UNIVERSIDAD DE LA FRONTERA

Facultad de Ingeniería y Ciencias

Doctorado en Ciencias de Recursos Naturales

"MECHANISMS INVOLVED IN SILICON UPTAKE AND TRANSPORT, AND GENE EXPRESSION OF SILICON TRANSPORTERS IN RESPONSE TO ALUMINIUM TOXICITY IN RYEGRASS PLANTS"

DOCTORAL THESIS IN FULFILLMENT OF THE REQUERIMENTS FOR THE DEGREE DOCTOR OF SCIENCES IN NATURAL RESOURCES

SOFÍA VALESKA PONTIGO SEGUEL

TEMUCO-CHILE

2019

“Mechanisms involved in silicon uptake and transport, and gene expression of silicon transporters in response to aluminium toxicity in ryegrass plants”

Esta tesis fue realizada bajo la supervisión de la Dra. PAULA CARTES INDO, perteneciente al Departamento de Ciencias Químicas y Recursos Naturales de la Universidad de La Frontera y es presentada para su revisión por los miembros de la comisión examinadora.

SOFÍA VALESKA PONTIGO SEGUEL

……

Dra. Paula Cartes Indo

…….

Dr. Andrés Quiroz Cortez

Director Programa de Doctorado en Ciencias de Recursos Naturales

…….

Dra. Alejandra Ribera Fonseca

.....

Dra. Mónica Rubilar Díaz

Directora Académica de Postgrado Universidad de La Frontera

…….

Dra. María de la Luz Mora

…….

Dra. Ana Gutiérrez Moraga

…….

Dra. Liliana Cardemíl

……

Dr. Luis Corcuera

To my parents, I owe it all to them.

Acknowledgements

First, I would like to thank to my parents and brothers for believing in me and for their unconditional love. Many thanks because they were always interested in what I was doing, although it is likely that they never understood it. I am also grateful to the other members of my family and friends who have supported me throughout this process.

A very special gratitude goes out to my Thesis advisor, Dr. Paula Cartes for her confidence, motivation, understanding and constant support during all these years.

I also want to thanks to the members of the evaluation committee Dr. Alejandra Ribera, Dr. María de la Luz Mora, Dr. Ana Gutiérrez, Dr. Liliana Cardemil and Dr. Luis Concuera, for their useful comments and suggestions in each Thesis Advance.

Besides, I am grateful to the Scientific and Technological Bioresource Nucleus of the Universidad de La Frontera (BIOREN-UFRO) as well as to the Plant-Soil Research Group for their collaboration and assistance in part of my experimental work.

A special mention to the members of the Plant Nutrition Research Group, belonging to the Institute for Multidisciplinary Research (IMSI) of the University of Belgrade, especially to Dr. Miroslav Nikolic for hosting me during my internship and for his valuable collaboration along my research.

Finally, further acknowledgments go to the National Commission of Scientific and Technological Research (CONICYT) for their financial support throughout the Doctoral Scholarship 21120704 and FONDECYT project 1120901. In addition, thanks to Dirección de Investigación of the Universidad de La Frontera for funding my participation at national and international congresses.

Summary and thesis outline

Silicon (Si) has been widely recognized as a beneficial element for many plant species, especially under stress conditions. Since several studies have shown that Si benefits are closely related with Si accumulation in plants, the elucidation of the mechanisms of Si uptake and transport is crucial for exploiting the Si-induced beneficial effects on agricultural systems. In this regard, it has been reported that Si can alleviate Al toxicity, which had led to suppose that the uptake and subsequent accumulation of this element could generate benefits for ryegrass, an important Si-accumulating forage species that commonly grow under Al stress. In Chapter I, we present a general introduction of this Doctoral Thesis, indicating the hypothesis and goals of this study.

In Chapter II, the importance of Si uptake in vascular plants and its influence on mineral stress under acidic conditions is presented as a review. Here, we present a comprehensive update about the considerable advances has been achieved aimed to improve the understanding of the mechanisms of Si uptake and transport in vascular plants. In addition, this work provides the new insights into the role of Si against mineral stresses that occur in acid soils.

