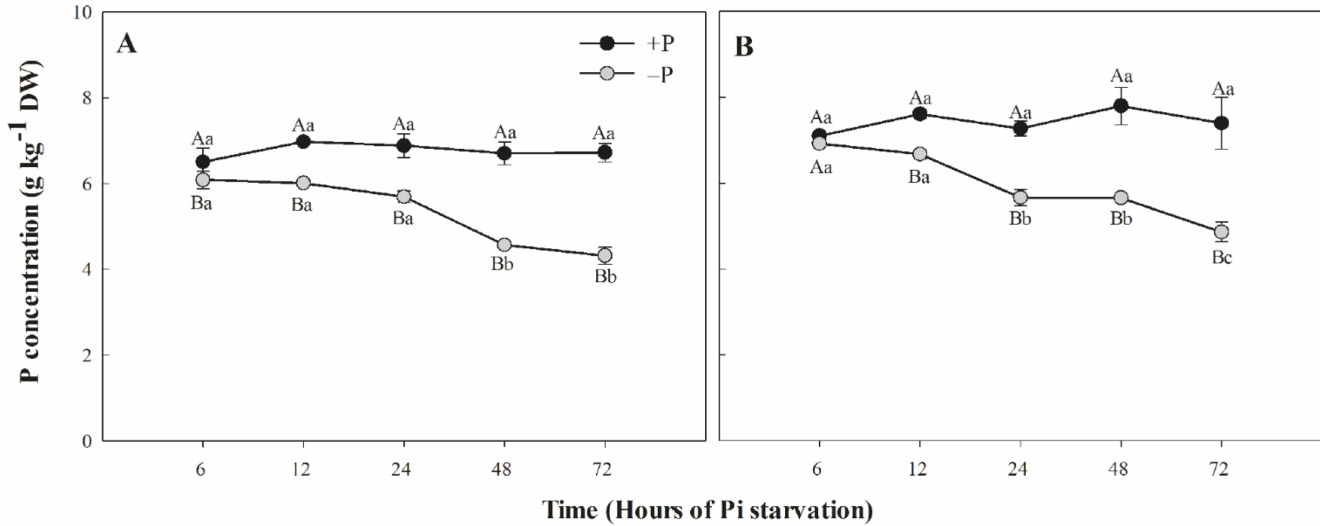

Figure 1. Phosphorus (P) concentration in shoots (A) and roots (B) of the Nui cultivar under P-optimal (+P, 100 μM) and P-starvation (–P, 0 μM) conditions at different times. Data are the means ± SD of three replicates. Different uppercase letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) between P treatments at each time. Different lowercase letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) between times for the same P treatment.

In order to study the regulation of PHT1 genes in response to this short-term Pi starvation period, we evaluated the expression of the ryegrass phosphate transporters *LpPHT1;1* and *LpPHT1;4*. In shoots, both Pi transporters (*LpPHT1;1/4*) showed the highest expression level at 72 h of P-starvation, whereas the highest expression levels in roots were found at 12 h and 24 h for *LpPHT1;1* and *LpPHT1;4*, respectively (Fig. 2). The expression of *LpPHT1;4* was up-regulated under P-starvation at all time points and was significantly

negatively correlated with P concentration in shoots and roots (Table 2), indicating that this transporter was highly influenced by P status. In contrast, the up-regulation of *LpPHT1;1* occurred only at 72 h in shoots and at 12 and 48 hours in roots under P-starvation (Fig. 2), and was not correlated with shoot or root Pi concentrations (Table 2).

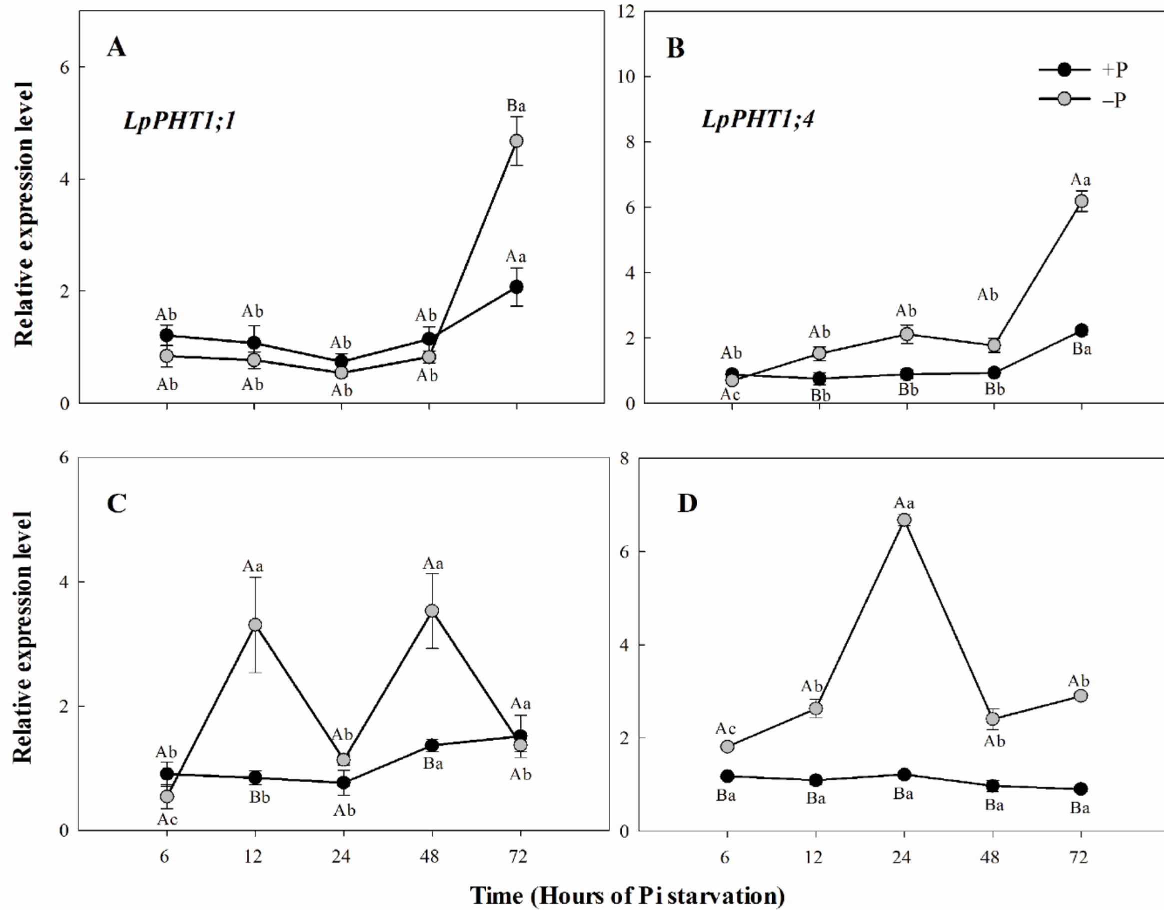

Figure 2. The relative expression levels of phosphate transporter genes *LpPHT1;1* in shoots (A) and roots (C) and *LpPHT1;4* in shoots (B) and roots (D) of the Nui cultivar under P-optimal (+P, 100 μM) and P-starvation (–P, 0 μM) conditions. The expression levels were normalized in relation to eEF1α(h) and eEF1α(s) gene expression. Data are the means ± SE of three replicates. Different uppercase letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) between P treatments at each time. Different

lowercase letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) between times for the same P treatment.

Table 2. Pearson's correlation matrix among chemical and molecular parameters of the Nui cultivar under different P supplies at each harvest time (6, 12, 24, 48 and 72 h).

	PS	PR	PU	ReS1	ReR1	ReS4	ReR4
PS	1.00						
PR	0.88**	1.00					
PU	0.59**	0.63**	1.00				
ReS1	0.35	0.35	0.24	1.00			
ReR1	-0.36	-0.32	-0.23	-0.35	1.00		
ReS4	-0.70**	-0.74**	-0.12	-0.24	0.11	1.00	
ReR4	-0.47*	-0.66**	-0.58**	-0.35	0.14	0.28	1.00

PS phosphorus concentration in shoots; **PR** phosphorus concentration in roots; **PU** phosphorus uptake in shoots; **ReS1** Relative expression level of *LpPHT1;1* in shoots; **ReR1** Relative expression level of *LpPHT1;1* in roots; **ReS4** Relative expression level of *LpPHT1;4* in shoots; **ReR4** Relative expression level of *LpPHT1;1* in roots. Pearson correlation coefficients (r) were calculated from three replicates of each sampling. $*P < 0.05$; $**P < 0.01$.

3.3.2 Heterologous expression of PHT1s in yeast

LpPHT1;1 and *LpPHT1;4* were tested for their ability to complement the growth defect of the *S. cerevisiae Δpho84* mutant strain deficient in high-affinity phosphate uptake. All transformed yeast lines, including the empty *Δpho84* mutant strain (negative control) and the empty wild-type (WT) strain (positive control) grew similarly under high Pi condition (10 mM) (Fig. 3A). In contrast, under Pi deficient condition (0.1 mM), the yeast mutant

carrying the *LpPHT1;1* gene and the empty vector control showed growth limitation, whereas the expression of LpPHT1;4 rescued yeast growth to that of the WT strain (Fig. 3A).

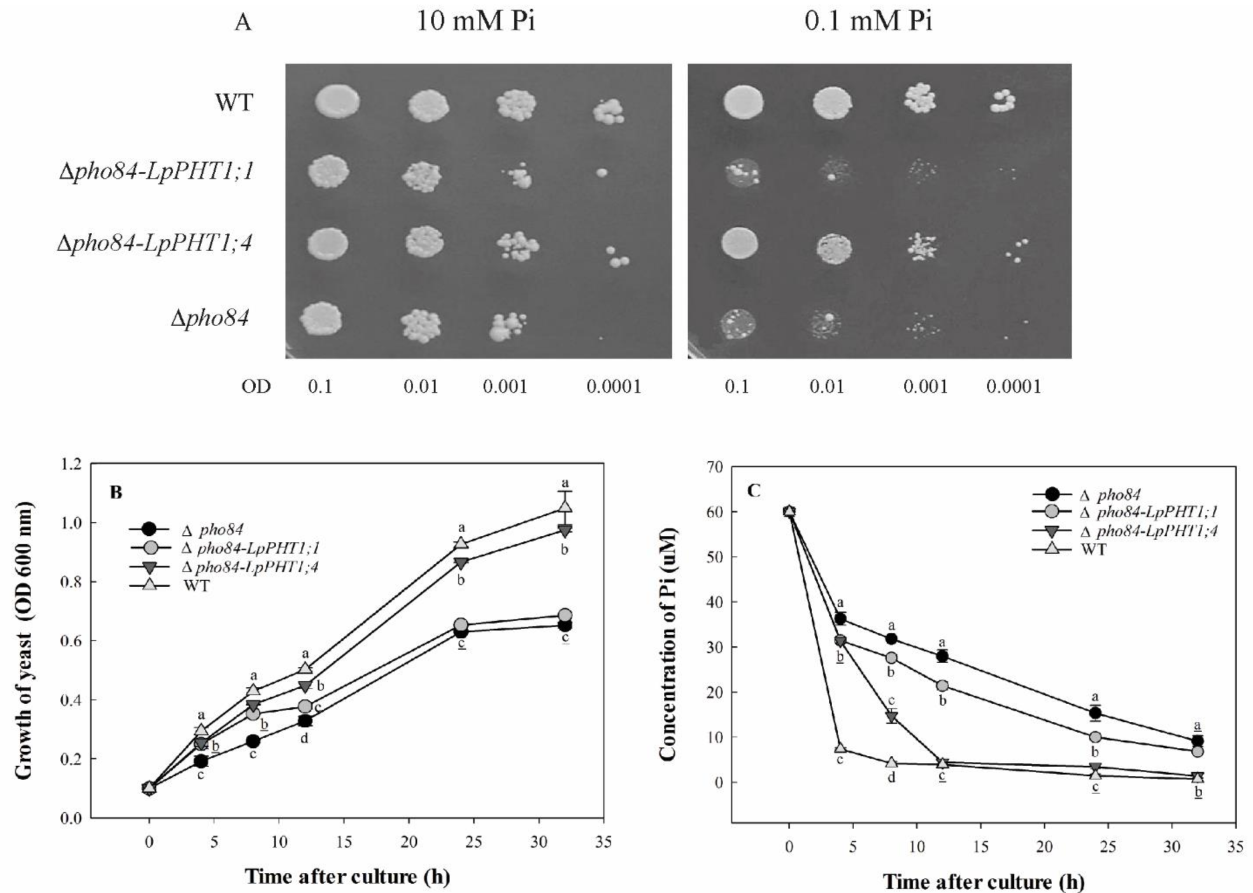

Figure 3. Functional complementation of Pi-uptake deficient Δ*pho84* mutant. **(A)** The yeasts wild-type (WT) strain, *Δpho84* mutant strain transformed with the constructs pDRf1-LpPHT1;1 (*Δpho84-LpPHT1;1*), pDRf1-LpPHT1;4 (*Δpho84-LpPHT1;4*) and mutant strain carrying empty vector (*Δpho84*) were spotted on SD-ura medium containing high (10 mM) and low (0.1 mM) Pi at different dilutions (OD). The plates were incubated at 28 ºC for 3 days. **(B)** Growth curves of yeasts grown with 60 µM of Pi. **(C)** The concentration of Pi remaining in SD-ura medium supporting yeasts grown with 60 µM Pi. Optical density was measured at 600 nm. Data are the means ± SE for three independent experiments. Different

letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) between yeast strains at the same time after culture.

The growth and Pi uptake of yeast cells expressing *LpPHT1;1* and *LpPHT1;4* were further examined in a time-course experiment for 32 h (Figs. 3B-C). The growth of the mutant strain expressing the *LpPHT1;1* gene was similar to the growth of the mutant *Δpho84* transformed with the empty vector, but lesser than the WT strain and the yeast mutant expressing *LpPHT1;4* (Fig. 3B). Similar growth curves were found between the yeast mutant expressing *LpPHT1;4* and the WT, reaching an OD_{600} of 0.9 after 20 h. These results were consistent with the uptake of Pi from the medium, where Pi uptake by the WT and yeast strains expressing *LpPHT1;4* was greater than that of *Δpho84* and the yeast mutant expressing LpPHT1;1 (Fig. 3C). Taken together, our results confirm that LpPHT1;4 restores the Pi transport activity of *Δpho84* mutant cells, whereas expression of LpPHT1;1 was not able to complement the growth defect of the *Δpho84* mutant under Pi deficient conditions.

3.3.3 Bioinformatic analysis of PHT1 genes

The Pi transporter proteins from *L. perenne* (LpPHT1;1 and LpPHT1;4) exhibited high similarity to a large number of previously identified Pi transporters from the Poaceae family, including wheat and barley (Table 3). Both sequences showed conserved phosphate: H^+ symporter domains, which belong to the major facilitator superfamily (MFS). These Pi transporters contain 12 membrane-spanning domains in a six-plus-six configuration with an intracellular central loop. LpPHT1;1 and LpPHT1;4 showed similar sizes of 527 and 536 amino acids, respectively, agreeing with other members of the Pht1 family (Supplementary Fig. S1).

Table 3. Sequences alignments for phosphate transporters from family 1 using Blastp-NCBI program.

Description	Identity	Number accession	Query protein Number accession
phosphate transporter 1 [*Hordeum vulgare* subsp. vulgare]	91%	*AAN37900.1*	
phosphate transporter protein [*Triticum aestivum*]	91%	*AIZ11193.1*	
phosphate transporter HvPT2 [*Hordeum vulgare* subsp. vulgare]	90%	*AAO72434.1*	*Lolium perenne* **LpPHT1;1** *AWR92645.1*
inorganic phosphate transporter 1-2 [*Sorghum bicolor*]	85%	*XP_002465845.1*	
inorganic phosphate transporter 1-2 [*Oryza sativa* Japonica Group]	80%	*XP_015630484.1*	
phosphate transporter protein 2 [*Zea mays*]	73%	*NP_001105816.1*	
phosphate transporter HvPT4 [*Hordeum vulgare* subsp. vulgare]	91%	*AA072437.1*	
phosphate transporter protein 4 [*Triticum aestivum*]	91%	*AIZ11183.1*	*Lolium perenne* **LpPHT1;4** *AWR92646.1*
probable inorganic phosphate transporter 1-8 [*Oryza sativa*] Japonica Group	86%	*XP_015614122.1*	
inorganic phosphate transporter 4 [*Zea mays*]	82%	*AAY42388.1*	

Phylogenetic analysis of the LpPHT1;1-4 protein sequences revealed that they belong to a well-defined family of previously identified phosphate transporters in other members of the Poaceae (Fig. 4). The LpPHT1;4 protein sequence is closely related to the orthologous PHT1;4 from wheat and barley. LpPHT1;1 is found in a different cluster than LpPHT1;4, showing a close relationship to the PHT1;1-2 from rice (Fig. 4).

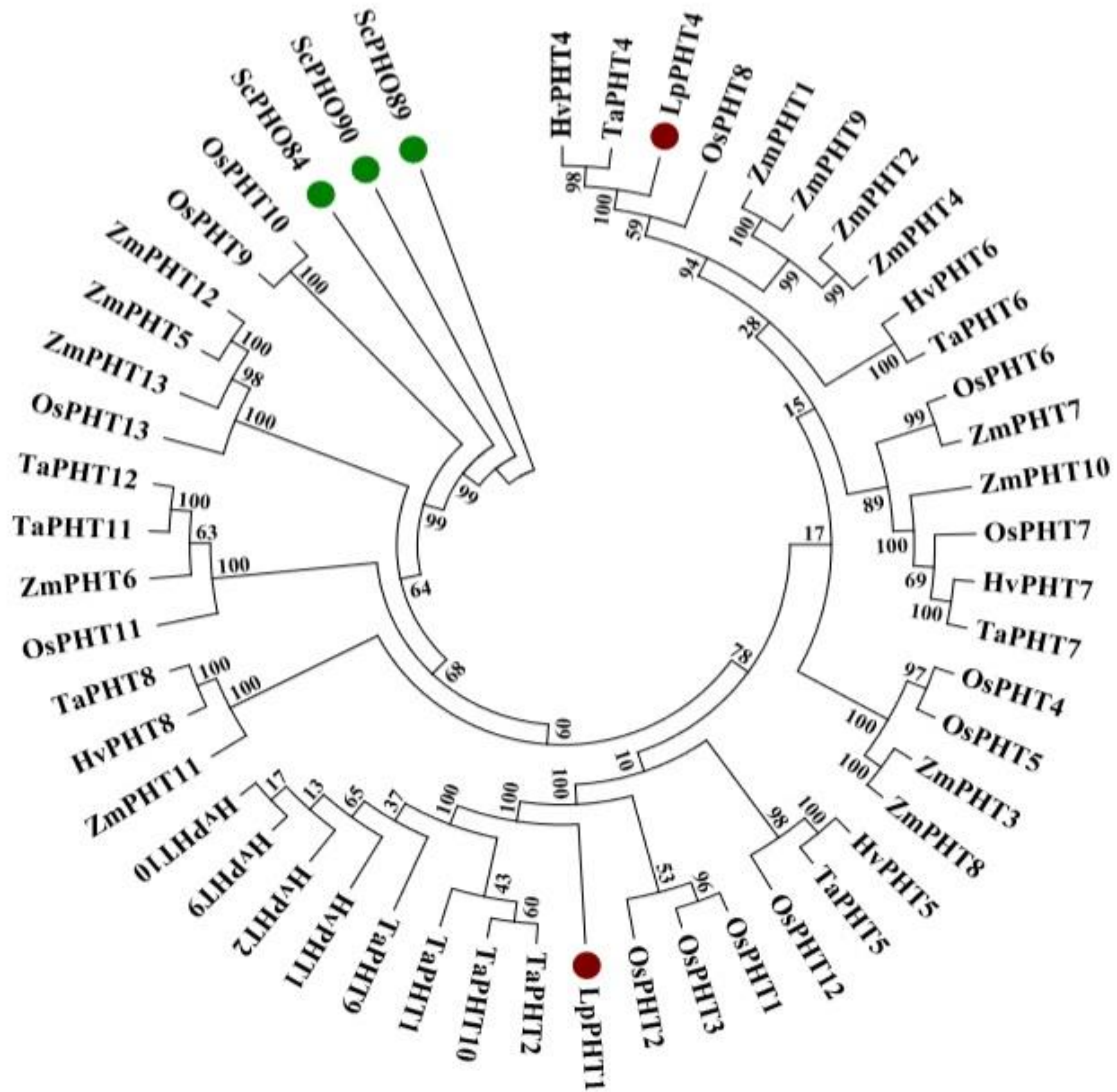

Figure 4. Phylogenetic relationship of the 51 amino acid sequences of the phosphate transporter family 1 in Poaceae family. A neighbor-joining unrooted tree was generated for *L. perenne* (Lp), rice (Os), barley (Hv), wheat (Ta), maize (Zm) and Phts from *S. cerevisiae* (ScPHO84-89-90). The bootstrap consensus tree was inferred from 1000 replicates. Evolutionary distances were calculated using the Poisson correction method. LpPHT1;1 and LpPHT1;4 from *L. perenne* are indicated by red circles. The high and low-affinity phosphate transporter systems from *S. cerevisiae* are indicated in a green circle.

Evolutionary conservation analysis by ConSurf server showed a highly conserved region inside the central cylindric structure of LpPHT1;1 and LpPHT1;4, which contains a substrate transport path perpendicular to the lipid membrane plane (Fig. 5). In this conserved structure it is also possible to identify critical residues such as Y145, Q172, W304, D311, Y315, N421 and K449 (Supplementary Fi. S1) which are fully conserved in phosphate-binding site of PHTs (Pedersen et al. 2013).