In Chapter III, we analyzed the kinetics of Si uptake in two ryegrass cultivars differing in Al tolerance (Al-sensitive, Jumbo; Al-semi-tolerant, Nui). To examine the concentration-dependent kinetics, plants were cultivated at five Si doses (0, 0.5, 1.0, 2.0 or 4.0 mM Si), and harvest was performed at 24 hours and 21 days after the initiation of treatments. To evaluate the time-dependent kinetics, plants were grown under 0, 0.5 or 2 mM Si doses during 0, 3, 6, 9, 12 and 24 hours. In general, both the concentration- and the time -dependent Si uptake experiments showed that Si accumulation in cv. Jumbo was higher than in cv. Nui. However, cultivar variation for Si concentration was only

observed at the short-term. In addition, differences in K_m values between Jumbo and Nui, but similar values of V_{max} were found. On the other hand, two putative Si transporter genes, *LpLsi1* and *LpLsi2*, were identified from ryegrass with characteristics highly conserved among Si transporters from different plant species. A gene expression analysis showed that both *LpLsi1* and *LpLsi2* were only expressed in roots, and the expression level was decreased by Si supply.

The role of Si for ryegrass cv. Nui subjected to Al toxicity was reviewed in the Chapter IV. Here, we assessed the effect of Si on the modulation of Si/Al uptake and the antioxidant performance of ryegrass plants hydroponically cultivated with Al (0 and 0.2 mM) in combination with Si (0, 0.5, and 2.0 mM). Exposure to Al significantly increased Al concentration, mainly in the roots, with a consequent reduction in root growth. However, Si applied to the culture media steadily diminished the Al concentration in ryegrass, which was accompanied by an enhancement in root dry matter production. A reduced concentration of Si in plant tissues was also observed when plants were simultaneously supplied with Al and Si. Likewise, Si transporter genes (*Lsi1* and *Lsi2*) were down-regulated in roots after Si or Al was applied alone; however, both *Lsi1* and *Lsi2* were up-regulated as a consequence of Si application to Al-treated plants. Complementary, this study also showed molecular and biochemical evidence supporting the role of Si on the improvement of the antioxidant performance of ryegrass cv. Nui subjected to Al toxicity. In this regard, Si uptake attenuated oxidative damage by increasing phenols concentration as well as by modulating the activity of SOD, CAT, APX and POD antioxidant enzymes and the gene expression of Mn-SOD, Fe-SOD and Cu/Zn-SOD isoforms under Al stress.

In order to extend our knowledge about Si/Al uptake under Al stress, studies involving ryegrass cv. Jumbo were discussed and compared with cv. Nui in Chapter V. Similar to cv. Nui; Si applied to Al-stressed plants of cv. Jumbo decreased both Al concentration and lipid peroxidation significantly improving the root matter production by about 118%. Interestingly, differences in both root Si concentration and gene expression pattern of Si transporters were also found between Jumbo and Nui under combined Al and Si treatments. These results might denote either a different Si requirement between cultivars to counteract Al stress or the involvement of unknown regulatory element(s) determining the function of Si transporters.

Finally, overall results are discussed in Chapter V, concluding the following: (1) Ryegrass has influx and efflux Si transporters with characteristics highly conserved among Si transporters from different plant species (2) Silicon alleviates Al stress by modulating Al/Si uptake and by reducing the Al-induced oxidative stress with the consequent improvement of root growth in ryegrass cultivars with contrasting Al-tolerance. Moreover, ryegrass cultivars differing in Al-tolerance exhibit differential gene expression pattern of Si transporters under Al stress.

TABLE OF CONTENTS

CHAPTER I

General Introduction

1.1 General Introduction

Several reports have highlighted the positive benefits of silicon (Si) on vascular plants. It increases the plant resistance against numerous biotic/abiotic stresses such as pathogen diseases and insect pests (Fauteux et al. 2005; Romero et al. 2011), drought (Hattori et al. 2005; Gong et al. 2005; Hosseini et al. 2017), salt (Ahmad et al. 1992; Zhu et al. 2004; Liang et al. 2007; Rios et al. 2017), extreme temperatures, UV-B radiation (Ma 2004; Liang et al. 2015), metal toxicity and nutrient imbalance (Wu et al. 2013; Adrees et al. 2015; Liang et al. 2015; Pontigo et al. 2015; Tripathi et al. 2015, 2016; Pontigo et al. 2017; Ribera et al. 2018).