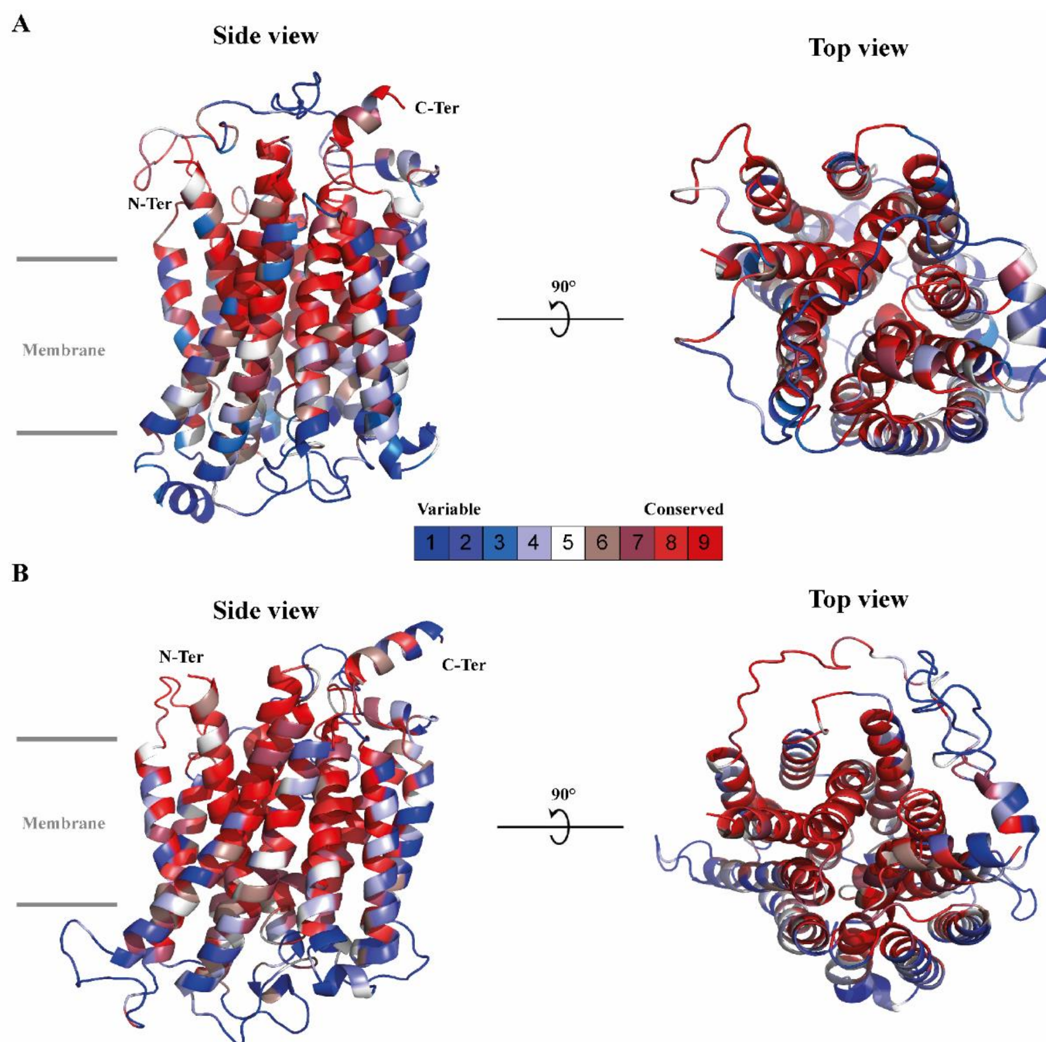

Figure 5. Evolutionary conservation analysis for LpPHT1;1 (A) and LpPHT1;4 (B). Structures were showed in two orientations in cartoon representation. Residues were collared by their conservation grade using a colour scale from blue to red. Being blue (1) the lowest conservation score and red (9) the highest.

3.4 Discussion

We identified and characterized two phosphate transporters from ryegrass, LpPHT1;1 and LpPHT1;4. The two systems exhibited different patterns of expression under Pi limitation *in planta* that may reflect their functional roles in the uptake and/or redistribution of Pi within the plant (Nussaume et al. 2011; Chien et al. 2018). Both high- and low-affinity systems may be up-regulated during P starvation (Smith et al. 2003; Wang et al. 2010; Ye et al. 2015). Our results indicated that both the *LpPHT1;1* and *LpPHT1;4* genes exhibit strong induction in as little as 6 h for LpPHT1;4 in both shoots and roots and 72 h and 12 h for LpPHT1;1 in shoots and roots, respectively, under Pi starvation. These patterns establish their roles in direct Pi uptake and in the remobilization of Pi within the plant. While the induction of *LpPHT1;4* was strongly and negatively correlated with tissue P concentration, that of *LpPHT1;1* was not, possibly suggesting different regulatory pathways. The maintenance of P homeostasis in plants is complex, and involves cellular Pi concentrations, shoot root signaling, transporter gene induction, and post-translational decay (Nussaume et al. 2011; Jain et al. 2012; Wang et al. 2017). The offset cycling of LpPHT1;4 mRNA suggests that these transporters may function in tandem to regulate PAE and PUE in P-limiting environments.

Complementation of *Δpho84* mutant yeast, which is defective in high-affinity Pi transport (Bun-Ya et al. 1991), is a widely used method to functionally characterize Pi transporters

from other species (Ai et al. 2009; Lapis-Gaza et al. 2014; Liu et al. 2016; Zheng et al. 2016). In general, our results showed that the Δ*pho84* mutant strain could grow under high Pi conditions, due to the activity of its low-affinity transporters PHO87 and PHO90 (Ghillebert et al. 2011), but, under low Pi conditions, the growth of the Δ*pho84* yeast mutant is limited by the lack of high-affinity Pi transporters (Ceasar et al. 2017). Complementation with LpPHT1;4, but not LpPHT1;1, can rescue the growth of *pho84* mutant under low Pi conditions, which suggests that LpPHT1;4 is a high-affinity Pi transporter and LpPHT1;1 might operate with a low-affinity system. Although both Pi transporters showed conserved Pi-binding residues, post-translational modifications may be important in regulating the affinities of PHT1 transporters (Ceasear et al. 2016). In this context, Ayadi et al. (2015) have argued that heterologous expression may not always reflect the natural regulatory mechanisms of PHT1s *in planta*, and further characterization of these Pi transport systems will be required to elucidate their regulatory connections in ryegrass.

Pi transporters clustering closely in a phylogenetic tree tend to have similar function and responses to Pi availability (Gu et al. 2016). In agreement, the close phylogenetic relationship of LpPHT1;4 with TaPHT1;4 from wheat or HvPHT1;4 from barley reveals a functional conservation in these Poaceae species. The TaPHT1;4 has been characterized as a high-affinity phosphate transporter with an important role in plant Pi acquisition under P starvation (Liu et al. 2013), and this is consistent with our complementation results for LpPHT1;4. The phylogenetic tree also shows that LpPHT1;1 has a close relationship with the PHT1s 1 and 2 from rice, which have been characterized as low-affinity Pi transporters (Ai et al. 2009; Sun et al. 2012). Low-affinity systems tend to be constitutively expressed

and are effective when the Pi concentration in the medium is relatively high (Liu et al. 2014; Qin et al. 2012; Wang et al. 2017), as supported by our complementation results for LpPHT1;1. Higher expression of low-affinity Pi transporters has also been associated with higher P utilization efficiency in crops (Huang et al. 2011; Wang et al. 2017), and these, therefore, play a key role in the growth and development of plants on P-limiting soils. In agreement, our previous results have shown that higher PHT1 transcript levels in both shoots and roots was linked with P efficient cultivar in ryegrass plants (Parra-Almuna et al. 2018).

Pi availability is a significant limitation to ryegrass production in Chile and on acidic soils worldwide. Improving uptake and internal utilization of P through the identification and manipulation of Pi transporters is a promising approach to produce pastures with enhanced PUE. Based on these results, the *LpPHT1;1* and *LpPHT1;4* genes can be used as potential molecular markers for cultivars with high Pi acquisition and use efficiencies, and may aid breeding programs and selection of ryegrass cultivars suited to acidic soils. As conclusion, both Pi transporter genes, *LpPHT1;1* and *LpPHT1;4*, were up-regulated by Pi starvation, similar to other PHT1s previously described in plants. Our data suggests that LpPHT1;1 functions as a low-affinity Pi transporter, whereas LpPHT1;4 acts as a high-affinity Pi transporter. Expression patterns in ryegrass shoots and roots under Pi limitation reveal that LpPHT1;1 may play a key role in Pi remobilization under both Pi-sufficient and Pi-deficient conditions, whereas LpPHT1;4 participates in direct Pi uptake and is induced by Pi starvation. These findings improve our knowledge of the molecular mechanism underlying P efficient ryegrass genotypes and further studies under field conditions will

establish the functional roles and differences in PHT1s on PAE and PUE in ryegrass on P-limiting soils.

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Author contribution

This research was carried out in collaboration between all authors. LPA, NF and MLM defined the research theme. LPA performed all the experiments with the assistance of SP, GL and JPT. NF and MLM supervised the study. LPA, SP, JC, NF, GL and MLM analysed the data and wrote the manuscript. All authors have contributed to and approved the manuscript.

CHAPTER IV

Aluminium toxicity and phosphate deficiency activates antioxidant systems and up-regulates expression of phosphate transporters gene in ryegrass (Lolium perenne L.)

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Aluminium toxicity and phosphate deficiency activates antioxidant systems and up-regulates expression of phosphate transporters gene in ryegrass (*Lolium perenne* L.) plants

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Abstract

Soil acidity, associated with aluminium (Al) toxicity and low phosphorus (P) availability, is considered the most important problem for agricultural production. Even though the Al-P interaction has been widely investigated, the impact of P-nutrition on Al-toxicity still remains controversial and poorly understood. To elucidate further insights into the underlying mechanisms of this interaction in ryegrass (*Lolium perenne* L.), P uptake, antioxidant responses and the gene expression of phosphate transporters were determined. Two ryegrass cultivars with different Al resistances, the Al-tolerant Nui cultivar and the Al-sensitive Expo cultivar were hydroponically grown under low (16 μM) and optimal (100 μM) P doses for 16 days. After P treatments, plants were exposed to Al doses (0 and 200 μM) under acidic conditions (pH 4.8) for 24 h. Al and P accumulation were higher in the roots of Nui than that of Expo. Moreover, lower Al accumulation was found in shoots of Nui independent of P supplies. Oxidative stress induced by Al-toxicity and P-deficiency was more severe in the Al-sensitive Expo. Expression levels of *L. perenne* phosphate transporters were higher in Nui than they were in Expo. While *LpPHT1* expression was up-regulated by P deficiency and Al toxicity in both cultivars, *LpPHT4* expression only increased in the Al-tolerant cultivar. This report shows that the higher Al-tolerance of Nui can be attributed to a greater antioxidant system under both P conditions. The observation of higher P and Al accumulation in roots of Nui might indicate that the Al-tolerance of Nui is a consequence of Al immobilization by P mediated by the high expression of phosphate transporters.

Keywords: aluminium toxicity; phosphate deficiency; phosphate transporter genes; radical scavenging activity; antioxidant enzymes.

4.1 Introduction

Soil acidity, associated with aluminium (Al) toxicity and low phosphorus (P) availability, is considered the main problem for agricultural production in different parts of the world (Kochian et al., 2004, 2015; Ma et al., 2014). Phosphorus is an essential macronutrient in plants, that is a structural component of nucleic acids and membranes and participates in enzyme regulation, the activation of signal transduction and in energy metabolism (Marschner, 1995; Wang et al., 2017). In contrast, Al toxicity (Al3+) causes the inhibition and reduction in root growth, limiting their capacity to uptake water and nutrients, including phosphate (Pi) (Kochian et al., 2004, 2005). Aluminium toxicity impairs several other physiological and metabolic functions, including the induction of oxidative damage of biomolecules by increasing the production of reactive oxygen species (ROS) in plant tissues (Achary et al., 2008; Inostroza-Blancheteau et al., 2011; Cartes et al., 2010, 2012).

Phosphorus fixation by the formation of aluminium-phosphate precipitates is a significant factor for low P availability in acidic soils (Bian et al., 2013). However, plants have shown several mechanisms to cope with P deficiency and Al toxicity. The most important mechanisms of Al resistance are the following: i) Al exclusion by means of organic acid anion exudation into the rhizosphere, preventing the entry of Al to the root, ii) internal Al detoxification by cytosolic chelation with organic acid anions or phenolic compounds, and subsequently compartmentalization of Al in the vacuoles, and iii) augmented scavenging of ROS and H+-ATPase plasma membrane activity (Kochian et al., 2005; Panda et al., 2009; Inostroza- Blancheteau et al., 2012; Daspute et al., 2017; Pontigo et al., 2017, Zhang et al., 2017). Similarly, under P deficient conditions, plants intensify phosphatase activity, modify root growth, induce secretion of organic acid anions, release phosphate (Pi) from vacuolar

stores and enhance the expression of phosphate transporters (Raghothama and Karthikeyan, 2005; Pariasca-Tanaka et al., 2009; Rouached et al., 2010).

Despite the evident Al-P interaction in acid soils, the influence of P nutrition on Al toxicity is contradictory between plant species and the underpinning mechanisms remain poorly understood. In this context, P supply has shown to alleviate Al toxicity in sorghum (Tan and Keltjens, 1990), buckwheat (Zheng et al., 2005), rice (Nakagawa et al., 2003), maize (Gaume et al., 2001), wheat (Iqbal, 2014) and bush clover (Sun et al., 2008). This alleviative effect has been related mainly to Al-P precipitation in the soil solution and Al-immobilization by P in root cell walls (Chen et al., 2012). In contrast, P deficiency reduced Al toxicity either by the replacement of membrane phospholipids with non-phosphate lipids, as observed in rice (Maejima et al., 2014), or by enhancing the exudation of organic acid anions, as observed in tomato (Ward et al., 2011). Other studies showed that beneficial effects of P nutrition on Al-tolerance are associated with P-efficient genotypes. For example, Al-tolerant cultivars of rice (Sivaguru and Paliwal., 1993), sorghum (Ramirez and Lopez, 2000) and soybean (Liao et al., 2006) showed more efficient P uptake than did Al sensitive cultivars.

The beef and dairy market in southern Chile is based on grazing pasture systems and the main forage species is perennial ryegrass (Lolium perenne) (Demanet et al., 2015). Soils used for this activity are derived from volcanic ash and are acidic (pH: 5.0-5.5), showing low available P (4-15 mgkg-1 Olsen P) and high levels of soluble Al. Both stresses affect yields, quality and the persistence of pastures (Mora et al., 2006; Redel et al., 2016). Previous reports have established that short and long-term Al exposure induced oxidative damage in perennial ryegrass plants, resulting in augmented enzymatic antioxidant activity

(Cartes et al., 2012; Wulff-Zottele et al., 2014). Furthermore, early response to P deficiency in ryegrass showed a significant change in metabolic response such as the use of alternative glycolytic bypasses and lipid membrane remodelling (Byrne et al., 2011). However, the P-Al interaction it has been scarcely explored in this species. In fact, the mechanisms of phosphate uptake associated with phosphate transporters under Al toxicity are still unknown. In this study, we aim to determine the effect of different Al-P interactions on phosphate uptake, antioxidant responses and gene expression of phosphate transporters in two ryegrass cultivars.

4.2 Materials and Methods

4.2.1 Plant material and growth conditions

Screening for aluminium sensitive evaluation was previously performed on four perennial ryegrass (*Lolium perenne* L.) cultivars, according to the method of Gallardo and Borie (1999). Plants were grown in nutrient solution Taylor and Foy (1985) at pH 4.8 with and without exposure to Al (200 µM) for one week. The results were expressed as relative root growth (RRG), considering the length of roots seedlings in solution without Al as 100% (Table 1). Based on these results, we selected the Nui cultivar as Al-tolerant and Expo cultivar as Al-sensitive. Seeds from the Expo andNuicultivarswererinsed with2% v/v sodium hypochlorite for 15 min and washed several times with distilled water. The seeds were germinated on filter paper moistened with deionized water for 10 days in a greenhouse with a photoperiod of 16 h light at 24°C. Seedlings were adapted for one week in a hydroponic culture using nutrient solution Taylor and Foy (1985) at pH 4.8, which contained the following nutrients (µM): 750 K, 1270 Ca, 100 P, 300 NH_4^+, 3710 NO_3^-, 270 Mg, 120 S, 2.4 Mn, 18 Fe ethylenediaminetetraacetic acid (EDTA), 46 Na, 6.6 B, 0.6 Zn,

0.2 Cu, and 0.1 Mo. Continuous aeration with an aquarium pump was applied. The first treatments were conducted after growth in the nutrient solution for 7 days. Phosphorus was administered as K_2HPO_4 (Merck reagent), with 16 µM as a low P-supply and 100 µM as an optimal P-supply. Ryegrass plants were grown in a greenhouse with three replicates, for 16 days under controlled conditions (22 ± 2 °C and 70–80% relative humidity). After 16 days, two Al doses (0 and 200 µM; as $AlCl_3$, Merck reagent) were applied in combination with P doses for 24 h. During the treatment period, pH was adjusted daily to 4.8 using diluted HCl or NaOH and the nutrient solution was replaced every week. Root and shoot samples were harvested and quickly stored for later analyses.

Table 1. Aluminium (Al) tolerance of four *Lolium perenne* cultivars grown in nutrient solution Taylor and Foy (1985). Data are the means ± SD of three replicates. Different letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) from % of root growth (*) with Al over root growth without Al for each cultivar.

Al (µM)	Nui	Expo	Bealey	One 50
0	100±9.8a	100±12.1a	100±10.1a	100±11.04a
200	98±1.2a	69±4.3b	79±1.4b	83±1.4b

(*) Root length of the plant grown for 7 days in nutrient solution without Al at pH 4.8 was considered 100% relative root growth (RRG_{Al}). The values were expressed as a percentage of relative root growth (RRG%).

4.2.2 Plant Al and P analyses

Shoots and roots were collected, washed with deionized water and weighed at harvest. Plants were ashed in a muffle at 500 ºC for10 h andafterward digested with 2 M HCl and filtered to determine Al and P concentrations. The Al concentration was measured by flame atomic absorption spectrophotometry (FAAS) at 324.7 nm and the P concentration was measured by the molybdovanadate method, both according to Sadzawka et al. (2007). A molybdovanadate solution was prepared by mixing equal parts 8 mM NH_4VO_3, 1.5 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ and 1.5 M HNO_3. Filtered solutions were mixed with 4 ml of the molybdovanadate solution, maintainedfor 1 h at room temperature and then spectrophotometrically analysed at 466 nm. Two reference samples with known Al and P concentrations were included for each analytical run as internal controls.

4.2.3 Radical scavenging activity

Ethanolic extracts of shoots and roots were used to determine radical scavenging activities (RSA) by the free radical 2.2-diphenyl-1-picrylhydrazyl (DPPH) method according to Chinnici et al. (2004). The DPPH method estimates antioxidant activity by measuring the elimination capacity of different antioxidant compounds to act as hydrogen donors or free radical scavengers (Kedare and Singh, 2011). The extract absorbance was followed at 515 nm in a spectrophotometer (SynergyTM HT, Biotek), using Trolox (hydrosoluble analogue of alpha-tocopherol) as the standard.

4.2.4 Lipid peroxidation measurements

Determination of lipid peroxidation of membranes was used as an oxidative stress indicator as described by the modified method of Du and Bramlage (1992). Fresh material of shoots and roots, were macerated with 0.2% of trichloroacetic acid solution (TCA) and then centrifuged at 4 ºC. The supernatant was collected and homogenized with thiobarbituric solution (TBA) containing 0.5% TBA and 20% TCA. An incubation of 30 min at 95 ºC was performed and the thiobarbituric acid reactive substances (TBARS) were determined spectrophotometrically at 532, 600 and 440 nm.

4.2.5 Superoxide dismutase and peroxidase activity

Shoot and root tissues previously frozen and stored at −80 °C were macerated with liquid nitrogen and then mixed with a 50 mM potassium phosphate buffer (pH 7.0). Samples were centrifuged at 11.000 g at 4°C for15 min. The supernatant was collected and used as a protein extract for the evaluation of both superoxide dismutase (SOD; EC. 1.15.1.1) and peroxidase (POD; EC. 1.11.1.7) activities.

Superoxide dismutase activity was determined by measuring the photochemical reduction of nitroblue tetrazolium (NBT) according to Donahue et al. (1997). The extract was mixed with a 0.1 M potassium phosphate buffer (K_2HPO_4-KH_2PO_4;pH 7.0), 10 mM EDTA, 260 mM methionine, 4.2 mM NBT and 130 µM riboflavin. The mixture was illuminated for 15 min and the reaction absorbance was measured by a multimode microplate reader (SynergyTM HT, Biotek) at 560 nm. The reactions without the protein extract under both illuminated and non-illuminated conditions were used as controls. The amount of enzyme was defined as 50% inhibition of the NBT reduction corresponding to one SOD unit.

Peroxidase activity was assayed by monitoring the oxidation of tetraguaiacol at 470 nm for 60 s according to Pinhero et al. (1997). The reaction was performed by mixing the extract with extraction buffer (K_2HPO_4-KH_2PO_4; pH 7.0), H_2O_2 (30% v/v) and guaiacol. POD activity was determined using a molar extinction coefficient of 26.6 mM^{-1} cm^{-1}. The amount of total proteins was measured for the calculated SOD and POD activities using bovine serum albumin (BSA) as the standard following the method of Bradford (1976).

4.2.6 Protoplasts isolation

Shoot (10-15 g) samples were maintained under dark conditions for 24 h before the procedure. Then, they were sterilized with 70% ethanol for 1 min and rinsed thoroughly with deionized water. Shoots were macerated in 40 mL of digestion buffer (0.33 M sorbitol, 0.1 M tris-Cl (pH 7.8), 5 mM $MgCl_2$, 10 mM NaCl, 2 mM EDTA and 0.1% BSA), contained 1.5% (w/v) cellulase and 0.4% (w/v) pectinase. Samples were filtered into 50 mL falcon tubes and then were centrifuged at 2000 rpm at 4°C for 10 min and the supernatant was discarded. The pellet was resuspended in 1X digestion buffer by gentle pipetting. The resulting protoplast suspension was mixed with 10 mL of 40% Percoll and centrifuged at 2000 rpm at 4°C for 10 min. The supernatant was carefully discarded, and the intact protoplasts were resuspended in 0.5 mL of 1X phosphate-buffered saline (PBS). The protoplasts suspension was kept on ice until use.

4.2.7 Detection of hydrogen peroxide (H_2O_2) production in shoot protoplasts by confocal microscopy

The protoplasts suspension was incubated for 30 min with 10 µM of the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) at 37°C according to the modified

method of Maxwell et al. (1999) in order to detect intracellular H_2O_2-ROS (reactive oxygen species) production. The intracellular H_2O_2 production was analysed and visualized by laser scanning confocal microscopy (CLSM) Olympus FV1000 (Arquimed, Japan). The 488 nm line of an Ar ion laser and the 530 nm line of a He–Ne laser were used for excitation and emission, respectively. Chlorophyll autofluorescence was detected at excitation/emission of 633/750 nm. The images were analysed and interpreted using the Image Processing software (software FV10-ASW v0.200c; Arquimed). A preparation of intact protoplasts with 100 µM H_2O_2 was used as a positive control.

4.2.8 Detection of hydrogen peroxide (H_2O_2) production in root tissues

Root (30 mg) samples previously frozen and stored at −80 °C were macerated with liquid nitrogen and then mixed with a 1 M potassium phosphate buffer (pH 7.0). The samples were centrifuged at 10.000 g at 4°C for 4 min. The supernatant was collected and used as a root extract. The H_2O_2 production was quantified using the Amplex® Red hydrogen peroxide/peroxidase assay kit (Invitrogen) according to the manufacturer's recommendations. The reaction was performed mixed 50 µL of root extract with 100 µM Amplex® Red reagent and 0.2 U mL^{-1} HRP (horseradish peroxidase). After incubation for 30 min at room temperature protected from light, fluorescence was detected using a multimode microplate reader (SynergyTM HT, Biotek) at excitation/emission of 548/590 nm. The standard curve was performed with known H_2O_2 concentrations, including a no-H_2O_2 control. The buffer without root extract was used as a negative control to corrected background fluorescence. The H_2O_2 content was expressed as pmol mg^{-1} FW (FW-fresh weight).

4.2.9 De novo assembly of ryegrass transcriptomes and search for phosphate transporter genes

The ryegrass transcriptome was obtained from the SRA (Sequence Read Archive) database hosted at NCBI (accession codes from SRR2034619 to SRR2034623, Blackmore et al., 2015). FASTQ files were filtered based on their Q-Score using NGSQCToolkit v2.3.3 (Patel and Jain, 2012) and assembled de novo with Trinity v2.1.1 (Grabherr et al., 2011). Local databases of transcriptomes were constructed using BLAST v2.2.30+ and searches for phosphate transporters were based on Uniprot sequences from *Oryza sativa* (Pht1.2 Q8GSD9, Pht1.4 Q8H6H2, Pht1.6 Q8H6H0). The identity and homology were confirmed using BLASTX on the NCBI host.

4.2.10 Phosphate transporters gene expression analyses

RNA was isolated from shoot and root tissues using the NucleoSpin® RNA Plant Kit (Macherey-Nagel GmbH and Co., KG, Düren, Germany). The RNA concentration was assessed spectrophotometrically using Synergy HT counts with a plate for microvolumes (take3, nanoq). The RNA purity was evaluated using the A260/280 and A260/230 ratios provided by the take3 software. Total RNA (1.0 µg) was synthesized to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Invitrogen).

Gene expressions of phosphate transporters were evaluated by real-time quantitative reverse transcription PCR (qRT-PCR). The expression levels were performed using the Brilliant II SYBR® Master Mix Kit (Agilent, USA) and qPCR Step One Plus (Applied Biossystems, Foster City, CA, USA). Specific primers were designed using Primer3 (V. 0.4.0) to amplify three ryegrass phosphate transporters (Table 2): *LpPHT1;1* (GeneBank

accession MF966998), *LpPHT1;4* (GeneBank accession MF966999) and *LpPHT1;6* (GeneBank accession MF967000). Two housekeeping genes, eukaryotic elongation factor 1 alpha eEF1α(h) (GeneBank accession GO924753) and eEF1α(s) (GeneBank accession GO924801) were used as internal controls (Table 2). The qPCR reaction was evaluated in 20 µL of final volume containing 10 µL of Brilliant II SYBR® Master Mix, 1 µL of cDNA and 2.4 µL of each gene-specific primer (600 nM). Cycling conditions were 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s, 59 °C for 1 min, and 72 °C for 5 s. The normalized values were subjected to the $2^{-\Delta\Delta Ct}$ method according to Pfaffl (2001). All qRT-PCR reactions were determined on three biological replicates with three technical replicates.

Table 2. Primer used for analysis of phosphate transporter genes from *Lolium perenne* L.

Gene name	Sequence forward primer (5´- 3´)	Sequence reverse primer (5´- 3´)
LpPHT1;1	CCTGGGATTGCTTTCTCAC	TGGTTGCGTCATCGTCATAG
LpPHT1;4	AACCAGCGTACCAGGACAAC	GAGGATGATGCGCCAGAC
LpPHT1;6	GAGAACGACGACGAGACGA	TCCGAACAGGGACACAGATAC
eEF1α(h)	ATGTCTGTTGAGCAGCCTTC	GCGGAGTATATAAAGGGGTAGC
eEF1α(s)	CCGTTTTGTCGAGTTTGGT	AGCAACTGTAACCGAACATAGC

4.2.11 Statistical analysis

The experimental design was a complete randomized block with three replications. Experimental data were analysed using the Sigma Stat 2.0 software (SPSS, Chicago, IL, USA) to perform analyses of variance (ANOVA). Comparisons of means were performed using Tukey’s test at $p \leq 0.05$. Pearson's correlations were also calculated.

4.3 Results

4.3.1 Phosphorus and aluminium concentrations

Significant differences were observed in root and shoot concentrations of P and Al among all treatments in both cultivars (Table 3). Independent of P supplies, P concentrations in shoots and roots from the Nui cultivar were 27% and 40% higher, respectively than those in Expo. The Al supply decreases shoot P concentration by approximately 10%, whereas in roots, P concentration increased by approximately 15% for both cultivars (Table 3). Root P and Al concentrations in the Nui cultivar were positively correlated, with r = 0.62 (Table 4). Although Al accumulation in the roots of Nui was higher than in that in Expo, Al accumulation in shoots was approximately 2.5-fold higher in Expo than that in Nui and was independent of P treatments. In addition, root Al accumulation in Nui was significantly smaller ($p \leq 0.05$) under P deficient conditions than that under P optimal conditions, whereas in Expo, Al accumulation was approximately 2-fold higher under the same conditions (Table 3).

Table 3. Phosphorus (P) concentration and aluminium (Al) concentration in shoots and roots of the ryegrass Nui cultivar (Al-tolerant) and Expo cultivar (Al-sensitive) under different P and Al treatments. +P: 100 µM, -P: 16 µM, +Al: 200 µM, -Al: 0 µM. Data are the means ± SD of three replicates. Different letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) between Al-P treatments and cultivars.

Cultivar	Treatments		P concentration ($g\ kg^{-1}$ DW)		Al concentration ($mg\ kg^{-1}$ DW)		Al content ($\mu g\ pot^{-1}$)	
			Shoot	Root	Shoot	Root	Shoot	Root
Nui	+P	+Al	5.9±0.11b	8.6±0.17a	84.1±4.4ab	1125 ±216.2a	125.7±16.7c	494.0±16.2b
		-Al	6.3±0.06a	6.5±0.15b	43.7±5.3cd	137.7±7.4d	65.9±8.8d	56.0±4.7f
	-P	+Al	3.6±0.14e	4.2±0.20d	82.3±5.7b	738.7±14.1b	141.7±9.8c	440.8±15.3c
		-Al	3.7±0.12e	3.8±0.21d	43.4±2.2cd	125.3±6.3d	77.1±9.3d	63.3±2.9ef
Expo	+P	+Al	4.1±0.10d	6.3±0.26b	84.2±10.6ab	569.9±46.2c	333.6±41.7a	354.1±20.6d
		-Al	4.6±0.03c	5.3±0.17c	51.8±0.9c	106.5±14.3d	235.0±0.6b	68.0±11.8ef
	-P	+Al	1.7±0.09f	2.2±0.06e	97.5±1.2a	710.6±12.4b	315.9±0.3a	682.7±11.3a
		-Al	1.9±0.04f	2.2±0.10e	32.3±1.4e	96.2±10.4d	108.9±0.5cd	98.0±0.3e

Table 4. Pearson's correlation matrix among chemical and biochemical parameters of the ryegrass Nui cultivar (below diagonal) and Expo cultivar (above diagonal, grey background) under different P and Al treatments. P S/R; phosphorus concentration in shoots and roots (g kg-1 DW), Al S/R; aluminum concentration in shoots and roots (mg kg-1 DW), TBARS S/R; lipid peroxidation in shoots and roots (nmol MDA g-1 FW), DPPH S/R; radical scavenging activity in shoots and roots (nmol TE mg-1 FW), SOD S/R; superoxide dismutase activity in shoots and roots (U mg-1 protein), POD S/R; peroxidase activity in shoots and roots (nmol mg-1 protein min-1). Pearson correlation coefficients (r) were calculated from three replicates of each sampling. *P < 0.05; **P < 0.01.

Nui/Expo	PS	AlS	TBARSS	DPPHS	SODS	PODS	PR	AlR	TBARSR	DPPHR	SODR	PODR
PS		-0.08	-0.37	-0.87**	-0.91**	-0.74**	0.93**	-0.28	-0.78**	0.62*	-0.73**	0.38
AlS	-0.06		0.69*	-0.23	0.20	0.12	0.17	0.96**	0.50	0.30	0.32	0.42
TBARSS	-0.15	0.85**		0.25	0.66*	-0.08	0.00	0.84**	0.35	0.34	0.83**	-0.33
DPPHS	-0.42	0.01	0.26		0.82**	0.55	-0.83**	-0.01	0.51	-0.57	0.64*	-0.60*
SODS	-0.30	0.90**	0.87**	0.32		0.45	-0.70**	0.43	0.63*	-0.31	0.92**	-0.59
PODS	-0.37	-0.73**	-0.33	0.46	-0.50		-0.85**	0.16	0.81**	-0.80**	0.21	0.23
PR	0.85**	0.41	0.13	-0.45	0.14	-0.79**		0.01	-0.74**	0.80**	0.80**	0.24
AlR	0.14	0.92**	0.65*	-0.08	0.80**	-0.89**	0.62*		0.57	0.25	0.56	0.20
TBARSR	0.90**	-0.14	-0.39	-0.56	-0.41	-0.43	0.81**	0.09		-0.53	0.43	0.21
DPPHR	0.37	0.21	0.01	-0.50	-0.02	-0.46	0.46	0.31	0.30		-0.01	-0.09
SODR	-0.20	-0.46	-0.05	0.71**	-0.19	0.80**	-0.55	-0.60*	-0.36	-0.64*		-0.64*
PODR	-0.71**	0.20	0.54	0.45	0.46	0.39	-0.66*	-0.04	-0.88**	-0.64*	0.31	

4.3.2 Lipid peroxidation and plant antioxidant responses

Lipid peroxidation, measured as malondialdehyde (MDA) accumulation, was assayed as an index of oxidative damage (Fig. 1). The Al supply significantly increased shoot lipid peroxidation in both cultivars independent of P treatments (Fig. 1A). Moreover, a positive correlation was detected between lipid peroxidation and the Al concentration in shoots of Nui (r = 0.85) and Expo (r = 0.69) (Table 4). When plants of both cultivars were grown under normal P conditions, no significant differences were found between root lipid peroxidation and Al treatments ($p \leq 0.05$). P deficient conditions resulted in significantly increased root lipid peroxidation in Expo but decreased lipid peroxidation in Nui ($p \leq 0.05$, Fig. 1B). Under P deficient conditions, root lipid peroxidation was not affected by Al treatments in Nui but was 27% higher in Expo (Fig. 1B).

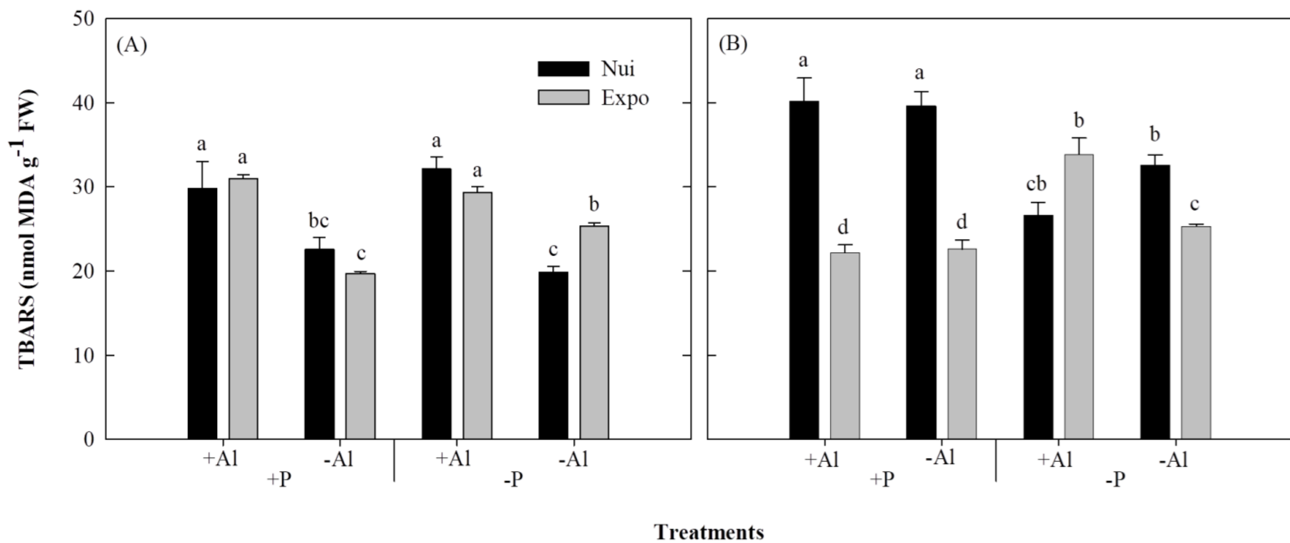

Figure 1. Lipid peroxidation of membranes (TBARS) in shoots (A) and roots (B) of Nui and Expo cultivars grown in nutrient solution with different Al and P treatments. Data are the means ± SD of three replicates. Different letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) among Al-P treatments and cultivars.

To determine the effect of the different Al-P treatmentson the enzymatic ROS scavenging system, total superoxide dismutase (SOD) and peroxidase (POD) activities were assessed in shoots and roots (Fig. 2). The shoot and root SOD activities were higher in Nui than those in Expo (Fig. 2A, B). Shoot SOD activity was enhanced by Al supply in Nui independent of P treatments, whereas the SOD activity was only higher with Al addition at the optimal P treatment in Expo (Fig. 2A). The root SOD activity in Nui showed no significant differences among all treatments ($p \leq 0.05$, Fig. 2B). In the roots of Expo, Al addition increased the SOD activity when plants were grown under optimal P conditions. Conversely, no significant changes were observed in the SOD activity under the P deficiency treatment by Al supply ($p \leq 0.05$, Fig 2B).

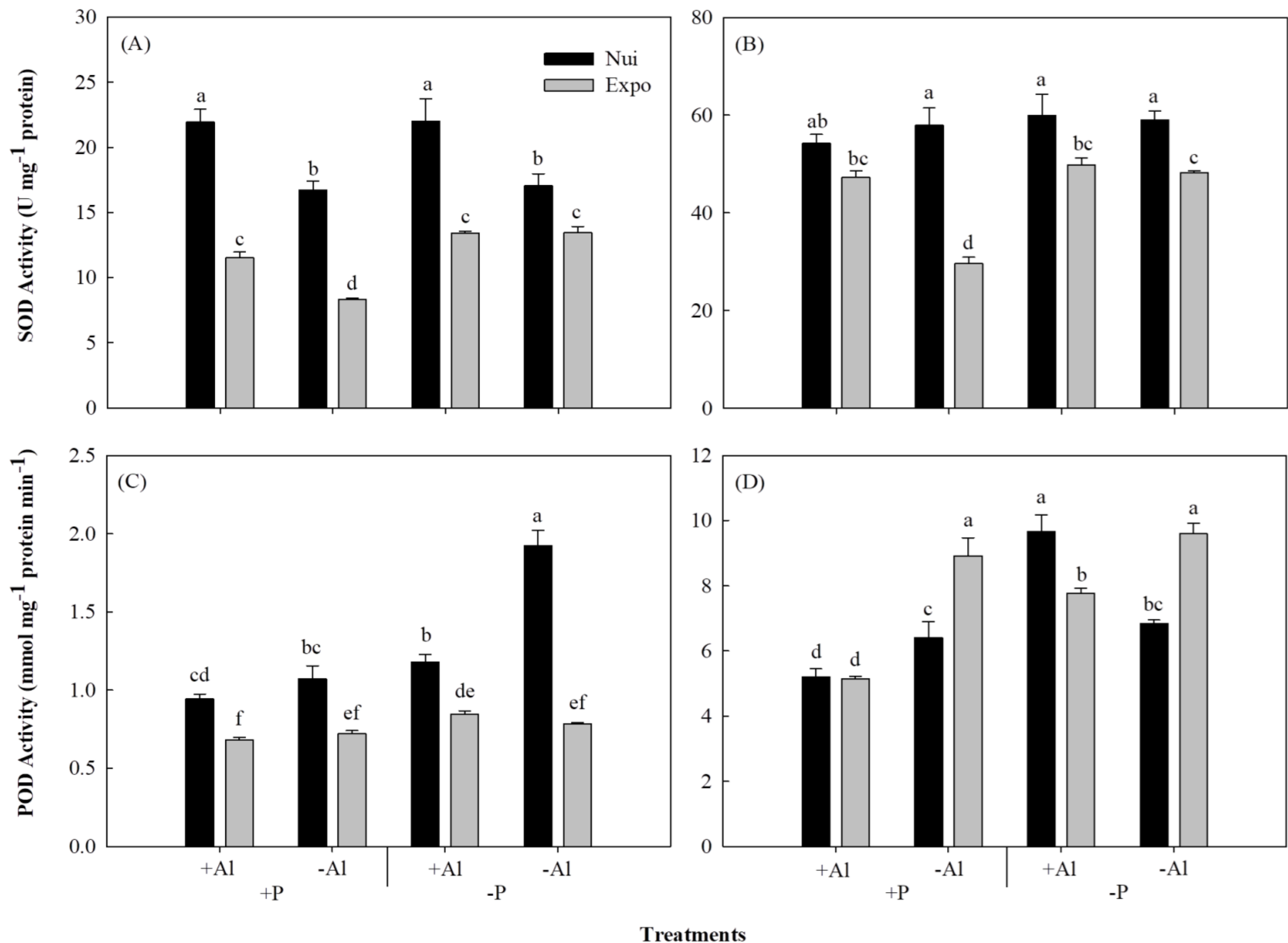

Figure 2. SOD activity and POD activity in shoots (A, C) and roots (B, D) of Nui and Expo cultivars grown in nutrient solution with different Al and P treatments. Data are the means ± SD of three replicates. Different letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) among Al-P treatments and cultivars.

The POD activity in shoots was significantly higher in Nui than it was in Expo for all treatments ($p \leq 0.05$, Fig. 2C). The highest shoot POD activity was observed in Nui grown under P deficiency without Al, whereas no difference was observed among all treatments in Expo ($p \leq 0.05$, Fig. 2C). The changes in root POD activity by Al supply depended on the plant P status and was differentially regulated in both cultivars. In the roots of Nui, the POD activity was increased when Al was supplied to P deficient plants but was decreased when it was supplied under optimal P conditions (Fig. 2D). The opposite trend was visualized in the roots of Expo.

The shoot and root radical scavenging activities (RSAs) were significantly higher (approximately 75%) in Nui than those in Expo independent of treatment ($p \leq 0.05$, Fig. 3). In both cultivars, the shoot RSA was higher than the root RSA ($p \leq 0.05$). The P deficiency treatment without Al only increased the shoot RSA in Nui (Fig. 3A). Al addition under optimal P conditions increased the root RSAs of both cultivars (Fig. 3B).

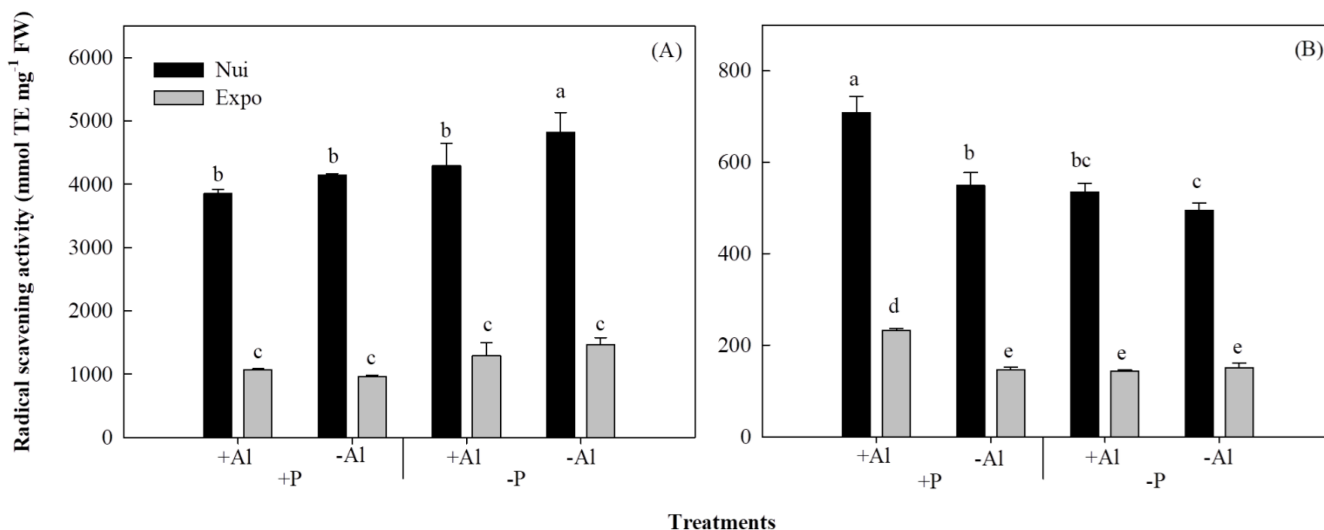

Figure 3. Radical scavenging activity (RSA) in shoots (A) and roots (B) of Nui and Expo cultivars grown in nutrient solution with different Al and P treatments. Data are the means ± SD of three replicates. Different letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) among Al-P treatments and cultivars.

4.3.3 Production of H_2O_2 in ryegrass shoot protoplasts and roots

Production of H_2O_2 was measured in the shoots and roots of Nui and Expo from the different Al-P treatments using a H_2DCFDA probe and Amplex® Red kit, respectively. Higher fluorescence intensity measured in relative fluorescence units (RFU) was found for Expo than that for Nui for all treatments (Fig. 4). The fluorescence intensity of the shoot protoplasts in both cultivars was not different for the Al treatments at any of the P conditions (Fig. 4A, B). While the fluorescence distribution in Expo protoplast was mainly found in the plasma membrane, an intracellular distribution was also observed in Nui. In addition, P deficiency increased H_2O_2 production in the shoots and roots from both cultivars (Figs. 4, 5). The H_2O_2 content in the roots of Nui was significantly higher when Al

was supplied independent of P treatments, while no significant difference was found in Expo cultivar ($p \leq 0.05$, Fig. 5).