Despite the benefits of Si on agriculture have been well documented, the use of Si amendments is still not well understood because of the many intricacies surrounding Si properties, uptake and efficacy (Deshmukh and Belanger 2015). Since the positive effects of Si on plants have been mostly associated to a high accumulation of this element in different tissues, Si uptake is arguably the most critical aspect of its benefits.

Rapid progress has been made in unveiling molecular mechanisms for Si uptake and transport in plants. The discovery of specific Si transporters (Ma et al. 2006) and the huge advances in high-throughput sequencing and genomics have opened ways to reach a clearer understanding of the molecular mechanisms underlying Si uptake in plants. Consequently, it has become easier to correctly classify plants on the basis of its Si-accumulating capacities and even to predict which plant species can be more prone to gain an advantage from Si uptake.

Plants take up Si by roots in the uncharged form of monosilicic acid ($H_4SiO_4^0$), which is then converted into hydrated amorphous silica and deposited on the cell walls. Two different protein families with distinct characters have been found to be responsible for

silicic acid transport from the soil solution to the shoots in varying plant species. Firstly, Si influx transporters, also known as Lsi1 and Lsi6, are passive channels belonging to the NIP III subfamily of aquaporins (Gomes et al. 2009; Deshmukh and Bélanger, 2015). The membrane channel Lsi1 facilitates the transport of Si from the external solution to the root cells, whereas Lsi6 has been implicated in unloading Si from the xylem to the shoots and in the inter-vascular transfer of Si (Ma et al. 2006; Yamaji et al. 2009). Functional Lsi1s have been reported to be essential for a plant species to accumulate Si (Deshmukh and Belanger 2015; Vatansever et al. 2017). Silicon efflux transporters, also called Lsi2, belong to the less-studied family of putative anion transporters (Ma et al. 2007; Yamaji et al. 2011). Different to aquaporins, Lsi2 is supposed to be an active transporter, driven by the proton gradient (Ma et al., 2007b) that facilitates the loading of Si into the xylem. In addition, a new gene has been identified in rice (Lsi3) that would be helpful in the process of distributing Si in panicles (Ma and Yamaji 2015). Although these findings have contributed substantially to understand the different levels of Si accumulation in plants, the expression of Si transporters genes and related mechanisms responsible for Si accumulation under stress conditions remains to be explored.

On the other hand, Si has been reported to alleviate Al toxicity in vascular plants (Adrees et al. 2015; Pontigo et al. 2015; Liang et al. 2015; Tripathi et al. 2017). Current evidence supports that Si can regulate plant resistance and/or tolerance to Al toxicity by means either external or internal mechanisms including: (i) Si-induced increase in solution pH (Li et al. 1996; Cocker et al.1998a), (ii) formation of Al-Si complexes in the growth media (Barcelo et al. 1993; Baylis et al. 1994; Ma et al. 1997; Cocker et al. 1998a) or/and within the plant (Corrales et al. 1997; Cocker et al. 1998b; Britez et al. 2002; Zsoldos et al. 2003; Wang et al. 2004; Prabagar et al. 2011), (iii) exudation of

organic acid anions and phenolic compounds (Barcelo et al., 1993; Cocker et al., 1998b; Kidd et al. 2001), (iv) increase in the chlorophyll and carotenoid contents of leaves (Singh et al. 2011) and (v) activation of the plant antioxidant system (Shahnaz et al. 2011; Shen et al. 2014). However, to our knowledge, there is a dearth of reports regarding the molecular aspects related with the uptake and transport of Si in plants subjected to Al stress.

In Southern Chile, ryegrass (*Lolium perenne* L.) is one of the main forage species used in intensive dairy and beef production systems (Mora et al. 1999; 2009). Nevertheless, it is commonly exposed to high amounts of phytotoxic Al^{+3} present on acid soils that limit its productivity. It has been reported that ryegrass is able to accumulate high Si concentrations (Jarvis, 1987; Nanayakkara et al. 2008), which had led to suppose that the uptake and subsequent accumulation of this element could generate benefits for ryegrass plants grown under Al stress. However, Si uptake and transport mechanisms are unknown for ryegrass, and the transporters involved in such processes have not been identified yet.

1.2 Hypothesis and research objectives

1.2.1Hypothesis

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

- In ryegrass, a gramineae species with high capacity to accumulate Si, the uptake and transport of Si are processes mediated by influx and efflux Si transporters.
- Silicon alleviates Al toxicity by modulating Al and Si uptake, oxidative damage and the gene expression of Si transporters in ryegrass plants. Ryegrass cultivars with contrasting Al-tolerance exhibit different gene expression pattern of Si transporters under Al stress.

1.2.2 Research objectives

1.2.2.1 General objective

- To study the mechanisms involved in the Si uptake and transport as well as the gene expression of Si transporters in response to Al toxicity in ryegrass plants (*Lolium perenne* L.)

1.2.2.2 Specific objectives

1 To identify genes involved in Si uptake and transport in ryegrass plants.

2 To evaluate the kinetics of Si uptake and gene expression of Si transporters at different Si doses in ryegrass.

3 To analyze gene expression of Si transporters in ryegrass cultivars subjected to Al toxicity.

CHAPTER II

Silicon in vascular plants: uptake, transport and its influence on mineral stress under acidic conditions

PUBLISHED

Pontigo, S., A. Ribera, L. Gianfreda, M.L. Mora, M. Nikolic, P. Cartes. 2015. Silicon in vascular plants: uptake, transport and its influence on mineral stress under acidic conditions. Planta 242(1): 23-37. doi: 10.1007/s00425-015-2333-1

Silicon in vascular plants: uptake, transport and its influence on mineral stress under acidic conditions

Sofía Pontigo[1], Alejandra Ribera[2,3], Liliana Gianfreda[4], María de la Luz Mora[3,5], Miroslav Nikolic[6], Paula Cartes[3,5*]

[1] Programa de Doctorado en Ciencias de Recursos Naturales, Universidad de La Frontera, Casilla 54-D, Temuco, Chile.

[2] Departamento de Producción Agropecuaria, Facultad de Ciencias Agropecuarias y Forestales Universidad de La Frontera, Casilla 54-D, Temuco, Chile.

[3] Scientific and Technological Bioresource Nucleus, Universidad de La Frontera, Casilla 54-D, Temuco, Chile.

[4]Dipartimento di Agraria, Università Degli Studi di Napoli Federico II, Naples, Italy.

[5] Departamento de Ciencias Químicas y Recursos Naturales, Universidad de La Frontera, Casilla 54-D, Temuco, Chile.

[6] Plant Nutrition Research Group, Institute for Multidisciplinary Research, University of Belgrade, PO Box 33, Belgrade, Serbia.

*Corresponding author: paula.cartes@ufrontera.cl

Abstract

Main conclusion So far, considerable advances have been achieved in understanding the mechanisms of Si uptake and transport in vascular plants. This review presents a comprehensive update about this issue, but also provides the new insights into the role of Si against mineral stresses that occur in acid soils. Such information could be helpful

to understand both the differential Si uptake ability as well as the benefits of this mineral element on plants grown under acidic conditions.

Silicon (Si) has been widely recognized as a beneficial element for many plant species, especially under stress conditions. In the last few years, great efforts have been made to elucidate the mechanisms involved in uptake and transport of Si by vascular plants and recently, different Si transporters have been identified. Several researches indicate that Si can alleviate various mineral stresses in plants growing under acidic conditions, including aluminium (Al) and manganese (Mn) toxicities as well as phosphorus (P) deficiency all of which are highly detrimental to crop production. This review presents recent findings concerning the influence of uptake and transport of Si on mineral stress under acidic conditions because knowledge of this interaction provides the basis for understanding the role of Si in mitigating mineral stress in acid soils. Currently, only four Si transporters have been identified and there is little information concerning the response of Si transporters under stress conditions. More investigations are therefore needed to establish whether there is a relationship between Si transporters and the benefits of Si to plants subjected to mineral stress. Evidence presented suggests that Si supply and its subsequent accumulation in plant tissues could be exploited as a strategy to improve crop productivity on acid soils.