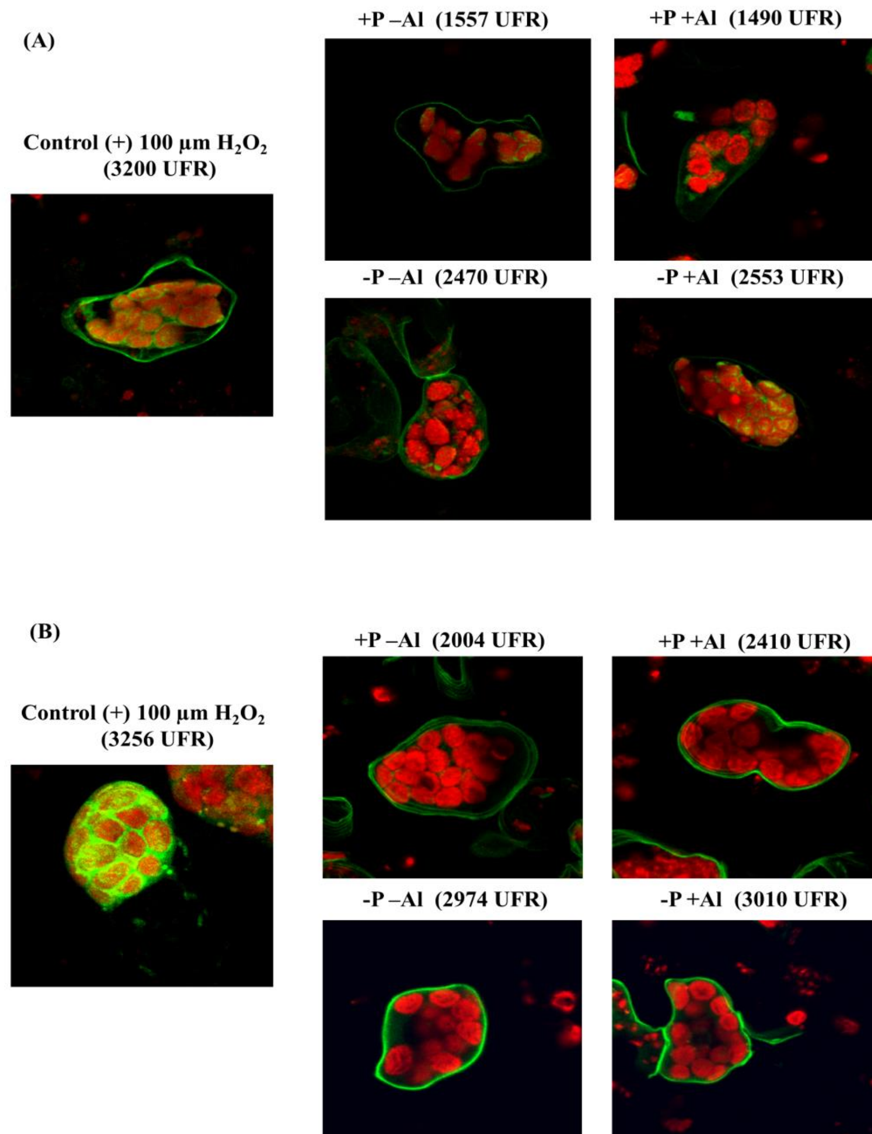

Figure 4. Fluorescence intensity measured in relative fluorescence units (RFUs) by means of isolated protoplasts incubated with the H_2DCFDA probe from Nui (A) and Expo (B) cultivars with different Al and P treatments. Hydrogen peroxide (H_2O_2) fluorescence was detected by laser-scanning confocal microscopy (LSCM). For the positive control, 100 µM H_2O_2 was used. Bars in all images represent 20 µm.

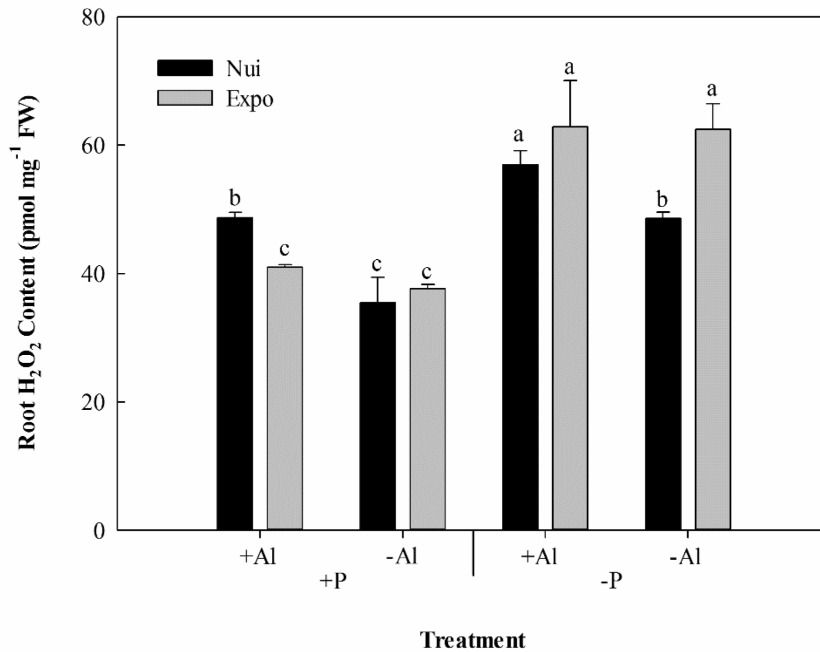

Figure 5. Production of H_2O_2 measured with Amplex® Red in roots of Nui and Expo cultivars grown in nutrient solution with different Al and P treatments. Data are the means ± SD of three replicates. Different letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) among Al-P treatments and cultivars.

4.3.4 Regulation of phosphate transporters gene expression

The gene expression of three ryegrass family 1 phosphate transporters was evaluated using qRT-PCR. In general, the transcript levels of *LpPHT1;1* and *LpPHT1;4* were higher in Nui than those in Expo (Fig. 6). While P deficiency up-regulated expression of *LpPHT1;1*, *LpPHT1;4* and *LpPHT1;6* in the roots of Nui, only *LpPHT1;1* and *LpPHT1;6* were up-regulated in Expo (Fig. 6B, D, F). The addition of Al under P deficiency conditions increased the expression level of *LpPHT1;1* in the roots of both cultivars ($p \leq 0.05$, Fig. 6B). Similarly, the expression level of *LpPHT1;4* was increased by Al supply only in the roots of Nui, whereas the expression level of *LpPTH1;6* was reduced in both cultivars ($p \leq$

0.05, Fig. 6D, F). Under optimal P conditions, the addition of Al up-regulated the expression of *LpPHT1;4* and *LpPHT1;6* in the roots of Nui and Expo, respectively (Fig. 6D, F). In shoots, P deficiency increased the expression levels of *LpPHT1;1, LpPHT1;4* and *LpPHT1;6* in Nui ($p \leq 0.05$, Fig. 6A, C, E). In Expo, only the expression level of *LpPHT1;1* was differentially regulated by P deficiency. The addition of Al under P deficiency conditions enhanced the expression level of *LpPHT1;1* in the shoots of Expo and *LpPHT1;4* in the shoots of Nui (Fig. 6A, C). Under optimal P conditions, the expression level of *LpPHT1;1* was up-regulated by Al in both cultivars (Fig. 6A), whereas the expression level of *LpPHT1;4* was only increased by Al in the shoots of Nui (Fig. 6C). In the shoots of both cultivars, *LpPHT1;6* was expressed at very low levels compared with those of the other phosphate transporters (Fig. 6E). In addition, the transcript level of *LpPHT1;6* was down-regulated by Al supply in Expo and Nui under optimal and P deficient conditions, respectively.

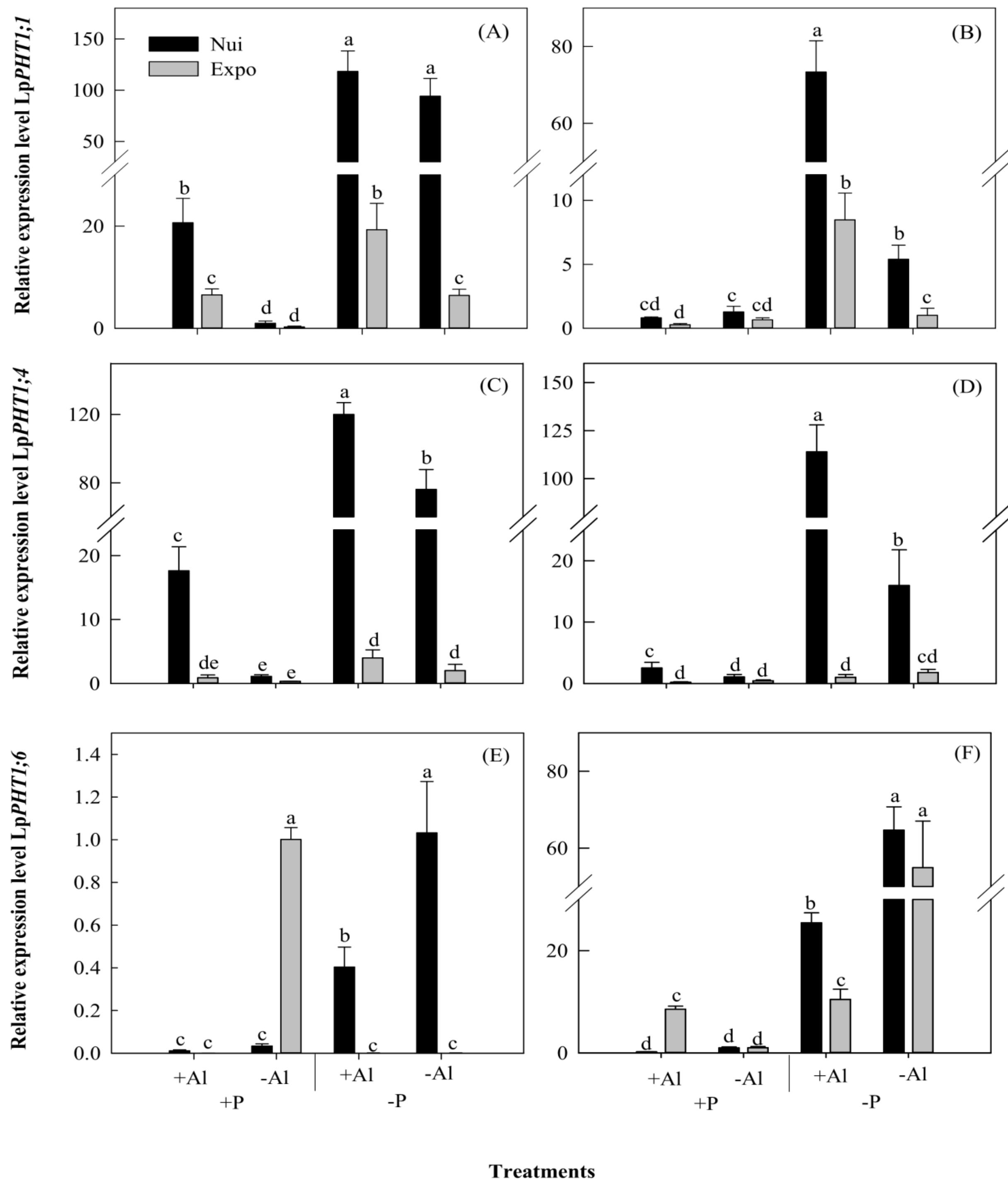

Figure 6. Relative expression of phosphate transporter genes *LpPHT1;1* (A, B), *LpPHT1;4* (C, D) and *LpPHT1;6* (E, F) in roots and shoots of Nui and Expo cultivars grown in

nutrient solution with different Al and P treatments. The expression levels were normalized in relation to *eEF1α(h)* or *eEF1α(s)* gene expression. Data are the means ± SD of three replicates. Different letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) among Al-P treatments and cultivars.

4.4 Discussion

In acid soils, several adaptive mechanisms have been described in plants to grow under phosphorus deficiency and aluminium toxicity conditions (Sun et al., 2016; Wang et al., 2017). It is well known that chemical interactions between P and Al occurs in plant tissues and growth media. However, the details of the interaction are still under debate because the consequences of P availability on Al toxicity have shown different results depending on the experimental method, plant species and genotypes (Chen et al., 2012; Zhao et al., 2014).

Significantly higher P and Al concentrations in roots were found in the Al-tolerant (Nui) cultivar than those in the Al-sensitive (Expo) cultivar subjected to optimal P conditions. This result is consistent with the previous report of Mora et al. (2006), which showed that in Al-semi-tolerant *Lolium multiflorum,* the formation of aluminium-phosphate complexes inside the roots was an important Al detoxification mechanisms. In addition, it has been demonstrated that both the decrease in Al uptake and a lower Al translocation from roots to shoots play key roles in Al tolerance (Gaume et al., 2001; Zheng et al., 2005). In fact, the Al accumulation in shoots was significantly lower in Nui than that in Expo, which added to the formation of complexes in the root, possibly explaining the greater tolerance of Nui. Our results also demonstrated that Al accumulation in the roots of Nui slightly decreased under P deficiency compared to the optimal P supply. The reduction in Al under P deficiency in Al-tolerant plants has been associated with a substitution of phospholipids

with galactolipids and sulfolipids and/or enhanced exudation of organic acid anion, leading to less Al binding to the plasma membrane and the cell wall (Gaume et al., 2001; Kochian et al., 2004; Maejima et al., 2014; Byrne et al., 2011; Ward et al., 2011). Nevertheless, it remains to be investigated whether Al-tolerant ryegrass has similar response under P deficiency. Under optimal P conditions, the observation of higher P and Al accumulation in roots of Nui might indicate that Nui`s Al-tolerance is a consequence of Al immobilization by P, followed by less Al translocation from roots to shoots as a result of this accumulation. Even though under P deficiency, the Expo cultivar accumulated more Al in roots than did Nui, the Al content was considerably higher in Nui, which could indicate that it is also operating an Al immobilization mechanism.

Although several studies have shown that Al toxicity induces an overproduction of ROS causing oxidative damage to membrane lipids (Kochian et al., 2005; Xu et al., 2010; Ma et al., 2012; Anjum et al., 2015), the oxidative stress response to the Al-P-interaction has been poorly studied in ryegrass. The decrease in root lipid peroxidation of Nui under P deficient conditions was accompanied by increased antioxidant activity (RSA). In contrast, in Expo Al-treated roots, lipid peroxidation increased under P deficient conditions, which was mainly explained by the higher Al content. These results could indicate that an efficient P supply reduces membrane oxidative damage induced by Al only in Al-sensitive ryegrass plants. Previous studies on many plants species have demonstrated that Al induces antioxidant enzymes as a critical mechanism for the elimination of ROS (Achary et al., 2008; Cartes et al., 2012; Inostroza-Blancheteau et al., 2012; Ma et al., 2012). SOD catalyses the dismutation of the superoxide anion to H_2O_2 and O_2, whereas POD utilizes H_2O_2 in the oxidation of various substrates (Sharma and Dubey, 2007). Independent of the

P supply, we found that SOD activity was increased in both cultivars subjected to the Al treatment and that this POD

activity was higher in Nui than that in Expo among all treatments. In fact, it is well known that Al tolerance in plants is due to an efficient antioxidant system (Ma et al., 2012). Previous reports have established that H_2O_2 generation not only induces oxidative damage but also plays an important role as a signalling molecule in many abiotic stresses, including nutrient deficiency (Shin and Schachtman, 2004; Ahmad et al., 2008; Hossain et al., 2015). We also found that H_2O_2 production increased under P deficiency in both cultivars and that the increase was visibly affected by Al addition only in the roots of Nui. Although the roots of Nui showed higher levels of H_2O_2 under both stresses, the activities of antioxidant enzymes were greater, allowing to less lipid peroxidation. Likewise, the higher generation of H_2O_2 in protoplasts in Expo than that in Nui under all treatments can also be related to the lower shoot SOD and POD activities of Expo. However, further studies are required to establish the long-term effects of Al on H_2O_2 production under different P statuses.

Up-regulation of phosphate transporter (*PHT1*) genes is an important mechanism that plants have developed under P deficient conditions (López-Arredondo et al., 2014; Wang et al., 2017). *PHT1* is expressed in epidermal and cortical root cells, leaves and flowers (Nagy et al., 2006; Jia et al., 2011), demonstrating their roles in phosphate uptake and phosphate translocation to different tissues and organs (Karthikeyan et al., 2002; Ai et al., 2009; Nussaume et al., 2011). In this study, three PHT1s members (LpPHT1;1/4/6) were identified and isolated from ryegrass plants. As expected, P deficiency up-regulated the *LpPHT1s* gene expression mostly in the roots of both cultivars, although the members showed different expression patterns. *LpPHT1;*1 and *LpPHT1;4* were highly expressed both in shoots and roots, whereas *LpPHT1;6* was expressed mostly in roots. Similar results

have been obtained for *PHT1;1* and *PHT1;4* in rice (Sun et al., 2012; Ye et al., 2015) maize (Liu et al., 2016) and barley (Rae et al., 2003) as well as for *PHT1;6* in rice and barley (Rae et al., 2003; Ai et al., 2009). These results suggest that LpPHT1;1/4 participate in the phosphate uptake and its translocation to different tissues, whereas LpPHT1;6 is involved mostly in phosphate uptake under P deficiency. The higher transcript levels of *LpPHT1;1* and *LpPHT1;4* in Nui than those in Expo could explain the higher P accumulation of Nui. In fact, Al-tolerant plants of wheat (Iqbal, 2014) and soybean (Liao et al., 2006; Liang et al., 2013) were characterized by efficient P uptake, translocation, and utilization. Similarly, wheat P uptake efficiency and barley P utilization efficiency have been attributed to the transcript abundance of the *PHT1* genes (Huang et al., 2000; Aziz et al., 2014). Our data also showed that the expression of the *LpPHT1;1* and *LpPHT1;4* genes was up-regulated by Al and was more marked in the Nui cultivar. The mechanisms by which phosphate transporters are involved in Al tolerance can be explained by an increase in P uptake leading to precipitation and immobilization of Al in roots (Gaume et al., 2001; Zheng et al., 2005). Moreover, the increased *PHT1* transcript levels in shoots of the Al-treated plants suggest that higher root-to- shoot P translocation is required to maintain optimal phosphate levels, leading to reduced Al stress. These data suggest that Al-tolerance in ryegrass is due to its efficient mechanism to transport P and greater antioxidant activity, allowing greater alleviation of Al-toxicity.

Author contributions

This research was carried out in collaboration between all authors. LPA and MLM defined the research theme. LPA performed all the experiments with the assistance of ADC and under the supervision of NF and MLM. LPA, NF and MLM wrote the manuscript. All authors have contributed and approved the manuscript.

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CHAPTER V

General discussion, concluding remarks and future directions

5. General discussion, concluding remarks and future directions

5.1 General discussion

As mentioned in the previous chapters, phosphorus (P) is an essential nutrient indispensable for plant growth and development. However, about 50% of agricultural soils in the world are P deficient (MacDonald et al., 2011). At a local scale, the agricultural production in Southern Chile is carried out in acid soils with large amount of P-fixed and toxic levels of aluminum (Al). Thus, enhancement of the ability to acquire P as well as improvement of Al tolerance of crops represents an emerging opportunity to reach agricultural sustainability on acid soils. According to these assumptions we studied the P uptake system and evaluated P nutrition and Al toxicity interaction on pasture grass (*Lolium perenne* L.) grown under acidic conditions.

Several studies have shown that an efficient mechanism of phosphate (Pi) transport from the soil and within the plant enhances phosphorus acquisition efficiency (PAE) and phosphors utilization efficiency (PUE) (Wang et al., 2010; Liu et al., 2016; Heuer et al., 2017; Luan et al., 2017; Maharajan et al., 2018). We successfully identified by the transcriptome assembly, three phosphate transporters (PHT1s) in ryegrass plants. The PHT1s found (LpPHT1;1/4/6) showed a high amino acid sequence similarity to a large number of previously identified Pi transporters from the Poaceae family (Figure S1 of Annex 1). Different studies of these three Pi transporters in the model plants *Arabidopsis* and rice have showed that they have a main role in Pi uptake and translocation within the plant. For example, *ArPHT1;1* and *ArPHT1;4* have the highest transcription rates in response to Pi deprivation, thus the double mutant plants *pht1;1/pht1;4* result in a 75% to

80% reduction of Pi uptake in *Arabidopsis* (Shin et al., 2004). Likewise, the OsPHT1;4 plays an important role in Pi uptake and in the development of the embryo in rice, whereas OsPHT1;1/6 plays a broad role in Pi uptake and translocation throughout the plant (Ai et al., 2009; Zhang et al., 2015).

In general, most of PHT1 members are induced by P deficiency in several plant species (Guo et al., 2011; Nussaume et al., 2011). As expected, our results have shown that all Pi transporter genes were up-regulated during Pi deficiency at long- or short-term in the shoot and root tissues (Chapter III-IV), indicating that they are involved in Pi uptake and Pi redistribution in ryegrass.

Complementation experiments in *S. cerevisiae* have been widely used to functionally characterize Pi transporters in plants (Ai et al., 2009; Liu et al., 2014; Ye et al., 2015). Based on this test, only two Pi transporters from ryegrass (LpPHT1;1 and LpPHT1;4) were successfully functionally characterized (chapter III, Fig. 3), which could mean that the LpPHT1;6 was toxic to competent cells. In fact, some genes that encode ion channels and transporters cannot be cloned by conventional techniques requiring *E. coli* (Vu et al., 2009). Our results showed that the LpPHT1;4, but not LpPHT1;1, can rescue the growth of yeast mutant defective in high-affinity Pi transport under low Pi conditions. These data suggest that LpPHT1;4 is a high-affinity Pi transporter and LpPHT1;1 might operate with a low-affinity system in ryegrass (chapter III). These findings are in agreement with the role of PHT1;1 from rice (Ai et al. 2009; Sun et al. 2012) and PHT1;4 from wheat (Liu et al. 2013; Teng et al., 2017). Likewise, phylogenetic analysis of LpPHT1s showed a high similarity with other Pi transporters from rice, wheat and maize. LpPHT1;1 was grouped in the phylogenetic tree with OsPHT1;1/2 and TaPHT1;1/2 which have been shown to be a low-

affinity transporter expressed under all Pi conditions. Similarly, LpPHT1;4 was clustered with orthologues from wheat (TaPHT1;4) and barley (HvPHT1;4), which act as high-affinity transporter. While the functional analysis of LpPHT1;6 has not been achieved, phylogenetic analysis suggested that is closely related to the HvPHT1;6 and TaPHT1;6 (Figure S2, Anexx 1). HvPHT1;6 is a low-affinity Pi transporter (Preusset al., 2010), which plays a role in Pi remobilization in the whole plant (Hunag et al., 2011). Thus, this Pi transporter could have the same function in ryegrass.

Although both Pi transporters showed conserved Pi-binding residues (Figure S1 of Annex 1), post-translational modifications may be important in regulating their Pi transport affinities.

In acid soils where low Pi availability coexist with Al toxicity, the plants have developed numerous adaptive strategies to cope with both stresses (Kochian et al., 2004; Sun et al., 2016), including exudation of organic acids (OAs) and changes in root growth. However, the impact of P nutrition on Al toxicity still remains controversial and poorly understood. In this sense, our results showed that expression of Pi transporters in ryegrass not only was up-regulated by P deficiency but also by Al toxicity (chapter IV, Fig. 6). This observation could explain the increased P accumulation in root and shoot tissues, which in turn will reduce Al stress, mostly on the Al-tolerant cv. Nui (chapter IV).