Keywords: Silicon uptake; Silicon transporters; Plant stress; Acidic soils; Phosphorus deficiency; Aluminium toxicity; Manganese excess.

2.1 Introduction

In recent years the beneficial role of silicon (Si) in agricultural systems has been increasingly recognized. Silicon is not an essential plant nutrient as defined by the first criterion of essentiality of Arnon and Stout (1939), since its absence does not prevent completion of the plant life cycle except for Equisetaceae (Chen and Lewin, 1969) and some algae (Likhoshway et al. 2006). Nevertheless, there is much evidence that Si is highly beneficial in improving crop yield, especially under conditions of stress. Numerous researches have shown that enhancing Si uptake has a beneficial influence on plant growth and development by alleviating several abiotic and biotic stresses. These include salt stress (Ahmad et al. 1992; Zhu et al. 2004; Liang et al. 2007), drought stress (Hattori et al. 2005; Gong et al. 2005), metal toxicity (Wu et al. 2013), nutrient imbalance (Hernandez-Apaolaza 2014; Liang et al. 2015), radiation damage, high temperature and freezing (Ma 2004; Liang et al. 2015), as well as raising tolerance to plant diseases and pest attack (Fauteux et al. 2005; Romero et al. 2011).

Many beneficial effects of Si on vascular plants have been associated with enhanced accumulation of this element in different tissues, although such increases may not be easily noticeable because Si accumulation varies widely within the plant kingdom, with Si concentrations among plant species ranging from 1 to 100 g kg^{-1} dry weight (Ma and Takahashi 2002). Consequently, several researches have been undertaken to establish how plants take up Si from the soil and transport it to different tissues within the plant. In terms of the ability to accumulate Si in the shoots, plants have been classified into Si accumulators, intermediate and non-accumulator species (Takahashi et al. 1990). Accordingly, Equisetaceae, Gramineae and Cyperaceae families can accumulate up to 100 g kg^{-1} of Si on dry weight basis, whereas most dicotyledonous species usually accumulate less than 1 g kg^{-1}. Additionally, Si tissue concentration can vary

substantially among genotypes of the same species as shown in rice (*Oryza sativa*) (Deren 2001; Ma et al. 2007a), sugarcane (*Saccharum officinarum*) (Deren 2001) and barley (*Hordeum vulgare*) (Ma et al. 2003).

Recent studies on molecular mechanisms involved in Si uptake and transport in plants have shown that these processes are mediated by different transporters that differ in function, expression and localization in plant cells (Ma et al. 2006; 2007b; Yamaji et al. 2008; 2009; Chiba et al. 2009; Mitani et al. 2009a; 2009b; 2011a; 2011b; Grégoire et al. 2012; Montpetit et al. 2012; Yamaji et al. 2012; 2013; Deshmukh et al. 2013). These Si transporters have only been identified in plants that are known to accumulate relatively high Si concentrations, including rice (Ma et al. 2006; 2007b; Yamaji et al. 2008), barley (Chiba et al. 2009; Mitani et al. 2009b; Yamaji et al. 2012), maize (*Zea mays*) (Mitani et al. 2009a; 2009b), and more recently, wheat (*Triticum aestivum*) (Montpetit et al. 2012) and horsetail (*Equisetum arvense*) (Grégoire et al. 2012). In addition, Si transporters in two dicot species, pumpkin (*Curcubita moschata*) (Mitani et al. 2011a; 2011b) and soybean (*Glycine max*) (Deshmukh et al. 2013) have been identified. Despite research progress in this area, however, only four Si transporters have been identified in the above-mentioned species and the mechanisms responsible for Si uptake and transport in other species remain poorly understood. It is important to point out that there is no similarity or homology between the genes involved in Si transport of vascular plants and other members of the eukaryotic kingdom, including diatoms (Hilderbrand et al. 1997; 1998; Marron et al. 2013), sponges (Schröder et al. 2004) and chrysophycean algae (Likhosway et al. 2006).

Silicon can influence availability of other mineral elements through complex interactions that can be achieved either outside or inside plant cells. Interestingly, it has been reported that some interactions involving Si can induce beneficial effects on plant

growth and development under acidic conditions. Since soil acidity is one of the main problems that limit agricultural production in many areas of the world, this positive influence is of major importance. Below pH (H_2O) 5.5, acidification generates increased availability of phytotoxic aluminium (Al) and manganese (Mn), as well as deficiency of some nutrients such as phosphorus (P), calcium (Ca), magnesium (Mg) and potassium (K) with detrimental impact on plant growth. However, it has been demonstrated that Si can ameliorate metal toxicity (Wu et al. 2013; Kim et al. 2014), resulting from Mn (Shi et al. 2005; Li et al. 2012) and Al (Cocker et al. 1998a; 1998b; Kidd et al. 2001), and also alleviate effects of P deficiency (Ma and Takahashi 1990a; 1990b, 1991; Owino-Gerroh and Gascho 2004). The present paper examines recent findings concerning Si uptake and transport as well as the influence of Si on mineral stress under acidic conditions. To our knowledge this is the only review to be published focused on evaluating these two aspects of Si function in plants.

2.2 Silicon bioavailability in soil

Silicon is the second most abundant mineral element of the earth's crust after oxygen (Epstein 1999) and is one of the main constituents of most soils. It is present as silica minerals in the form of primary and secondary silicates. Primary silicates occur mainly in sand and silt fractions whereas secondary silicates are concentrated in the clay fraction as a result of pedogenic processes. Additionally, various amorphous forms of inorganic and biogenic silica (including phytoliths) are found in soils (Cornelis et al. 2011). All these Si forms undergo chemical and physical weathering, resulting in the release of Si into soil solution which is then transferred to rivers and oceans (Guntzer et al. 2012).

Monosilicic acid is weakly acidic (pKa_1 = 9.83 and pKa_2 = 13.17) and represents the soluble Si form in soil solution. It is commonly found as an uncharged monomer molecule ($H_4SiO_4^0$) over a pH range from 2 to 9, or in ionized form ($H_3SiO_4^-$ / $H_2SiO_4^{2-}$) at pH values greater than 9.0 (Knight and Kinrade 2001). Thus, considering that the pH of most soils is below 9.0, undissociated silicic acid is the most common Si form present in soils with concentrations varying between 0.1 to 0.6 mM (Epstein 1999).

Silicon concentration in soil solution is mainly affected by the dissolution of the siliceous compounds and by sorption reactions between soluble silica and soil constituents (Wickramasinghe and Rowell 2006). Silicate can be adsorbed by ligand exchange on to iron (Fe) and aluminium (Al) oxides and hydroxides, and can also compete for sorption sites with other anions on mineral surfaces. Additionally, Si can also be complexed by heavy metals, but scarcely forms complexes with dissolved organic matter (Cornelis et al. 2011). Accordingly, despite the abundance of Si in soils, the amount of soluble Si available for plant uptake may be limited. Usually, less weathered, geologically younger mineral soils tend to supply more Si to plants than typically acid, weathered, leached and low base saturation soils. For instance, Oxisols and Ultisols tend to be highly weathered and have low Si availability to plants (Foy 1992). Likewise, due to the high content of organic matter and low mineral content, Histosols are also low in available Si content (Snyder 1991). Additionally, despite of the high content of quartz in Entisols, Si is only slightly soluble and thus virtually unavailable to plants (Datnoff et al. 1997).

2.3 Silicon uptake and transport

Silicon uptake by vascular plants is a very complex process characterized by selectivity of transport and Si accumulation in specific tissues; this can differ both between species

and within each plant species. (Fig.1). Briefly, once silicic acid is taken up by the roots, it is transported from cortical cells to the stele. Subsequently, Si is released into the xylem and translocated through the transpiration stream to the shoots (Ma and Yamaji 2006). Here it is concentrated during loss of water associated with the transpiration process and transformed to amorphous silica (SiO_2-nH_2O) by Si polymerization (Ma and Yamaji 2006). Consequently, amorphous silica is accumulated mainly in the cell wall of leaves, stems, and hulls (Prychid et al. 2004; Ma and Yamaji 2006). Silica can also be deposited in cells of roots, tubers and inflorescences of a variety of plant species (Hodson and Sangster 1988; 1989; Lux et al. 1999; Chandler-Ezell et al. 2006). It has been suggested that silica can interact with cell wall components such as polysaccharides, lignins or proteins, but the nature of this association is not yet fully understood (Currie and Perry 2007; Guerriero et al. 2016; Głazowska1 et al. 2018).