Several studies have demonstrated that Al-tolerant genotypes showed higher P uptake and utilization efficiency than the Al-sensitive ones (Kochian et al., 2015; Heuer et al., 2017; Seguel et al., 2017). Similarly, P uptake efficiency in barley has been attributed to the transcript abundance of the *PHT1* genes (Huang et al., 2011). These reports agree with our

results, as the Al-tolerant Nui cultivar displayed higher transcript levels of Pi transporters than the Al-sensitive Expo cultivar.

Ryegrass cultivars with contrasting Al-tolerance also exhibited different antioxidant activities (chapter IV, Figs. 2-3). The enhanced antioxidant activity (RSA) of the Al-tolerant cultivar Nui under both stresses was accompanied by higher activity of antioxidant enzymes, allowing to less lipid peroxidation (chapter IV, Fig. 1). In this context, it is well known that Al tolerance in plants is due to an efficient antioxidant system (Ma et al., 2012; Hernández and Munné-Bosch, 2015).

Currently, molecular mechanisms underlying P efficiency and Al resistance in crops have also emerged (Magalhaes et al., 2018). The genes encoding membrane transporters which facilitate the efflux of malate (ALMT) and citrate (MATE) across the plasma membrane are the primary genes involved in Al resistance in plants (Ryan et al., 2001; Kochian et al., 2015). Besides, other membrane transporters have been implicated in Al-resistance such as ABC transporter (Huang et al., 2009) and Nramp (Nrat1) (Xia et al., 2010). An enhanced expression of these genes could result in improved Pi uptake efficiency when plants grown on acid soils (Heuer et al., 2017). Hence, we also evaluated the expression level of the putative *Nrat1* transporter in ryegrass, which transports Al^{+3} into root cells to finally sequester it into the vacuole (Annex 2). Our results showed that the expression level of *Nrat1* was enhanced by P deficiency and Al toxicity (Figure S2.2 of Annex 2). These results could indicate that P deficiency enhanced Al transport, by a common signalling mechanism between Al toxicity and P deficiency, as it has been previously described (Sun et al., 2016). Therefore, improved P acquisition and utilization through the identification

and manipulation of Al resistance genes is a promising approach to produce pastures with enhanced PAE.

5.2 Concluding remarks and future directions

Our research showed that *LpPHT1;1*, *LpPHT1;4* and *LpPHT1;6* encode typical Pi transporters in ryegrass plants, with characteristics conserved among Pi transporters from Poaceae family. The functional analysis in yeast also reveals that LpPHT1;1 migth function as a low-affinity Pi transporter, whereas LpPHT1;4 acts as a high-affinity Pi transporter, demonstrating their roles in phosphate uptake and phosphate translocation under both Pi-sufficient and Pi-deficient conditions.

We report for the first time that *LpPHT1* genes were up-regulated by Al, mostly in Al-tolerant ryegrass cultivar. This finding can be explained by an increase of P uptake leading to precipitate and immobilize Al in roots. Our data also suggest that Al-tolerance in ryegrass is due to its efficient mechanism to transport P from growth solution and greater antioxidant activity allowing a more alleviation of Al-toxicity.

This thesis project contributes to improving our knowledge of the molecular and biochemical mechanisms underlying P-efficient and Al-tolerant ryegrass genotypes. Thus, a new model including these mechanisms was elaborated (chapter V, Fig 1). Further studies under field conditions are needed to establish the functional roles and differences in PHT1s on P uptake and utilization efficiency in ryegrass grown on acid soils. Understanding the function of Al resistance genes on P stress responses could also promote further improvement of crops adaptation to acidic soils.

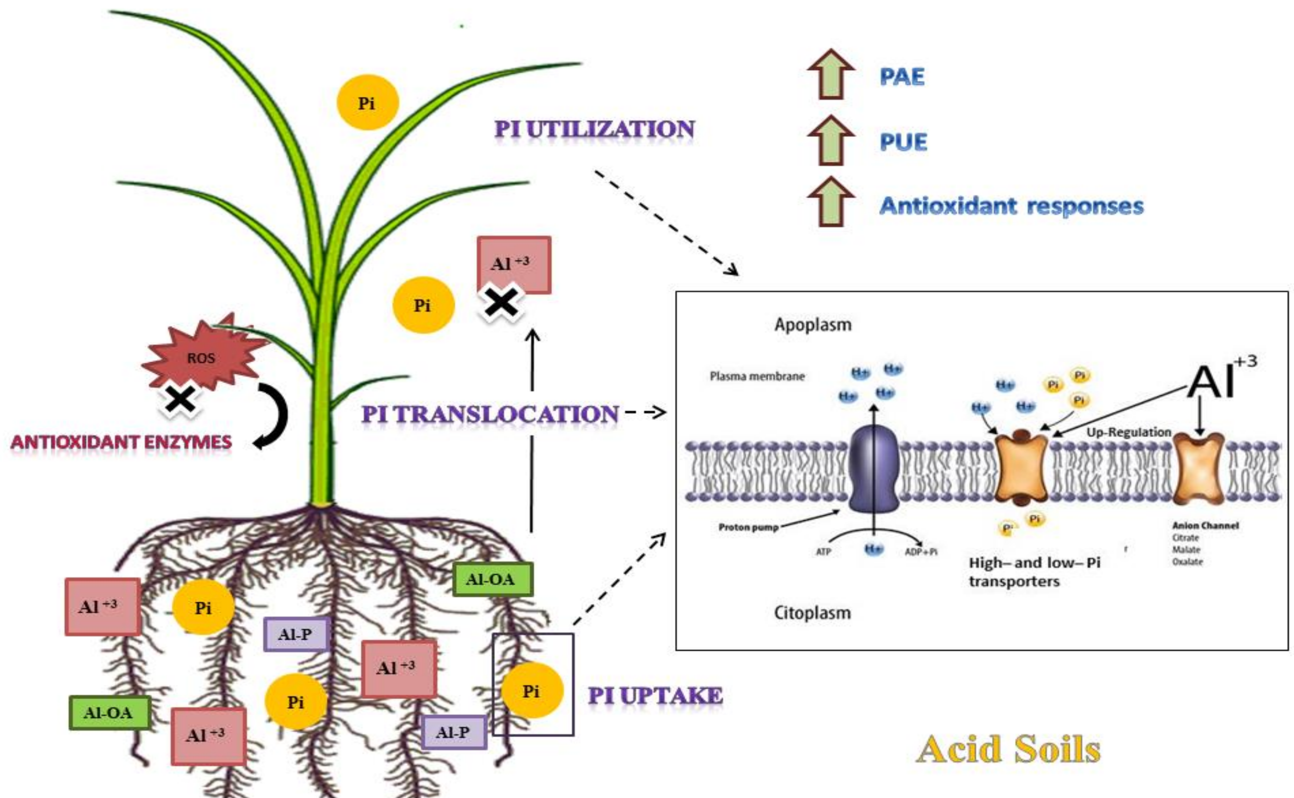

Figure 1. Proposed model illustrating the mechanisms of aluminum (Al) tolerance and phosphorus (P) use efficiency in ryegrass (*Lolium perenne* L.) plants when grown in acid soils. Under low phosphate (Pi) availability and Al toxicity, ryegrass plants enhanced P aquisition efficiency (PAE) and P utilization effIciency (PUE) mediated by up-regulation of high- and low-Pi transporters (*LpPHT1;1*, *LpPHT1;4* and *LpPHT1;6*). Higuer Pi uptake increases the formation of Al-P complexes in root tissues leading to less Al translocation in shoots. Al tolerance involves greater antioxidant responses and Al-OA complex formation allowing an increased alleviation of Al-toxicity.

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Annex 1

Supporting information about bioinformatic analysis of Pi transporters from ryegrass

Figure S1. Multiple sequence alignment of LpPHT1;1 and LpPHT1;4 and PHT1 transporters reported in Poaceae family. Identical amino acid residues were shaded in blue. Conserved residues of LpPHT1;1 and LpPHT1;4 involved in Pi binding transport were indicated in red letters.

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OsPHT13     --------MAGNQQLRVLHALDIARTQLYHFIAIVIAGMGFFTDAYDLFSISLVADLLGH   52
ZmPHT5      --------MALGQQLRVLHALDVARTQLYHFMATAIAGMGFFTDAYDFFAISLVMDLISY   52
ZmPHT12     --------MALGQQLRVLHALDVARTQLYHFMATAIAGMGFFTDAYDFFAISLVMDLISY   52
ZmPHT13     --MLIESRQMVRRQLRVLSALDVARTQLYHFMAIVIAGMGFFTDAYDFFSISLVIDLISF   58
OsPHT9      ----------MAPRIRVLAALDQARTQYYHFKAIVIAGMGLFTDSYDLFCISPVMKIFGR   50
OsPHT10     -----------MAPIGVLTALDQARTQYYHFKAIVIAGMGLFTDSYDLFCIAPVMKIVGR   49
TaPHT11     MAENGAGGGGGGQNLAVLDALDSARTQMYHMKAIVIAGMGFFTDAYDLFCITTVSKLLGR   60
TaPHT12     MAENGAGGGGGGQNLAVLDALDSARTQMYHMKAIVIAGMGFFTDAYDLFCITTVSKLLGR   60
OsPHT11     -----MADADGGSNLAVLDALDSARTQMYHMKAIVIAGMGFFTDAYDLFCISTVSKLLGR   55
ZmPHT6      ------MAAPGGSNLAVLDALDSARTQMYHMKAIVIAGMGFFTDAYDLFCISTVSKLLGR   54
ZmPHT11     ---------MALEKLQVLHALDVADTQRYHVRAVVIAGMGFFADAYDLFCITLVTKLLGR   51
HvPHT8      ---------MARQQLQVLHALDVARTQRYHAWAVVIAGMGFFADAYDIFCITLVTKLLGR   51
TaPHT8      ---------MARQQLQVLHALDVARTQRYHAWAVVIAGMGFFADAYDIFCITLVTKLLGR   51
LpPHT1      ---------MAGEQLNVLKALDQAKTQWYHFTAVVIAGMGFFTDAYDLFCISLVTRLLGR   51
TaPHT2      ---------MATEQLNVLKALDVAKTQLYHFKAVVIAGMGFFTDAYDLFCIALVTKLLGR   51
TaPHT10     ---------MATEQLNVLKALDVAKTQLYHFKAVVIAGMGFFTDAYDLFCIALVTKLLGR   51
HvPHT1      ---------MATEQLNVLKALDVAKTQLYHFKAVVIAGMGFFTDAYDLFCIALVTKLLGR   51
HvPHT9      ---------MATEQLNVLKALDVAKTQLYHFKAVVIAGMGFFTDAYDLFCIALVTKLLGR   51
HvPHT2      ---------MATEQLNVLKALDVAKTQLYHFKAVVIAGMGFFTDAYDLFCIALVTKLLGR   51
HvPHT10     ---------MATEQLNVLKALDVAKTQLYHFKAVVIAGMGFFTDAYDLFCIALVTKLLGR   51
TaPHT1      ---------MATEQLNVLKALDVAKTQLYHFKAVVIAGMGFFTDAYDLFCIALVTKLLGR   51
TaPHT9      ---------MATEQLNVLKALDVAKTQLYHFKAVVIAGMGFFTDAYDLFCIALVTKLLGR   51
OsPHT2      ---------MAGSQLNVLVKLDQAKTQWYHFMAIVIAGMGFFTDAYDLFCIALVTKLLGR   51
OsPHT1      ---------MAGGQLNVLSTLDQAKTQWYHFMAIVIAGMGFFTDAYDLFCISLVTKLLGR   51
OsPHT3      ---------MADGQLKVLTTLDHARTQWYHFMAIVIAGMGFFTDAYDLFCISLVSKLLGR   51
ZmPHT10     ------MAMAGGQNMQVLSALDGAKTQRYHFTAIVVAGMGFFTDAYDLFCISLVTKLIGR   54
OsPHT7      ---------MAGDQMHVLSALDSAKTQWYHFTAIVIAGMGFFTDAYDLFCISLVTKLIGR   51
HvPHT7      ---------MAGDQVHVLAALDGAKTQWYHFTAIVVAGMGFFTDAYDLFCISLVTKLIGR   51
TaPHT7      ---------MAGDQVHVLSALDGAKTQWYHFTAIIVAGMGFFTDAYDLFCISLVTKLIGR   51
HvPHT6      ---------MAREQLEVLSALDTAKTQWYHFTAIVIAGMGFFTDAYDLFCISLVTKLLGR   51
TaPHT6      ---------MAREQLEVLTALDAAKTQWYHFTAIVIAGMGFFTDAYDLFCISLVTKLLGR   51
OsPHT6      ----MGGGGGEQQQLEVLHALDVAKTQWYHFTAIVVAGMGFFTDAYDLFCISLVTKLLGR   56
ZmPHT7      ---------MAAGDLEVLTALDTAKTQWYHFTAIVVAGMGFFTDAYDLFCISLVTKLLGR   51
ZmPHT3      --------------------------------------MGFFTDAYDLFSISLVTKLLGR   22
ZmPHT8      --------------------------------------MGFFTDAYDLFSISLVTKLLGR   22
OsPHT4      ----------MAGELKVLNALDSAKTQWYHFTAIVIAGMGFFTDAYDLFSISLVTKLLGR   50
OsPHT5      ----------MVQDRKVLDALDTAKTQWYHFTAVVIAGMGFFTDAYDLFSISLVTKLLGR   50
ZmPHT1      -------MARGGDGLQVLSALDAAKTQWYHFTAIIVAGMGFFTDAYDLFCISLVTKLLGR   53
ZmPHT9      -------MARGGDGLQVLSALDAAKTQWYHFTAIIVAGMGFFTDAYDLFCISLVTKLLGR   53
ZmPHT2      -------MARGGDGLQVLSALDAAKTQWYHFTAIIVAGMGFFTDAYDLFCISLVTKLLGR   53
ZmPHT4      -------MARGGDGLQVLSALDAAKTQWYHFTAIIVAGMGFFTDAYDLFCISLVTKLLGR   53
OsPHT8      -----MARQEQQQHLQVLSALDAAKTQWYHFTAIVVAGMGFFTDAYDLFCISLVTKLLGR   55
LpPHT4      ------MARSEQQGLQVLSALDAAKTQWYHFTAIVVAGMGFFTDAYDLFCISLVTKLLGR   54
HvPHT4      ------MARSEQQGLQVLSALDAAKTQWYHFTAIVVAGMGFFTDAYDLFCISLVTKLLGR   54
TaPHT4      ------MARSEQQGLQVLSALDAAKTQWYHFTAIVVAGMGFFTDAYDLFCISLVTKLLGR   54
OsPHT12     -------MGRQDQQLQVLNALDAAKTQWYHFTAIIVAGMGFFTDAYDLFCISLVTKLLGR   53
HvPHT5      ------MASRQQQQLQVLSALDGAKTQLYHFRAVVVAGMGFFTDAYDLFCISLVTKLLGR   54
TaPHT5      ---------MASRQLQVLSALDGAKTQWYHFRAIVVAGMGFFTDAYDLFCISLVTKLLGR   51

OsPHT13     VYYHG------------ELPRNIHAAVTGIALCGTVPGQLVFGWLGDKMGRKRVYGITLL   100
ZmPHT5      LYNEE------------QIDRGVKATINGIALCGAVPGQLVFGWLGDKMGRKRIYGVTLL   100
ZmPHT12     LYNEE------------QIDRGVKATINGIALCGAVPGQLVFGWLGDKMGRKRIYGVTLL   100
ZmPHT13     QYYDG------------HMRSAVKAAINGIALCGALPGQLVFGWLGDKMGRKRIYGVTLV   106
OsPHT9      VYYAPSGSVDGSGSGPGVTPPAVVSATVGVALLGAVAGNVVFGALGDRVGRRRVYGACLL   110
OsPHT10     VYYSDG------GARPGVTPPAVVSATVGVALLGAVIGNVVFGALGDRVGRRRVYGACLL   103
TaPHT11     LYYPDSNA---DIGKPGTMPVRINNMVTGVALVGTLMGQLVFGYFGDKLGRKRVYGITLV   117
TaPHT12     LYYPDSNA---DIGKPGTMPVRINNMVTGVALVGTLMGQLVFGYFGDKLGRKRVYGITLV   117
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OsPHT11     LYYQPDGS---TDSKPGALSKTANNMVIGVALVGTLMGQLVFGYFGDKLGRKRVYGVTLI   112
ZmPHT6      IYYPDDNLYI-DKPKPGTLPVSVNNMVTGVALVGTLMGQLVFGYFGDKLGRKRVYGITLV   113
ZmPHT11     IYYHVPG-----REEPGRLPPRLEAAINGVTFCGMIVGQLLFGWLGDKVGRKKFYGKTIM   106
HvPHT8      IYYHVPG-----QPDPGMLPRRIEAAINGVTFCGMIVGQLLFGWLGDKVGRKMFYGKTIM   106
TaPHT8      IYYQVPG-----QREPGMLPRRIEAAINGVTFCGMIVGQLLFGWLGDKVGRKMFYGKTIM   106
LpPHT1      IYYTEAG-----SNEPGHLPANVSAAVNGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLI   106
TaPHT2      IYYTDPA-----LNEPGHLPANVSAAVNGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLI   106
TaPHT10     IYYTDPA-----LNEPGHLPANVSAAVNGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLI   106
HvPHT1      IYYTDPA-----LNEPGHLPANVSAAVNGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLI   106
HvPHT9      IYYTDPA-----LNEPGHLPANVSAAVNGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLI   106
HvPHT2      IYYTDPA-----LNEPGHLPANVSAAVNGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLI   106
HvPHT10     IYYTDPA-----LNEPGHLPANVSAAVNGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLI   106
TaPHT1      IYYTDPA-----LNEPGHLPANVSAAVNGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLI   106
TaPHT9      IYYTDPA-----LNEPGHLPANVSAAVNGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLI   106
OsPHT2      LYYTDIT-----KPNPGTLPPNVSSAVTGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLI   106
OsPHT1      IYYTDDS-----KDTPGALPPNVSAAVTGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLI   106
OsPHT3      IYYTDLA-----GDNPGSLPPNVSAAVNGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLV   106
ZmPHT10     IYYTVDG-----SPRPGSLPPHVSAAVNGVAFVGTLSGQLFFGWLGDRVGRKSVYGMTLL   109
OsPHT7      VYYTADG-----ASKPGSLPPNVSAAVNGVAFVGTLTGQLFFGWLGDRVGRKSVYGMTLL   106
HvPHT7      IYYTVPG-----SPSPGSLPPTVSAVVNGVAFVGTLSGQLFFGWLGDKVGRKSVYGMTLM   106
TaPHT7      IYYTVPG-----SSRPGSLPPTVSAVVNGVAFVGTLSGQLFFGWLGDKVGRKSVYGMTLM   106
HvPHT6      IYYYREG-----ADAPGSLPPNVAAAVNGVAFCGTLSGQLFFGWLGDRMGRKRVYGMTLM   106
TaPHT6      IYYYREG-----ADAPGSLPPNVAAAVNGVAFCGTLSGQLFFGWLGDRMGRKRVYGMTLM   106
OsPHT6      IYYRVDG-----SPSPGTLPPHVSASVNGVAFVGTLSGQLFFGWLGDKLGRKRVYGITLM   111
ZmPHT7      IYYTVEG-----SATPGTLPPHVSASVNGVAFVGTLSGQLFFGWLGDKLGRKKVYGMTLM   106
 ZmPHT3      IYYSDPS-----SKTPGSLPPNVSAAVNGVAFCGTLAGQLFFGWLGDKMGRKKVYGMTLM    77
 ZmPHT8      IYYSDPS-----SKTPGSLPPNVSAAVNGVAFCGTLAGQLFFGWLGDKMGRKKVYGMTLM    77
OsPHT4      IYYFNPA-----SKSPGSLPPNVSAAVNGVAFCGTLAGQLFFGWLGDKMGRKKVYGMTLM   105
OsPHT5      IYYFNPA-----SKSPGSLPPNVSAAVNGVAFCGTLAGQLFFGWLGDKMGRKKVYGMTLM   105
ZmPHT1      IYYTDTS-----KDNPGSLPPNVAAAVNGVAFCGTLAGQLFFGWLGDKLGRKSVYGMTLM   108
ZmPHT9      IYYTDTS-----KDNPGSLPPNVAAAVNGVAFCGTLAGQLFFGWLGDKLGRKSVYGMTLM   108
ZmPHT2      IYYTDTS-----KDSPGSLPPNVAAAVNGVAFCGTLAGQLFFGWLGDKLGRKSVYGMTLM   108
ZmPHT4      IYYTDTS-----KDSPGSLPPNVAAAVNGVAFCGTLAGQLFFGWLGDKLGRKSVYGMTLM   108
OsPHT8      IYYTDLA-----KENPGSLPPNVAAAVNGVAFCGTLAGQLFFGWLGDKLGRKSVYGMTLL   110
LpPHT4      IYYTDPS-----LPNPGTLPPGVAAAVNGVAFCGTLSGQLFFGWLGDKLGRKSVYGMTLI   109
HvPHT4      IYYTDLS-----KPDPGSLPPSVAAAVNGVAFCGTLAGQLFFGWLGDKMGRKSVYGMTLL   109
TaPHT4      IYYTDLS-----KPDPGTLPPGVAAAVNGVAFCGTLAGQLFFGWLGDKMGRKSVYGMTLI   109
OsPHT12     IYYTDPA-----SPTPGSLPPNIAAAVNGVALCGTLSGQLFFGWLGDKLGRKSVYGMTLL   108
HvPHT5      IYYADPS-----SPNPGSLPPNVAAAVNGVALCGTLAGQLFFGWLGDRLGRKSVYGMTLI   109
TaPHT5      IYYADPS-----SPTPGSLPPNVAAAVNGVALCGTLVGQLFFGWLGDRLGRKSVYGMTLM   106


OsPHT13     LMVVSSLASGLSFSKHEGMNIIAVLCFFRFWLGVSIGGDYPLSATIMSEYANKRTRGAFI   160
ZmPHT5      LMVVTSLASGLYFGTNEASNVVAVLCFFRFWLGFGIGGDYPLSATIMSEYANKRRRGAFI   160
ZmPHT12     LMVVTSLASGLYFGTNEASNVVAVLCFFRFWLGFGIGGDYPLSATIMSEYANKRRRGAFI   160
ZmPHT13     LMVVTSLASGLSFSKRRGKNVVTVLCFFRFWLGVSIGGDYPLSATIMSEYANKRRRGAFI   166
OsPHT9      LMVCSSVGSGLSVCRT-RRCALASLCFFRFLLGVGVGGDYPLSATIMSEFANRRTRGAFI   169
OsPHT10     LMVCSSVGSGFSVCRT-RRCALASLCFFRFLLGVGVGGDYPLSATIMSEFANRRTRGAFI   162
TaPHT11     LMAVCAIGSGLSFGSS-ASAVIGTLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFI   176
TaPHT12     LMAVCAIGSGLSFGSS-ASAVIGTLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFI   176
OsPHT11     LMAACAIGSGLSFGSS-RKAVIGTLCFFRFWLGFGIGGDYPLSATIMSEYSNKKTRGAFI   171
ZmPHT6      LMAACAIGSGLSFGSS-AHAVIGTLCFFRFWLGFGIGGDYPLSATIMSEYSNKKTRGAFI   172
ZmPHT11     LMIMGSFLSGLSFGNS-ADGVMATLCFFRFWLGVGIGGDYPLSATIMSEYANKRTRGSFV   165
HvPHT8      LMIMGSFLSGLSFGNT-ADGVMATLCFFRFWLGVGIGGDYPLSATIISEYSNKRSRGSLI   165
TaPHT8      LMIMGSFLSGLSFGNT-ADGVMATLCFFRFWLGVGIGGDYPLSATIISEYSNKRSRGSLI   165
LpPHT1      LMVLCSIASGLSFGHE-AKGVIGTLCFFRFWLGFGVGGDYPLSATIMSEYANKKTRGTFI   165
TaPHT2      LMVLCSIASGLSFGHE-AKGVMGTLCFFRFWLGFGVGGDYPLSATIMSEYANKKTRGTFI   165
TaPHT10     LMVLCSIASGLSFGHE-AKGVMGTLCFFRFWLGFGVGGDYPLSATIMSEYANKKTRGTFI   165
HvPHT1      LMVLCSIASGLSFGHE-AKGVMGTLCFFRFWLGFGVGGDYPLSATIMSEYANKKTRGTFI   165
HvPHT9      LMVLCSIASGLSFGHE-AKGVMGTLCFFRFWLGFGVGGDYPLSATIMSEYANKKTRGTFI   165
HvPHT2      LMVLCSIASGLSFGHE-AKGVMGTLCFFRFWLGFGVGGDYPLSATIMSEYANKKTRGTFI   165
HvPHT10     LMVLCSIASGLSFGHE-AKGVMGTLCFFRFWLGFGVGGDYPLSATIMSEYANKKTRGTFI   165
TaPHT1      LMVLCSIASGLSFGHE-AKGVMGTLCFFRFWLGFGVGGDYPLSATIMSEYANKKTRGTFI   165
TaPHT9      LMVLCSIASGLSFGHE-AKGVMGTLCFFRFWLGFGVGGDYPLSATIMSEYANKKTRGTFI   165
OsPHT2      LMVVCSIASGLSFGHT-PKSVIATLCFFRFWLGFGIGGDYPLSATIMSEYASKKTRGAFI   165
OsPHT1      LMVVCSVASGLSFGSS-AKGVVSTLCFFRFWLGFGIGGDYPLSATIMSEYANKRTRGAFI   165
OsPHT3      LMVVCSVASGLSFGRT-AKGVVATLCFFRFWLGFGIGGDYPLSATIMSEYANKRTRGAFI   165
ZmPHT10     TMVVCSVASGLSFGRS-PACVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKRTRGAFV   168
OsPHT7      LMIICSVASGLSFGDT-PTSVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKRTRGAFI   165
HvPHT7      LMIICSVASGLSFGRT-PTSVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKRTRGAFI   165
TaPHT7      LMILCSVASGLSFGNT-PTSVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKRTRGAFI   165
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HvPHT6        CMVLCSIASGLSFGST-PGSVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFI   165
TaPHT6        CMVLCSIASGLSFGST-PGSVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFI   165
OsPHT6        LMVLCSLASALSFGHT-PTSVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFI   170
ZmPHT7        LMVLCSVASGLSFGHT-PASVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFI   165
ZmPHT3        LMAICCLASGLSFGST-PKDVMVTLCFFRFWLGVGIGGDYPLSATIMSEYANKRTRGAFI   136
ZmPHT8        LMAICCLASGLSFGST-PKDVMVTLCFFRFWLGVGIGGDYPLSATIMSEYANKRTRGAFI   136
OsPHT4        LMVICCLASGLSFGSS-AKGVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKRTRGAFI   164
OsPHT5        LMVICCLASGLSFGSS-AKGVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKRTRGAFI   164
ZmPHT1        LMVICSVASGLSFGHT-PTGVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFI   167
ZmPHT9        LMVICSVASGLSFGHT-PTGVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFI   167
ZmPHT2        VMVICSVASGLSFGHT-PTGVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKRTRGAFI   167
ZmPHT4        VMVICSVASGLSFGHT-PTGVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKRTRGAFI   167
OsPHT8        MMVICSIASGLSFSHT-PTSVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFI   169
LpPHT4        LMVICSLGSGLSLAHT-PKSVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFI   168
HvPHT4        LMVICSIGSGLSFAHT-PKSVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFI   168
TaPHT4        LMVICSIGSGLSFAHT-PKSVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFI   168
OsPHT12       LMVICSIASGLSFSHT-PTSVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFI   167
HvPHT5        LMVVCSVASGLSFGHT-PASVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFI   168
TaPHT5        LMVVCSVASGLSFGHT-PASVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFI   165


OsPHT13       AAVFAMQGFGNLAAGIIGMIVSAAFKHSSA-------------------SKIDYAWRIIL   201
ZmPHT5        AAVFAMQGFGNLAAGIVAVVVSASFLRTNP------------------RRNANFVWRIVL   202
ZmPHT12       AAVFAMQGFGNLAAGIVAVVVSASFLRTNP------------------RRNANFVWRIVL   202
ZmPHT13       AAVFAMQGFGNLAAGIVGMVVSAAFTNSAP-------------------SNADFVWRIVL   207
OsPHT9        AAVFSMQGFGILVSSAVTMAVAAAFDHYTGYPA-----------PLDTPECADLAWRIIL   218
OsPHT10       AAVFSMQGFGILASSAVTMAVAAAFDHYTGYPA-----------PLDTPECADLAWRIIL   211
TaPHT11       AAVFAMQGVGIIFAGLVSMIVSAIFLHYNPAPAWNVHHGWTVDHQIEQWPGADYMWRVVL   236
TaPHT12       AAVFAMQGVGIIFAGLVSMIVSAIFLHYNPAPAWDAHHGLTVDGQMDQWPGADYMWRVVL   236
OsPHT11       AAVFAMQGVGIIFAGLVSMIVSSIFLTYNKAPSYKGNHDLS-----RQMPAADYVWRIVL   226
ZmPHT6        AAVFAMQGVGIIFAGLVSMIVSGILLHYHPAPAWKENHDRSWQ---DQMPAADYMWRIVL   229
ZmPHT11       AAVFAMEGFGVLAGCIVTLVVSATFQARFSAPAYEEDPAAS------TPPQADYAWRIVL   219
HvPHT8        AAVFAMEGFGILAGCIVTLVVSATFQARFNPPAYEEDPMAS------VPPQADYVWRIIL   219
TaPHT8        AAVFAMEGFGILAGCIVTLVVSATFQARFNPPAYDEDHMAS------VPPQADYVWRIIL   219
LpPHT1        AAVFAMQGFGILFGTIVTIVVSSAFRNAFPAPPFYVDATSS------IGPEADYVWRIIV   219
TaPHT2        AAVFAMQGFGILFGTIVTIIVSSAFRHAFPAPPFYINAAAS------IGPEADYVWRIIV   219
TaPHT10       AAVFAMQGFGILFGTIVTIIVSSAFRHAFPAPPFYIDAAAS------IGPEADYVWRIIV   219
HvPHT1        AAVFAMQGFGILFGTIVTIIVSSAFRHAFPAPPFYIDAAAS------IGPEADYVWRIIV   219
HvPHT9        AAVFAMQGFGILFGTIVTIIVSSAFRHAFPAPPFYIDAAAS------IGPEADYVWRIIV   219
HvPHT2        AAVFAMQGFGILFGTIVTIIVSSAFRHAFPAPPFYIDAAAS------IGPEADYVWRIIV   219
HvPHT10       AAVFAMQGFGILFGTIVTIIVSSAFRHAFPAPPFYIDAAAS------IGPEADYVWRIIV   219
TaPHT1        AAVFAMQGFGILFGTIVTIIVSSAFRHAFPAPPFYIDAAAS------IGPEADYVWRIIV   219
TaPHT9        AAVFAMQGFGILFGTIVTIIVSSAFRHAFPAPPFYIDAAAS------IGPEADYVWRIIV   219
OsPHT2        AAVFAMQGFGILFGAIVALVVSAGFRHAYPAPSYAQNPAAS------LAPQADYTWRLIL   219
OsPHT1        AAVFAMQGFGILFGAIVALAVSAGFRHAYPAPSYSDNHAAS------LVPQADYVWRIIL   219
OsPHT3        AAVFAMQGFGILFGAIVALVVSAGFRNAYPAPSYADGRAAS------LVPEADYVWRIIL   219
ZmPHT10       AAVFAMQGFGILAGGAVAIGVTALFRSRFPAPPYDVDPAAS------TPAEADLVWRIIL   222
OsPHT7        AAVFAMQGFGILAGGAVAIGITAIFRSRFPAPPFAADPAAS------TPPQADYVWRLIL   219
HvPHT7        AAVFAMQGFGILAGGGVAIGITALFRALFPAPPYAADPVAS------TPDQADYVWRIVL   219
TaPHT7        AAVFAMQGFGILAGGGVAIGITALFRDLFPAPPYAADPAAS------TPAQADYVWRIVL   219
HvPHT6        AAVFAMQGFGILTGGVVTLIVSAAFRAAFHAPAYEKGAVAS------TPPQADFVWRFIL   219
TaPHT6        AAVFAMQGFGILTGGVVTLIVSAAFRAAFPTPAYKDGALAS------TPPQADFVWRFIL   219
OsPHT6        AAVFAMQGFGIITGGLVAILVSASFRAAFPAPPYGEDPVAS------TPPQADFVWRIIL   224
ZmPHT7        AAVFAMQGFGIMAGGLVAIVVSASFKARFPAPAYAVDPAGS------TPPQADFVWRIIL   219
ZmPHT3        AAVFAMQGFGNLAGGIVAIAVSAAFKSRFDAPAYKDDPAGS------TVPQADYVWRIVL   190
ZmPHT8        AAVFAMQGFGNLAGGIVAIAVSAAFKSRFDAPAYKDDPAGS------TVPQADYVWRIVL   190
OsPHT4        AAVFAMQGFGNLTGGIVAIIVSAAFKSRFDAPAYRDDRTGS------TVPQADYAWRIVL   218
OsPHT5        AAVFAMQGFGNLTGGIVAIIVSAAFKLRFDAPAYRDDRAGS------TVPQADYAWRIVL   218
ZmPHT1        AAVFAMQGFGILAGGIVTLVISAAFRAGYPAPAYRDDHFNS------TVPQADYVWRIIL   221
ZmPHT9        AAVFAMQGFGILAGGIVTLVISAAFRAGYPAPAYRDDHFNS------TVPQADYVWRIIL   221
ZmPHT2        AAVFAMQGFGILAGGIVTLVISAAFRAAYPSPAYRDDHFTS------TVPQADFVWRVIL   221
ZmPHT4        AAVFAMQGFGILAGGIVTLVISAAFRAAYPSPAYRDDHFTS------TVPQADIVWRVIV   221
OsPHT8        AAVFAMQGFGILAGGIVTLIISSAFRAGFPAPAYQDDRAGS------TVRQADYVWRIIL   223
LpPHT4        AAVFAMQGFGILAGGIVTLIISAAFRAAYPKPAYQDNATGS------IGSEADFVWRIIL   222
HvPHT4        AAVFAMQGFGILAGGIVTLIISSAFRAGFHEPAYQDDRVAS------TGTEADFVWRIIL   222
TaPHT4        AAVFAMQGFGILAGGIVTLIISSAFRAGFHEPAYQDDRVAS------TGTEADFVWRIIL   222
OsPHT12       AAVFAMQGFGILAGGVVTLAMSAGFQAAFPAPAYEVNAAAS------TVPQADYVWRIIL   221
HvPHT5        AAVFAMQGFGILAGGVVTLVLSTVFRSAFPAPAYQTDAAAS------TVPQADYVWRIIL   222
TaPHT5        AAVFAMQGFGILAGGVVTLVLSTVFRNAFPAPAYQVDAAAS------TVPQADYVWRIIL   219
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OsPHT13     MFGAIPAALTYHWRMKMPETARYTALISKNAKKAAKDMSAVLNVNITPDDEVINELA---   258
ZmPHT5      MLGAVPAILTYYWRMKMPETARYTALVAKDARKAASDMSSVLHVEIIPEDEAV-------   255
ZmPHT12     MLGAVPAILTYYWRMKMPETARYTALVAKDARKAASDMSSVLHVEIIPEDEAV-------   255
ZmPHT13     MFGAVPATLTYYWRMKMPETARYTALVAKDAKKVASDMSAVLNMHIVPEDEAVYELA---   264
OsPHT9      MAGAVPAALTYYWRMSMPETARYTALVERDVVKATNDIGRVLADLDLAAVAEEEVAA-AA   277
OsPHT10     MAGAVPAALTYYWRMSMPETARYTALVERDVVKATNDIGRVLADLDLGAVAEEEVAA-AL   270
TaPHT11     MLGAFPALATFYWRMKMPETARYTALIEGNAKQATNDMQKVLEIRIDEEQEKLSKFR---   293
TaPHT12     MLGAFPAVATFYWRMKMPETARYTALIEGNAKQATNDMQKVLEIRIDEEQEKLSKFR---   293
OsPHT11     MIGAFPALATFYWRMKMPETARYTAIIDGNAKQAANDMQKVLSIEIEAEQEKLAKFN---   283
ZmPHT6      MIGAFPALATFYWRMKMPETARYTALIEGNAKQAANDMQKVMDVEIQAEQDKLARYK---   286
ZmPHT11     MVGAIPACFTYHWRMRMPETARYTALVARDADKAARDMSRVLQVDITGEPDKVENIT---   276
HvPHT8      MVGAIPAVFTYRWRVMMPETARYTALVARDAEKAARDMSKVLKVEFTGEQDKIESFT---   276
TaPHT8      MVGAIPAVFTYRWRVMMPETARYTALVARDAEKAARDMSKVLKVELSGEQDKIESFT---   276
LpPHT1      MFGTIPAALTYYWRMKMPETARYTALITRNTKQATADMSRVLNKDITEEEEKVQLQV---   276
TaPHT2      MFGTIPAALTYYWRMKMPETARYTALIAGNTKQATSDMSKVLNKEISEE--NVQGER---   274
TaPHT10     MFGTIPAALTYYWRMKMPETARYTALIAGNTKQATSDMSKVLNKEISEE--NVQGER---   274
HvPHT1      MFGTIPAALTYYWRMKMPETARYTALIAGNTKQATSDMSKVLNKEISEE--AGQGER---   274
HvPHT9      MFGTIPAALTYYWRMKMPETARYTALIAGNTKQATSDMSKVLNKEISEE--AGQGER---   274
HvPHT2      MFGTIPAALTYYWRMKMPETARYTALIAGNTKQATSDMSKVLNKEISEE--AGQGER---   274
HvPHT10     MFGTIPAALTYYWRMKMPETARYTALIAGNTKQATSDMSKVLNKEISEE--AAQGER---   274
TaPHT1      MFGTIPAALTYYWRMKMPETARYTALIAGNTKQATSDMSKVLNKEILEE--NVQGER---   274
TaPHT9      MFGTIPAALTYYWRMKMPETARYTALIAGNTKQATSDMSKVLNKEISEE--DVQGER---   274
OsPHT2      MFGTIPAGLTYYWRMKMPETARYTALVARNAKQAAADMSKVLHAEIEERPEVVESQV---   276
OsPHT1      MFGTVPAALTYYWRMKMPETARYTALIARNAKQAAADMSKVLHTQIEESADRAETVA---   276
OsPHT3      MFGTVPAALTYYWRMKMPETARYTALIARNAKQAAADMSKVLDTEIQEDADRAEAVA---   276
ZmPHT10     MFGAVPAALTFYSRMKMPETARYTALVARNAERAAADMSRVLQVDIGNKEQPGAEEEATT   282
OsPHT7      MFGALPAALTFYWRMRMPETARYTAIVAKNAERAAADMSKVLQVKITAEQAEMAS-----   274
HvPHT7      MLGALPAALTFYWRMKMPETPRYTALIAKNAERAAADMSKVLNVEITKEQAGDLETA---   276
TaPHT7      MLGALPAALTFYWRMKMPETARYTALIAKNAERAAADMSKVLQVEITKEQAGDLETV---   276
HvPHT6      MFGAVPALLTYYWRMKMPETARYTALVAKNAKQAAADMSKVLQVEIAAEDETKD------   273
TaPHT6      MFGAVPALMTYYWRMKMPETARYTALVAKNAKQAAADMSKVLQVEIGAEEEDPKAN-DG-   277
OsPHT6      MLGALPAALTYYWRTKMPETARYTALVANNAKQAAADMSKVLQVVEMRNIGN--------   276
ZmPHT7      MLGAMPAALTYYWRTKMPETARYTALVAKNAKQAAADMSKVLQVEISAGAPEEDAAAAA-   278
ZmPHT3      MFGAVPALLTYYWRMKMPETARYTALVAKNAKQATSDMARVLDVDLAEERQKPV------   244
ZmPHT8      MFGAVPALLTYYWRMKMPETARYTALVAKNAKQATSDMARVLDVDLAEERQKPV------   244
OsPHT4      MFGAIPALLTYYWRMKMPETARYTALVAKNAKQAAADMTQVLNVEIVEEQEKA-------   271
OsPHT5      MFGAIPALLTYYWRMKMPETARYTALVAKNDKKAAADMARVLNVELVDEQEKAAAATAAA   278
ZmPHT1      ILGAAPAMLTYYWRMKMPETARYTALVAKNAKQAAADMSRVLQTEIVDEQEKLDEMV---   278
ZmPHT9      ILGAAPAMLTYYWRMKMPETARYTALVAKNAKQAAADMSRVLQTEIVDEQEKLDEMV---   278
ZmPHT2      MLGAAPALLTYYWRMKMPETARYTALVAKNAKQAAADMSKVLHTEILDEQEKLDGMV---   278
ZmPHT4      MLGAAPALLTYYWLMKMPETARYTALVAKNAKQAAADMSKVLHTEIVDEQEKLD------   275
OsPHT8      MLGAMPALLTYYWRMKMPETARYTALVAKNAKQAAADMSKVLQVEIQEEQDKLEQMV---   280
LpPHT4      MLGAVPALLTYYWRMKMPETARYTALVAKDAKLAAADMSKVLNVQLEDESKKMEEMV---   279
HvPHT4      MLGALPALLTYYWRMKMPETARYTALVAKNAKLAAADMSKVLQVELEDETEKMDEMV---   279
TaPHT4      MLGAVPALLTYYWRMKMPETARYTALVAKNAKLAAADMSKVLQVELEDETEKMDEMV---   279
OsPHT12     MLGALPAILTYYWRMKMPETARYTALVAKDAKQASSDMAKVLQVEIEVEEEKLQDI----   277
HvPHT5      MLGALPAALTYYWRTKMPETARYTALVAKNAKKASLDMSKVLQSEVEAEPEKLDEIM---   279
TaPHT5      MLGALPAALTYYWRTKMPETARYTALVAKNAKQASLDMSKVLQSEIEAEPEKLDEIM---   276