It is widely known that mineral elements can be transported through the roots by both energy-independent (passive) and energy-dependent (active) processes, which occur down and against an electrochemical potential gradient, respectively. As reported above, three different ways of Si uptake in different plant species are responsible for high, medium and low Si concentration (Takahashi et al. 1990). Plants with an energy-dependent Si uptake mechanism show a significant decrease of Si concentration in the uptake solution, whereas in plants in which Si uptake is mediated by an energy-independent transport system, no significant changes are observed. By contrast, Si excluder plants tend to increase Si concentration in the uptake solution.

Most monocots, e.g. rice (Takahashi et al. 1990; Tamai and Ma 2003), wheat (Van der Vorm 1980; Jarvis 1987; Rains et al. 2006), ryegrass (Lolium perenne) (Jarvis 1987; Nanayakkara et al. 2008) and barley (Liang et al. 2006; Nikolic et al., 2007) and some cyperaceous plants, take up larger Si amounts compared with other species, indicating

involvement of an energy-dependent transport system. By contrast, most dicots take up less Si following the concentration gradient (Takahashi et al. 1990). However, there are some dicot species such as cucumber (*Cucumis sativus*) that take up Si more efficiently (Liang et al. 2005), whereas tomato (*Solanum lycopersicum*) (Nikolic et al. 2007) and bean (*Phaseolus vulgaris*) (Liang et al. 2005) exclude Si from uptake.

Energy-dependent and energy-independent Si uptake processes can coexist in Si accumulator species such as rice, maize and in intermediate type species such as sunflower (*Helianthus annuus*) and wax gourd (*Benincasa hispida*), with their relative contribution being dependent upon plant species and external Si concentrations (Liang et al. 2006). In addition, Van der Vorm (1980) suggests that plants may show a gradual transition from an energy-dependent uptake to Si exclusion as the external Si concentration rises. Similarly, a study on Si uptake in banana (*Musa* spp.) showed that uptake occurred essentially by an energy-independent transport system at the highest Si supply (Henriet et al. 2006); nevertheless, at lower Si concentrations, Si depletion in the nutrient solution was demonstrated, suggesting the existence of an energy-dependent process for Si transport. Neumann et al. (2002) proposed another mechanism of direct Si absorption into the leaf vacuole by a process of endocytosis. Although the authors found Si inside membrane invaginations and the vacuolar vesicles in leaf cells of Si accumulator plants, further evidence is needed to confirm this supposition.

Other studies have confirmed that uptake of silicic acid implies an energy-dependent process (Raven 2003; Tamai and Ma 2003; Mitani et al. 2005). Results of Raven (2001) support the findings that the low permeability of the plasma membrane for silicic acid could not explain the high Si concentration found in the rice shoots, thereby indicating that Si uptake in this species requires metabolic control. Moreover, it has been demonstrated that Si uptake can be significantly decreased by metabolic inhibitors such

as NaCN and 2,4-dinitrophenol (2,4-DNP) and also by $HgCl_2$, which is a specific inhibitor of water channels (Ma et al. 2002; Tamai and Ma 2003; Liang et al. 2005; Nikolic et al. 2007). For example, both 2,4-DNP and $HgCl_2$ severely inhibited the uptake and translocation of Si in rice, barley and cucumber, but in tomato both inhibitors produced the reverse effect (Nikolic et al. 2007). Experimental results have led to the supposition that Si uptake involves two components: a transporter mediated component and diffusion (Mitani et al. 2005).