OsPHT13     --------------RQDEYGLFSFEFLHRHGLHLLGTTVCWFVLDVTFYSLNIFMKNIFT   304
ZmPHT5      --------------RQDKYGLFSAQFLRYHGTHLLATSACWLAVDITFYSLNLYMKDIFA   301
ZmPHT12     --------------RQDKYGLFSAQFLRYHGTHLLATSACWLAVDITFYSLNLYMKDIFA   301
ZmPHT13     --------------RHDQYGLFSAEFLRRHGAHLVGTSMCWLALDITFYSLNLFMKDFFT   310
OsPHT9      LS---PPPVTTPPPPRPSYGLFSRRFVRQHGRDLFACAAAWFLLDIPYYSSTLFQSQIYR   334
OsPHT10     SR---P-----PPPPRPSYGLLSRRFVRQHGRDLFACAAAWFLLDIPYYSSTLFQSQIYR   322
TaPHT11     --------------AANEYSLLSMEFARRHGLHLIGTTTTWFLLDIAFYSQNLTQKDIFP   339
TaPHT12     --------------AANEYSLLSMEFARRHGLHLIGTTTTWFLLDIAFYSQNLTQKDIFP   339
OsPHT11     --------------AANNYPLLSMEFARRHGLHLIGTTTTWFLLDIAFYSQNLTQKDIFP   329
ZmPHT6      --------------AANDYPLLSREFARRHGLHLIGTATTWFLLDIAFYSQNLTQKDIFP   332
ZmPHT11     -------------RDRGDYGAFSRRFARRHGAHLAGAVACWFVLDVVFYSQNILQEGIFS   323
HvPHT8      -------------RDR-DYGVFSRRFARRHGWHLVGAVASWFVLDIVFYSQIILQEEIFR   322
TaPHT8      -------------RDR-DYGVFSRRFARRHGWHLVGAVASWFVLDIVFYSQIILQEEIFR   322
LpPHT1      -----------A--SGDTWGLFSRQFMSRHGLHLLATTSTWFLLDVAFYSQNLFQKDIFT   323
TaPHT2      -----------A--TGDTWGLFSRQFMKRHGVHLLATTSTWFLLDVAFYSQNLFQKDIFT   321
TaPHT10     -----------A--TGDTWGLFSRQFMKRHGVHLLATTSTWFLLDVAFYSQNLFQKDIFT   321
HvPHT1      -----------A--TGDTWGLFSRQFMKRHGVHLLATTSTWFLLDVAFYSQNLFQKDIFT   321
HvPHT9      -----------A--TGDTWGLFSRQFMKRHGVHLLATTSTWFLLDVAFYSQNLFQKDIFT   321
HvPHT2      -----------A--TGDTWGLFSRQFMKRHGVHLLATTSTWFLLDVAFYSQNLFQKDIFT   321
HvPHT10     -----------A--TGDTWGLFSRQFMKRHGVHLLATTSTWFLLDVAFYSQNLFQKDIFT   321
TaPHT1      -----------A--TGDTWGLFSRQFMKRHGVHLLATTSTWFLLDVAFYSQNLFQKDIFT   321
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TaPHT9     -----------A--TGDTWGLFSRQFMKRHGVHLLATTSTWFLLDVAFYSQNLFQKDIFT   321
OsPHT2     -----------V--AGETWGLFSRQFMKRHGMHLLATTSTWFLLDIAFYSQNLFQKDIFS   323
OsPHT1     -----------VGG--ESWGLFSRQFLRRHGLHLLATTSTWFLLDIAFYSQNLFQKDIFS   323
OsPHT3     -----------AGGAGNEWGLFSRQFVRRHGVHLVATTSTWFLLDIAFYSQNLFQKDIFS   325
ZmPHT10    GTEARLRGEQEEATTGPPFGLFSREFAHRHGLHLVGTASTWLLLDIAYYSQNLFQKDIFS   342
OsPHT7     --------PVDKPFTSKPFGLFSGEFARRHGFHLLGTTSTWLLLDIAYYSQNLFQKDIFS   326
HvPHT7     -------ISIKSHTS-PSFGLFSREFMRRHGLHLLGTASTWLLLDIAYYSQNLFQKDIFS   328
TaPHT7     -------ITIKSHTPPPSFGLFSGEFVRRHGLHLVGTASTWLLLDIAYYSQNLFQKDIFS   329
HvPHT6     --------NDGAGEDRNSFGLFSGEFLRRHGLHLLGTATCWFLLDIAFYSQNLFQKDIFT   325
TaPHT6     --------GAGAADDRNSFGLFSGEFLRRHGLHLLGTATCWFLLDIAFYSQNLFQKDIFT   329
OsPHT6     -----------NGGSRRPFGLFSGEFVRRHGLHLVGTSATWLLLDIAFYSQNLFQKDIFS   325
ZmPHT7     --------ATAPAPASASFGLFSGEFLRRHGLHLLGTSSTWFLLDIAFYSQNLFQKDIFS   330
ZmPHT3     ---------EELERRREEFGLFSRQFAKRHGLHLLGTTVCWFTLDIAFYSQNLFQKDMYA   295
ZmPHT8     ---------EELERRREEFGLFSRQFAKRHGLHLLGTTVCWFTLDIAFYSQNLFQKDMYA   295
OsPHT4     ----------DEVARREQFGLFSRQFLRRHGRHLLGTTVCWFVLDIAFYSSNLFQKDIYT   321
OsPHT5     A--------EEEAARREQYGLFSREFARRHGHHLLGTTVCWFVLDIAYYSQNLFQKDIYT   330
ZmPHT1     -----------TAE-SNTFGLFSREFARRHGLHLVGTSTTWFLLDIAFYSQNLFQKDIFT   326
ZmPHT9     -----------TAE-SNTFGLFSREFARRHGLHLVGTSTTWFLLDIAFYSQNLFQKDIFT   326
ZmPHT2     -----------AAEGANSFGLFSREFARRHGLHLVGTATTWFLLDIAFYSQNLFQKDIFT   327
ZmPHT4     -----------AAEGANSFGLFSREFARRHGLHLVGTATTWFLLDIAFYSQNLFQKDIFT   324
OsPHT8     -----------TR-NSSSFGLFSPQFARRHGLHLVGTATTWFLLDIAFYSQNLFQKDIFT   328
LpPHT4     -----------SR-GNNEFGLFSPQFARRHGLHLVGTATTWFLLDIAFYSQNLFQKDIFT   327
HvPHT4     -----------SR-GANDFGLFSPQFARRHGLHLVGTATTWFLLDIAFYSQNLFQKDIFT   327
TaPHT4     -----------SR-GANDFGLFSPQFARRHGLHLVGTATTWFLLDIAFYSQNLFQKDIFT   327
OsPHT12    -----------TR--GRDYGLFSARFAKRHGAHLLGTAATWFLVDVAYYSQNLFQKDIFT   324
HvPHT5     -----------AR--GEDYGLLTSRFAKRHGLHLLGTATAWFLVDVAYYSQNLFQKDIFG   326
TaPHT5     -----------AR--GEDYGLFTSRFAKRHGLHLLGTATAWFLVDVAYYSQNLFQKDIFG   323


OsPHT13    EVGLLPRLDSEYHHTLQRMITMTAVHTFISLCGALPGYFFTVAFVDRIGRVKIQLIGFTM   364
ZmPHT5     DVGLIDPPGN--NDLFTRMTVTTLLHTGIALCGTLPGYFFTVAFVDRIGRVRIQLLGFTM   359
ZmPHT12    DVGLIDPPGN--NDLFTRMTVTTLLHTGIALCGTLPGYFFTVAFVDRIGRVRIQLLGFTM   359
ZmPHT13    KVRLLGAPHQ-GDEPFKRMVHTTAMHTILVLSFSLPGYFFSVAFVDRIGRVRIQLLGFAM   369
OsPHT9     --PWFPPAAK--VNAFQEAFNVAKFQAVIAVASTIPGYFAAMLLIERAGRRRLQMAGFLL   390
OsPHT10    --PLFPAPGL--INAFQEAFNVAKFQAVIAVASTIPGYFVAVLLIDRVGRRCLQMAGFLL   378
TaPHT11    AINLTGPPGT--MSALKEVFVISRAMFLIALFGTFPGYWVTVALIDKMGRYLIQLLGFFM   397
TaPHT12    AINLTGTPGS--MNALKEVFVISRAMFLIALFGTFPGYWVTVALIDKMGRYLIQLLGFFM   397
OsPHT11    AMGLISGAAE--VNALTEMFQISKASFLVALLGTFPGYWVTVALIDKMGRYMIQLIGFFM   387
ZmPHT6     AIKLTSPVDD--INALKEVFEISKAMFLVALLGTFPGYWVTVALIDKMGRYLIQLIGFFM   390
ZmPHT11    DVNWVPRART--MSALEETYRVGRAHAIIALCGTLPGYWFTIAFVDVVGRKAIQFLGFAM   381
HvPHT8     DVKWIPEART--MSALEEAYRVARGQAIIALCGTLPGYWFTVAFVDVVGRKAIQFLGFTM   380
TaPHT8     DIKWIPEANS--MSALEEAYRVARAQAIIALCGTLPGYWFTIAFVDVVGRKAIQFLGFTM   380
LpPHT1     KVGWIPPART--MSALEELYRIARAQALIALCGTVPGYWFTVAFIDIIGRFWIQLMGFAM   381
TaPHT2     KIGWIPPAKT--MNALEELYRIARAQALIALCGTVPGYWFTVAFIDIIGRFWIQLMGFTM   379
TaPHT10    KIGWIPPAKT--MNALEELYRIARAQALIALCGTVPGYWFTVAFIDIIGRFWIQLMGFTM   379
HvPHT1     KIGWIPPAKT--MNALEELYRIARAQALIALCGTVPGYWFTVAFIDIIGRFWIQLMGFTM   379
HvPHT9     KIGWIPPAKT--MNALEELYRIARAQALIALCGTVPGYWFTVAFIDIIGRFWIQLMGFTM   379
HvPHT2     KIGWIPPAKT--MNALEELYRIARAQALIALCGTVPGYWFTVAFIDIIGRFWIQLMGFTM   379
HvPHT10    KIGWIPPAKT--MNALEELYRIARAQALIALCGTVPGYWFTVAFIDIIGRFWIQLMGFTM   379
TaPHT1     KIGWIPPAKT--MNALEELYRIARAQALIALCGTVPGYWFTVAFIDIIGRFWIQLMGFTM   379
TaPHT9     KIGWIPPAKT--MNALEELYRIARAQALIALCGTVPGYWFTVAFIDIIGRFWIQLMGFTM   379
OsPHT2     KVGWIPPAKT--MNALEELYRISRAQALIALCGTIPGYWFTVAFIDIVGRFWIQIMGFFM   381
OsPHT1     KVGWIPPAKT--MNALEELYRIARAQALIALCGTIPGYWFTVAFIEIMGRFWIQIMGFAM   381
OsPHT3     KVGWIPPART--MNAVEEVFRIARAQALIALCGTIPGYWFTVAFIDVAGRFAIQLMGFAM   383
ZmPHT10    AVGWIPAAET--ISALDELFHIARAQTLIALCGTVPGYWFTVAFIDIVGRFAIQAVGFLM   400
OsPHT7     AIGWIPEAKT--MSALDELYHIARAQTLIALCGTVPGYWFTVALIDVVGRFKIQAAGFFV   384
HvPHT7     AIGWIPPAPT--MSALDELYHIARAQILIALCGTVPGYWFTVAFIDSVGRFKIQLMGFFM   386
TaPHT7     AIGWIPPAPT--MSALDELFHIARAQILIALCGTVPGYWFTVAFIDSVGRFKIQLMGFFM   387
HvPHT6     AINWIPKAKT--MSALEEVHRIARAQTLIALCGTVPGYWFTVALIDRIGRFWIQLGGFFF   383
TaPHT6     AINLIPEAKK--MSALEEVHRIARAQTLIALCGTVPGYWFTVALIDRIGRFWIQLGGFFF   387
OsPHT6     AVGWIPKAAT--MSALEELFRIARAQTLIALCGTVPGYWFTVALIDVVGRFKIQAVGFFM   383
ZmPHT7     AVGWIPKAAT--MNALEELFSIARAQSLIALCGTVPGYWFTVALIDVLGRFAIQVTGFLM   388
ZmPHT3     AVNWLPRADT--MNALEEMFRISRAQTLVALCGTIPGYWFTVFFIDIVGRFAIQLGGFFF   353
ZmPHT8     AVNWLPRADT--MNALEEMFRISRAQTLVALCGTIPGYWFTVFFIDIVGRFAIQLGGFFF   353
OsPHT4     AVQWLPKADT--MSALEEMFKISRAQTLVALCGTIPGYWFTVFFIDIIGRFVIQLGGFFF   379
OsPHT5     AVQWLPKADT--MSALEEMFKISRAQTLVALCGTIPGYWFTVLFIDIVGRFAIQLGGFFL   388
ZmPHT1     SINWIPKANT--MSALEEVFRISRAQTLIALCGTVPGYWFTVALIDVVGRFAIQLLGFFM   384
ZmPHT9     SINWIPKANT--MSALEEVFRISRAQTLIALCGTVPGYWFTVALIDVVGRFAIQLLGFFM   384
ZmPHT2     SINWIPKANT--MSALEEVYRISRAQTLIALCGTVPGYWFTVALIDVVGRFAIQLLGFFM   385
ZmPHT4     SINWIPKANT--MSALEEVYRISRAQTLIALCGTVPGYWFTVALIDVVGRFAIQLLGFFM   382
OsPHT8     SINWIPKAKT--MSALEEVFRIARAQTLIALCGTVPGYWFTVFLIDIVGRFAIQLLGFFM   386
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LpPHT4      AINWIHKAKT--MSALDEVFRISRAQTLLALCGTVPGYWFTVFLIDVVGRFKIQLMGFFM   385
HvPHT4      SINWIPKART--MSALDEVFRISRAQTLIALCGTVPGYWFTVFLIDVVGRFAIQLMGFFM   385
TaPHT4      SINWIPKART--MSALDEVFRISRAQTLIALCGTVPGYWFTVFLIDVVGRFAIQLMGFFM   385
OsPHT12     SIHWIPKART--MSELEEVFRISRAQTLIALCGTVPGYWFTVFLIDIIGRFKIQLLGFAG   382
HvPHT5      SIGWIPKART--MDALEEVFRISRAQTLIALCGTVPGYWFTVFLIDVIGRFWIQLVGFAM   384
TaPHT5      SIGWIPKART--MDALEEVFRISRAQTLIALCGTVPGYWFTVFLIDVVGRFWIQLVGFAM   381


OsPHT13     MTVFMLCLAIPYDQWLRH---KNKYGFAVMYGLTFFFANFGPNTTTFIIPAEIFPARLRS   421
ZmPHT5      MSVLTAILAATYAYWKRQETIQRKMGFAVLYGLTNFFANFGPNTTTFIVPAEIFPARMRA   419
ZmPHT12     MSVLTAILAATYAYWKRQETIQRKMGFAVLYGLTNFFANFGPNTTTFIVPAEIFPARMRA   419
ZmPHT13     MSAFILCLAIPYDHWTDDK--NNKYGFAVMYGLTSFFANFGPNTTTFIIPAEIFPARLRS   427
OsPHT9      MAVFLFALAGPYDGYWRDH--AKTAGYIVLYSLTFFSANLGPNTTTFILPAELFPARFRS   448
OsPHT10     MAVFLFALAGPYDGYWRDH--GAHAGYIVLYSLTFFSANLGPNTTTFILPAELFPARFRS   436
TaPHT11     MSLFMLVMGIKYEYLKDKG----HALFAILYALTFFFANFGPNSTTFVLPAELFPTRVRS   453
TaPHT12     MSLFMLVMGIKYEYLKDKG----HALFAILYALTFFFANFGPNSTTFVLPAELFPTRVRS   453
OsPHT11     MSMFMLAMGILYDYLKT-H----HFLFGLLYALTFFFANFGPNSTTFVLPAELFPTRVRS   442
ZmPHT6      MSVFMLLMGVMYNDLKNKH----TTLFALFYALTFFFANFGPNSTTFVLPAELFPTRVRS   446
ZmPHT11     MMGFMLLIAAFYDSLISPG--R-RIWLVLMYTFTFFFANFGPNSTTFILPAEIFPAHLRT   438
HvPHT8      MKGLMLVVAAFYHHLTQPG--R-RIWLVVMYAFTFFFANFGPNSTTFIIPAEIFPAHVRT   437
TaPHT8      MKGLMLVVAGFYHQLTQPG--R-RIWLVVMYAFTFFFANFGPNSTTFIIPAEVFPAHVRT   437
LpPHT1      MTIFMLAIAVPYDYLVKPG--H-HTGFVVLYGLTFFFANFGPNSTTFIVPAEIFPARLRS   438
TaPHT2      MTIFMLAIAIPYDYLVKPG--H-HTGFVVLYGLTFFFANFGPNSTTFIVPAEIFPARLRS   436
TaPHT10     MTIFMLAIAIPYDYLVKPG--H-HTGFVVLYGLTFFFANFGPNSTTFIVPAEIFPARLRS   436
HvPHT1      MTIFMLAIAIPYDYLVKPG--N-HTGFVVLYGLTFFFANFGPNSTTFIVPAEIFPARLRS   436
HvPHT9      MTIFMLAIAIPYDYLVKPG--N-HTGFVVLYGLTFFFANFGPNSTTFIVPAEIFPARLRS   436
HvPHT2      MTIFMLAIAIPYDYLVKPG--N-HTGFVVLYGLTFFFANFGPNSTTFIVPAEIFPARLRS   436
HvPHT10     MTIFMLAIAIPYDYLVKPG--H-HTGFVVLYGLTFFFANFGPNSTTFIVPAEIFPARLRS   436
TaPHT1      MTIFMLAIAIPYDYLVKPG--H-HTGFVVLYGLTFFFANFGPNSTTFIVPAEIFPARLRS   436
TaPHT9      MTIFMLAIAIPYDYLVKPG--H-HTGFVVLYGLTFFFANFGPNSTTFIVPAEIFPARLRS   436
OsPHT2      MTVFMLALGVPYDHWTHPA--H-HTGFVVLYALTFFFANFGPNSTTFIVPAEIFPARLRS   438
OsPHT1      MTAFMLGLAIPYHHWTTPG--H-HTGFIVMYGFTFFFANFGPNSTTFIVPAEIYPARLRS   438
OsPHT3      MTVFMLGLAAPYHHWTTPG--N-HTGFVVMYGFTFFFANFGPNATTFIVPAEIYPARLRS   440
ZmPHT10     MTAFMLGLAVPYRQWTRPG--N-QTGFVVMYALTFFFANFGPNATTFIVPAEIYPARLRA   457
OsPHT7      MTAFMLALAVPYDHWTAAG--N-QIGFVVLYALTFFFANFGPNATTFIVPAEIYPARLRA   441
HvPHT7      MTAFMLGLAGPYDYWTGQG--H-QVGFVVMYALTFFFANFGPNATTFIVPAEIYPARFRA   443
TaPHT7      MTTFMVGLAVPYDYWTGQG--H-QAGFVVMYALTFFFANFGPNATTFIVPAEIYPARLRA   444
HvPHT6      MAVFMLGLAFPYHHWTTPG--N-HIGFVVLYALTFFFANFGPNSTTFIVPAEIFPARLRS   440
TaPHT6      MAVFMLGLAFPYHHWTTPG--N-HIGFVVLYALTFFFANFGPNSTTFIVPAEIFPARLRS   444
OsPHT6      MTLFMLTLALPYHHWTAPG--KNHVGFLLLYGLTFFFANFGPNSTTFIVPAEIFPARLRA   441
ZmPHT7      MTVFMLGLAVPYEHWTTPG--H-HIGFIVMYGLTFFFANFGPNATTFIVPAEIFPARLRS   445
ZmPHT3      MTAFMLGLAIPYHHWTTPG--H-HVGFVVMYALTFFFANFGPNSTTFIVPAEIFPARLRS   410
ZmPHT8      MTAFMLGLAIPYHHWTTPG--H-HVGFVVMYALTFFFANFGPNSTTFIVPAEIFPARLRS   410
OsPHT4      MTAFMLGLAVPYHHWTTPG--N-HIGFVVMYAFTFFFANFGPNSTTFIVPAEIFPARLRS   436
OsPHT5      MTAFMLGLAVPYHHWTTPG--N-HVGFVVMYAFTFFFANFGPNSTTFIVPAEIFPARLRS   445
ZmPHT1      MTVFMLGLAIPYHHWTTPG--N-HIGFVVMYAFTFFFANFGPNSTTFIVPAEIFPARLRS   441
ZmPHT9      MTVFMLGLAIPYHHWTTPG--N-HIGFVVMYAFTFFFANFGPNSTTFIVPAEIFPARLRS   441
ZmPHT2      MTVFMLGLAIPYHHWTTPG--N-HIGFVVMYAFTFFFANFGPNSTTFIVPAEIFPARLRS   442
ZmPHT4      MTVFMLGLAIPYHHWTTPG--N-HIGFVVMYAFTFFFANFGPNSTTFIVPAEIFPARLRS   439
OsPHT8      MTVFMLGLAVPYHHWTTKG--N-HIGFVVMYAFTFFFANFGPNSTTFIVPAEIFPARLRS   443
LpPHT4      MTVFMLGLAIPYHHWTTPG--N-QVGFVIMYGFTFFFANFGPNATTFVVPAEIFPARLRS   442
HvPHT4      MTVFMLGLAVPYHHWTTPG--N-QIGFVVMYGFTFFFANFGPNATTFVVPAEIFPARLRS   442
TaPHT4      MTVFMLGLAVPYHHWTTPG--N-QIGFVVMYAFTFFFANFGPNATTFVVPAEIFPARLRS   442
OsPHT12     MTAFMLGLAIPYHHWTMPG--N-QVIFVFLYGFTFFFANFGPNATTFIVPAEIFPARLRS   439
HvPHT5      MAVFMLGLAVPYHHWTTPG--N-HVGFVVMYGLTFFFANFGPNATTFIVPAEIFPARLRS   441
TaPHT5      MTVFMLGLALPYHHWTTPG--N-HVGFVVMYGLTFFFANFGPNATTFIVPAEIFPARLRS   438


OsPHT13     TCHGISGAVGKIGAIVGVFGFLYTE---YH-------------IRIFLFVLIGCNLVGFI   465
ZmPHT5      TCHGIAGAFGKIGAIIGVFGFMSNMEEHGV-------------VPRKLWALFASNLVGLV   466
ZmPHT12     TCHGIAGAFGKIGAIIGVFGFMSNMEEHGV-------------VPRKLWALFASNLVGLV   466
ZmPHT13     TCHGISGAFGKLGAIIGVFAFVYA-EKHNN-------------NRSMLFALVGCNLVGLV   473
OsPHT9      TCHGLSGAAGKLGALVGSIGFLWASQQKDGA---AAGHLPGIGMMYALFVLGGICLLGLA   505
OsPHT10     TCHGLSGAAGKLGALVGSIGFLWASQQKDGA---AAGHLPGIGMMYALFVLGGICLLGLA   493
TaPHT11     TCHAISAASGKAGAIVAAFGVQTLTLKGDPKH-----------MKQALILLSVTNMFGFF   502
TaPHT12     TCHAISAASGKAGAIVAAFGVQTLTLKGDPKH-----------MKQALILLSVTNMFGFF   502
OsPHT11     TCHAISAAAGKAGAIVAAFGIQKLTYNSQVKS-----------IKKALIILSITNMLGFF   491
ZmPHT6      TCHAISAASGKAGAIVAAFGVQSLTLKGDVGH-----------IKKALIILSVTNILGFF   495
ZmPHT11     TCHGISAAAGKVGAIVGTFGFMYAAQKADGSEAAETGYPSGIGVRASLFVLAASNALGIL   498
HvPHT8      TCHGISSAAGKVGAIVGTFGFLYASQRADGSNEVKSGYPSGIGVRASLFVLAACNVLGII   497
TaPHT8      TCHGISSAAGKVGAIVGTFGFLYASQRADGSNERETGYPSGIGVRASLFVLAACNVLGII   497
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LpPHT1      TCHGVSAAAGKAGAIIGAFGFLYASQDQK--KP-DKGYSAGIGMRNALFVLAGTNFLGLL   495
TaPHT2      TCHGISAATGKAGAIIGAFGFLYASQDQK--KP-ETGYSRGIGMRNALFVLAGTNFLGLL   493
TaPHT10     TCHGISAATGKAGAIIGAFGFLYASQDQK--KP-ETGYSRGIGMRNALFVLAGTNFLGLL   493
HvPHT1      TCHGISAATGKAGAIIGAFGFLYASQDQK--KP-ETGYSRGIGMRNALFVLAGTNFLGLL   493
HvPHT9      TCHGISAATGKAGAIIGAFGFLYASQDQK--KP-ETGYSRGIGMRNALFVLAGTNFLGLL   493
HvPHT2      TCHGISAATGKAGAIIGAFGFLYASQDQK--KP-ETGYSRGIGMRNALFVLAGTNFLGLL   493
HvPHT10     TCHGISAATGKAGAIIGAFGFLYASQDQK--KP-ETGYSRGIGMRNALFVLAGTNFLGLL   493
TaPHT1      TCHGISAATGKAGAIIGAFGFLYASQDQK--KP-DTGYSRGIGMRNSLFVLAGTNFLGLL   493
TaPHT9      TCHGISAATGKAGAIIGAFGFLYASQDQK--KP-ETGYSRGIGMRNALFVLAGTNFLGLL   493
OsPHT2      TCHGISAASGKAGAIIGAFGFLYAAQDQH--NP-DAGYSRGIGIRNALFVLAGTNFLGML   495
OsPHT1      TCHGISAAAGKAGAIIGAFGFLYAAQDQH--KP-EPGYPRGIGIKNALFVLAGTNFLGTI   495
OsPHT3      TCHGISAAAGKAGAIVGAFGFLYAAQDPH--KP-EAGYKPGIGIRNALFVLAGTNFLGML   497
ZmPHT10     TCHGISAASGKVGAIIGSFGFLYLAQSQDPAKT-AHGYAPGIGVRNSLLLLAGCSFLGFL   516
OsPHT7      TCHGISAASGKVGAIVGSFGFLYLAQSPVPAKAAAHGYPPGIGVRNSLFALAGCSLLGFL   501
HvPHT7      TCHGISAASGKVGAIIGSFGFLYLAQSPDPAKT-AHGYHPGIGVRYSLFVLALCSLLGFM   502
TaPHT7      TCHGISAASGKVGAIIGSFGFLYLAQSPDPAKT-AHGYHPGIGVRYSLLVLAGCSLMGFL   503
HvPHT6      TCHGISAAAGKLGAIVGSFGFLYLAQNQDPSKV-DHGYKAGIGVKNSLFILAACNFLGMA   499
TaPHT6      TCHGISAAAGKLGAIVGSFGFLYLAQNKDPAKV-DHGYKAGIGVRNSLFILAACNFLGMG   503
OsPHT6      TCHGISAASGKLGAIVGSFGFLYLAQSPDRSKT-EHGYPPGIGVRNSLFLLAACNLLGLL   500
ZmPHT7      TCHGISAASGKLGAIVGSFGFLYLAQNRDPAKT-DHGYPAGIGVRNSLFLLAGCNLLGLA   504
ZmPHT3      TCHGISSAAGKCGAIVGSFGFLYAAQSTDPTKT-DSGYPPGIGVRNSLFMLTGCNVIGFL   469
ZmPHT8      TCHGISSAAGKCGAIVGSFGFLYAAQSTDPTKT-DSGYPPGIGVRNSLFMLTGCNVIGFL   469
OsPHT4      TCHGISAAAGKAGAIVGSFGFLYAAQSTDASKT-DAGYPPGIGVRNSLFFLAGCNVIGFF   495
OsPHT5      TCHGISSAAGKMGAIVGSFGFLYAAQSTDPSKT-DAGYPRGIGVRNSLFLLAGCNVVGFL   504
ZmPHT1      TCHGISAASGKAGAIIGAFGFLYAAQNQDKSKA-DAGYPAGIGVRNSLFVLAASNLLGFI   500
ZmPHT9      TCHGISAASGKAGAIIGAFGFLYAAQNQDKSKA-DAGYPAGIGVRNSLFVLAASNLLGFI   500
ZmPHT2      TCHGISAASGKAGAIIGAFGFLYAAQNQDKSKT-DAGYPAGIGVRNSLFVLAASNMLGFV   501
ZmPHT4      TCHGISAASGKAGAIIGAFGFLYAAQNQDRSKT-DAGYPAGIGVRNSLFVLAASNMLGFV   498
OsPHT8      TCHGISAAAGKAGAIIGSFGFLYAAQD--PHKP-DAGYKPGIGVRNSLFVLAGCNLLGFI   500
LpPHT4      TCHGISAAAGKAGAMIGAFGFLYAAQD--PHKP-DAGYKPGIGIRNSLFVLAGINILGFA   499
HvPHT4      TCHGISAAAGKAGAMIGAFGFLYAAQD--PHKP-DAGYRPGIGVRNSLFVLAGVNLLGFM   499
TaPHT4      TCHGISAAAGKAGAMIGAFGFLYAAQD--PHKP-DAGYRPGIGVRNSLFVLAGVNLLGFM   499
OsPHT12     TCHGISAASGKAGAIIGAFGFLYAAQPQDKAHV-DAGYKPGIGVRNALFVLAGCNLVGFL   498
HvPHT5      TCHGISAAAGKAGAIIGAFGFLYAAQSPDLAHV-DAGYKPGIGVQKALYVLAACNLLGFL   500
TaPHT5      TCHGISAAAGKAGAIIGAFGFLYAAQSPDPAHV-DAGYKPGIGVQKALYVLAACNLLGFL   497


OsPHT13     FTL-LLPESKGKSLEDLTGEIEEFQEEDEGSEVALSR--------------PIHTVPL--   508
ZmPHT5      FTF-LLPDSKGKSLEEMAGETEEQQQQDAAVVAAAD--------------HINLVPI--   509
ZmPHT12     FTF-LLPDSKGKSLEEMAGETEEQQQQDAAVVAAAD--------------HINLVPI--   509
ZmPHT13     FTL-LLPESKGKALEEISGEMEEQHPEQDAAAVAAAE--------------YVNVVPV--   516
OsPHT9      LTYAFTPETMTRSLEENESSVQAQSQVGDGGSDAGNGSDGLRFHELNVLMEAATKSPVSM   565
OsPHT10     LTYVFTPETMMRSLEENESD-RAQTQVGDGGSDTE-----------------AAKSPASM   535
TaPHT11     FTF-LVPETMGQSLEEISGEDGNVAGAAAGH--V-------------DK--DVEKAPPSS   544
TaPHT12     FTF-LVPETMGRSLEEISGEDGNVAGAAAGH--V-------------DK--DVEKAPPSS   544
OsPHT11     FTF-LVPETMGRSLEEISGEDGNTGAGGGGA--PAAANAGVGVSASDVS--RDEKFPASS   546
ZmPHT6      FTF-LVPETMGRSLEEISGEDGNVENGPGA-------PAGVAMGVADVS--KDDKMPVSS   545
ZmPHT11     FTC-FLPEPKGRSLEEVCGEGAEPTIRDDTD--VGDSK----------------ILPL--   537
HvPHT8      FTC-LLPEPNGRSLEEVSGE---PINREDAD--LGDSR----------------VLPL--   533
TaPHT8      FTC-LLPEPNGRSLEEVSGE---PINGEDAD--LGDSK----------------VLPL--   533
LpPHT1      FSL-LVPESKGRSLEEISKENYDDDATITPA--GA-------------------------   527
TaPHT2      FSL-LVPESKGKSLEELSKENVGDDDAIAPA--GV-------------------------   525
TaPHT10     FSL-LVPESKGKSLEELSKENVGDDDTIAPT--GV-------------------------   525
HvPHT1      FSL-LVPESKGKSLEELSKENVGDDGIDA-------------------------------   521
HvPHT9      FSL-LVPESKGKSLEELSKENVGDDGIDA-------------------------------   521
HvPHT2      FSL-LVPESKGKSLEELSKENVGDDDAIAPT--GV-------------------------   525
HvPHT10     FSL-LVPESKGKSLEELSKENVGDDDAIAPT--GV-------------------------   525
TaPHT1      FSL-LVPESKGKSLEELSKENVGDDGIEA-------------------------------   521
TaPHT9      FSL-LVPESKGKSLEELSKENVGDDGIEA-------------------------------   521
OsPHT2      MTL-LVPESKGLSLEEMSKDNVVDETAQEAI--AQA------------------------   528
OsPHT1      MTL-LVPESKGMSLEVISQEVADGDDEEAAY--PK-------------------------   527
OsPHT3      MTL-LVPESKGMSLEEVSKENVADDEEATA------------------------------   526
ZmPHT10     LTF-LVPEPKGKSLEEMSRETEPGAAEP--------------------------------   543
OsPHT7      LTF-LVPEPKGKSLEEMSRENEVGQP----------------------------------   526
HvPHT7      LTF-LVPEPKGKSLEEMSRETEPDHC----------------------------------   527
TaPHT7      LTF-LVPEPKGKSLEEMSR-----------------------------------------   521
HvPHT6      FTF-CAPESNGISLEELSGENDDEAPAPAT-----HAR----------------TVPV--   535
TaPHT6      FTF-CAPESNGISLEELSGENDDGEAAAPA-----HAR----------------TVPV--   539
OsPHT6      FTF-LVPESKGKSLEEMSGDAEAQEEAPP----PLQTV----------------L-----   534
ZmPHT7      FTF-LVPESKGKSLEEMSGENDEAAAAAATP--NYNNR----------------TVPV--   543
ZmPHT3      FTF-LVPESKGKSLEELSGENDEEAAPQQQQ--TV---------------------PTD   504
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ZmPHT8       FTF-LVPESKGKSLEELSGENDEEAAPQQQQ--TV----------------------PTD   504
OsPHT4       FTF-LVPESKGKSLEELSGENEDDDDVPEAP--ATADH----------------RTAPAP   536
OsPHT5       FTF-LVPESKGKSLEELSGENEMEAEPAAAT--NS-YR----------------QTVPDS   544
ZmPHT1       LTF-LVPESKGKSLEEMSGEADDAEDDAV-----GTR-----------------AVRPSG   537
ZmPHT9       LTF-LVPESKGKSLEEMSGEADDAEDDAV-----GTR-----------------AVRPSG   537
ZmPHT2       LTF-LVPESKGKSLEEMSGEAEDSEEEPV-----GAR-----------------AVRPSE   538
ZmPHT4       LTF-LVPESKGKSLEEMSGEAEDSEEEPV-----GAR-----------------AVRPSE   535
OsPHT8       CTF-LVPESKGKSLEEMSGEAEDDDDEVAAA--GGGA-----------------AVRPQT   540
LpPHT4       FTF-LVPEANGKSLEEMSGEAEDNED--EAR--E--P-----------------KVQPSM   535
HvPHT4       FTF-LVPEANGKSLEEMSGEAQDNENEDQAR--T--A-----------------AVQPSM   537
TaPHT4       FTF-LVPEANGKSLEEMSGEAQDNE--DQAR--A--A-----------------AVQPST   535
OsPHT12      MTWMLVPESKGKSLEEMSGEADDEEA--SAN--GGAT-----------------AVNSSG   537
HvPHT5       VTF-LVPESKGKSLEEMSGEADAEEG--NG-----AN-----------------NVRPSG   535
TaPHT5       VTF-LVPESKGKSLEEMSGEADAEEG--NG-----AN-----------------KVRPSG   532

             OsPHT13      -----------------        508
             ZmPHT5       -----------------        509
             ZmPHT12      -----------------        509
             ZmPHT13      -----------------        516
             OsPHT9       ASSHLSMSPILPHRMSL        582
             OsPHT10      ASSHLSMSPILPARVSV        552
             TaPHT11      TEWQPPSSMN-------        554
             TaPHT12      TEWQPPSSMN-------        554
             OsPHT11      TEWQTSMHA--------        555
             ZmPHT6       TEWQSSMHA--------        554
             ZmPHT11      -----------------        537
             HvPHT8       -----------------        533
             TaPHT8       -----------------        533
             LpPHT1       -----------------        527
             TaPHT2       -----------------        525
             TaPHT10      -----------------        525
             HvPHT1       -----------------        521
             HvPHT9       -----------------        521
             HvPHT2       -----------------        525
             HvPHT10      -----------------        525
             TaPHT1       -----------------        521
             TaPHT9       -----------------        521
             OsPHT2       -----------------        528
             OsPHT1       -----------------        527
             OsPHT3       -----------------        526
             ZmPHT10      -----------------        543
             OsPHT7       -----------------        526
             HvPHT7       -----------------        527
             TaPHT7       -----------------        521
             HvPHT6       -----------------        535
             TaPHT6       -----------------        539
             OsPHT6       -----------------        534
             ZmPHT7       -----------------        543
             ZmPHT3       LSE--------------        507
             ZmPHT8       LSE--------------        507
             OsPHT4       PA---------------        538
             OsPHT5       GQSE-------------        548
             ZmPHT1       TQMV-------------        541
             ZmPHT9       TQMV-------------        541
             ZmPHT2       TQMV-------------        542
             ZmPHT4       TQMV-------------        539
             OsPHT8       A----------------        541
             LpPHT4       A----------------        536
             HvPHT4       A----------------        538
             TaPHT4       A----------------        536
             OsPHT12      VEMV-------------        541
             HvPHT5       EQLV-------------        539
             TaPHT5       EQLV-------------        536
```

Table S1. Sequences alignments for phosphate transporters from family 1 using Blastp-NCBI program.

Description	Identity	Number accession	Protein found
Lolium perenne LpPHTI	100%	AIB04040.1	
Triticum aestivum TaPHT1	91%	AIZ11194.1	
Hordeum vulgare HvPHT1	90%	CAY56583.1	PHT1 from *Lolium perenne*
Oriza sativa OsPHT1	80%	XP015630484.1	
Zea mays ZmPHT1	72%	NP001105816.1	
Triticum aestivum TaPHT4	91%	AIZ11183.1	
Hordeum vulgare HvPHT4	91%	AA072437.1	PTH4 from *Lolium perenne*
Oriza sativa OsPHT4	86%	XP015614122.1	
Zea mays ZmPHT4	82%	AAY42388.1	
Triticum aestivum TaPHT6	92%	AIZ11187.1	
Hordeum vulgare HvPHT6	93%	AAN37901.1	PHT6 from *Lolium perenne*
Oriza sativa OsPHT6	78%	XP015649112.1	

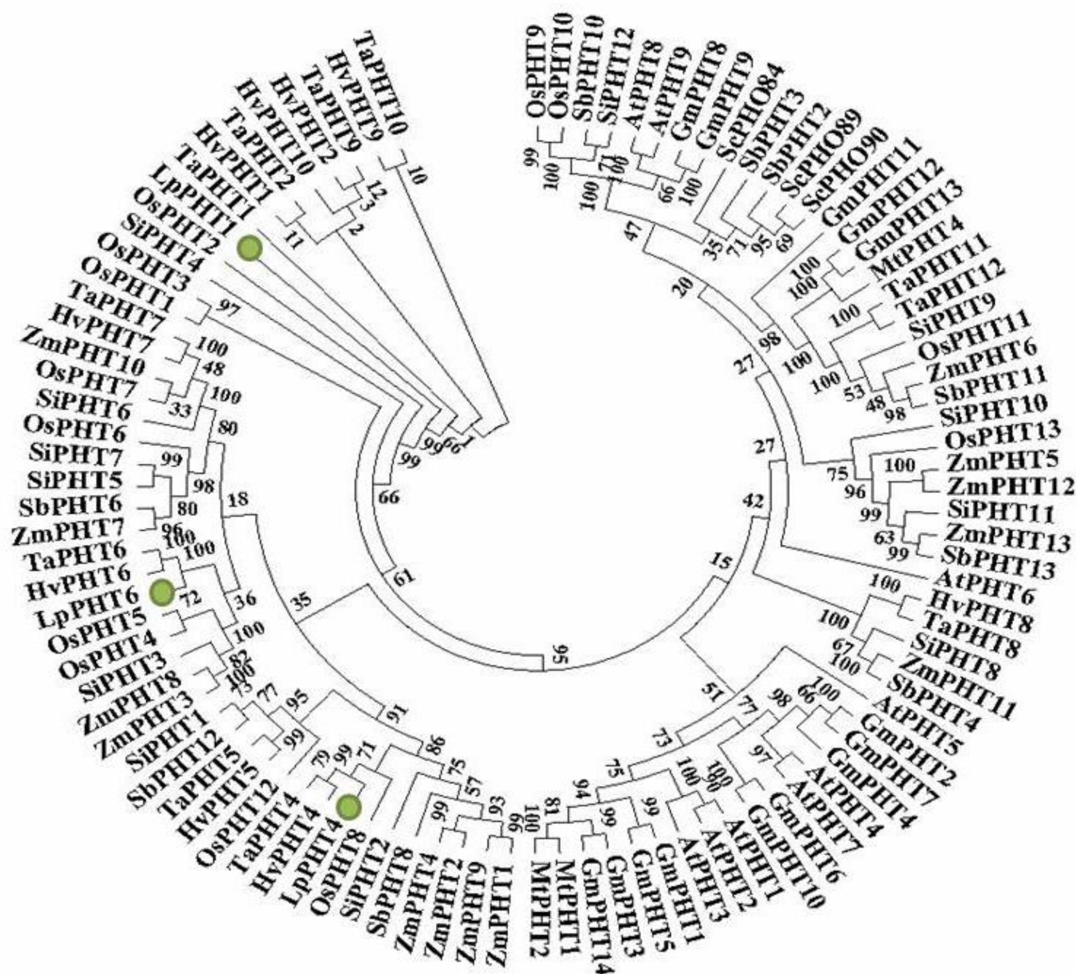

Figure S2. Phylogenetic relationship of the 52 amino acid sequences of the phosphate transporter family 1 in Poaceae family. A neighbor-joining unrooted tree was generated for *L. perenne* (Lp), rice (Os), barley (Hv), wheat (Ta) and maize (Zm). The bootstrap consensus tree was inferred from 1000 replicates. Evolutionary distances were calculated using the Poisson correction method. LpPHT1;1, LpPHT1;4 and LpPHT1;6 from *L. perenne* are indicated by green circles.

Annex 2

Supporting information about Al transporter (LpNRAT1) from ryegrass

Table 1. NCBI BLASTp search of full-length cDNA LpNrat1 obtained from *Lolium perenne*, showing the highest identity sequences from protein database.

Description	Length	Accession number	E-Value	Query cover	Identity
Metal transporter NRAT1 (*Oryza brachyantha*)	542	XP006646836.1	0.0	99%	87%
Metal transporter NRAT1 (*Oryza sativa Japonica*)	545	XP015625418.1	0.0	100%	87%
Metal transporter NRAT1 (*Setaria italica*)	543	XP004952002.1	0.0	100%	86%
Metal transporter NRAT1 (*Dichanthelium oligosanthes*)	544	OEL35611.1	0.0	100%	86%
Metal transporter NRAT1 (*Sorghum bicolor*)	547	XP002451480.2	0.0	100%	82%
Metal transporter NRAT1 (*Zea mays*)	551	NP001334019.1	0.0	100%	77%

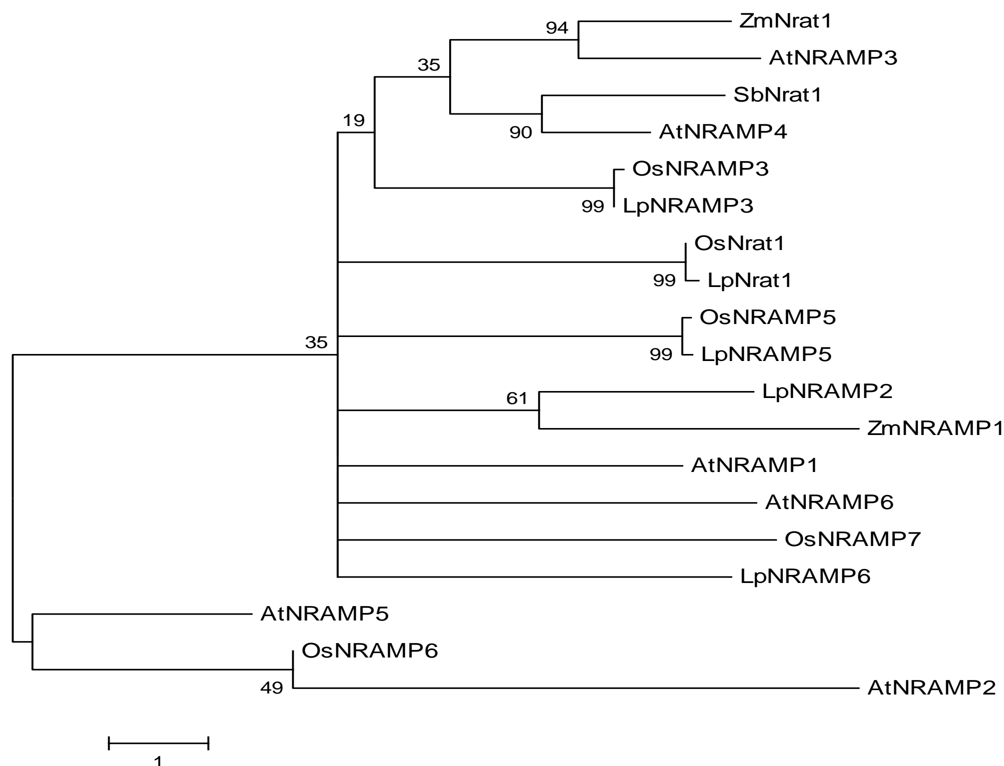

Figure S2.1. Phylogenetic relationship of the amino acid sequences of LpNramp homologues in Arabidopsis (At), rice (Os), sorghum (Sb) and maize (Zm). The bootstrap consensus tree was inferred from 1000 replicates. The 1 scale shows substitution distance. Phylogenetic analysis was performed using MEGA version 6 (Tamura et al., 2013) using the Poisson correction method.

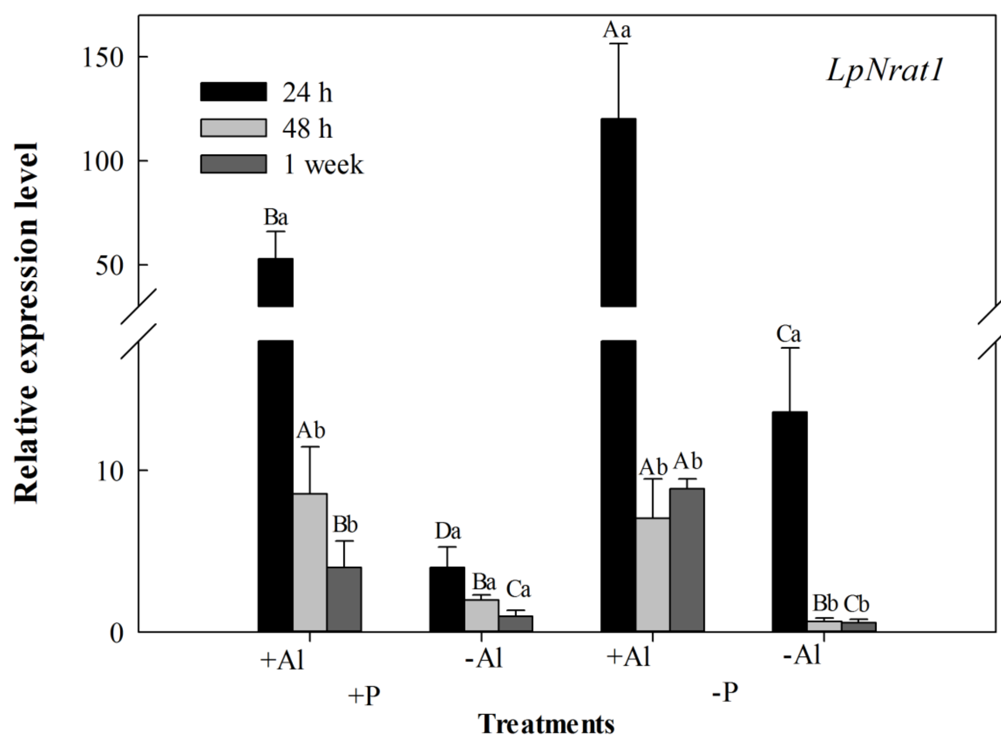

Figure S2.2. Relative expression of aluminum (Al) transporter gene in roots of Nui cultivar grown in nutrient solution with different Al and P treatments. The expression levels were normalized in relation to eEF1α(h) and eEF1α(s) gene expression. Data are the means ± SE of three replicates. Different uppercase letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) between P-Al treatments at each time. Different lowercase letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) between times for the same P-Al treatment.