In addition, a kinetic study in rice indicated that Si uptake was mediated by a transporter, which exhibited a low affinity for silicic acid (Tamai and Ma 2003). Also, other studies have revealed that Si transport from the external solution to the cortical cells in three species with different Si accumulation abilities (rice, cucumber and tomato) was mediated by a transporter, which showed a similar affinity to silicic acid in all species. However, differences in maximum velocity (V_{max}) values for Si transport suggest that the density of the Si transporter on the root cell membranes differs among plant species (Mitani et al. 2005; Nikolic et al 2007). Likewise, kinetics of Si uptake in two rice cultivars (Nipponbare and Kasalath) revealed a similar Michaelis constant (K_m) value in both varieties, suggesting that similar transporters involved in the Si uptake are present in the roots of both varieties. However, the V_{max} value was higher in cv. Nipponbare than in cv. Kasalath, suggesting a greater abundance of transporters in the Nipponbare cultivar (Ma et al 2007a).

All these findings support the view that Si uptake is brought out by both energy-dependent and energy-independent processes, which apparently involve the presence of Si transporters. In some species Si transport is predominantly energy-independent, whereas in others Si transport is mainly energy-dependent but despite this, both processes can occur simultaneously in plants.

2.3.1 Silicon transporters

Even though the process of Si uptake in plants is not yet fully understood, clarification has been helped by the discovery of specific genes in gramineous species (Fig. 1). Thus, recent studies have shown that plant Si accumulation is attributed to an efficient uptake system mediated by influx and efflux transporters (Ma and Yamaji 2015; Vatansever et al. 2017; Guo-chao et al. 2018). Accordingly, four membrane proteins that transport silicic acid have been identified to date and are known as Lsi1 (or NIP2;1), Lsi2, Lsi3 and Lsi6 (or NIP2;2) (Ma et al. 2006; 2007b; Yamaji et al. 2008; 2009; Chiba et al. 2009; Mitani et al. 2009a; 2009b; 2011a; 2011b; Grégoire et al. 2012; Montpetit et al. 2012; Yamaji et al. 2012; 2013; Deshmukh et al. 2013).

The first approach to identify Si transporters was carried out on rice mutants defective in Si uptake using map-based cloning. Accordingly, it was discovered that the uptake of silicic acid from the external solution to the root cortical cells is a process carried out by a Si influx transporter named Low silicon rice 1 (*OsLsi1*) (Tamai and Ma 2003; Ma et al. 2006). Low silicon rice 1 gene (*OsLsi1*) was predicted to encode a membrane channel that shows high homology with the nodulin-26 intrinsic protein (NIP), a subgroup of plant aquaporins. The predicted amino acid sequence of OsLsi1 has six transmembrane domains and two NPA (Asn-Pro-Ala) motifs, which are highly conserved among aquaporins (Ma et al. 2006). NIPs subfamilies of aquaporins are exclusive to plants and have been subdivided into three subgroups (NIP I, II, and III) according to the sequence similarity of the aromatic/arginine (ar/R) selectivity filter, which exerts a great influence on the substrate specificity (Wallace et al. 2004; Mitani et al. 2008). NIPIII subgroup, which includes silicon influx transporters, has a distinctive ar/R selectivity filter that consists of Gly (G), Ser (S), Gly (G) and Arg (R) and generates a larger constriction pore compared with other NIP subgroups. This

characteristic allows relatively large molecules, such as silicic acid to permeate the channel (Wu et al. 2007; Mitani et al. 2011c).

The subsequent transport of silicic acid into the stele is mediated by a high-affinity efflux transporter of silicic acid Lsi2 in rice (OsLsi2) (Fig. 1), which is driven by the proton gradient (Ma et al. 2007b). Lsi2 belongs to a putative anion transporter family containing 11 transmembrane domains (Ma et al. 2007b). A homolog of *OsLsi1*, named *OsLsi6* has also been identified in rice. Despite the fact that both *Lsi1* and *Lsi6* genes code for the Si permeable channels, they play different roles in Si uptake. Thus, OsLsi6 is responsible for the export of silicic acid from the xylem and for subsequent Si distribution into the shoots (Fig. 1) (Yamaji et al. 2008). Additionally, it was found that OsLsi6 controls the inter-vascular transport of Si at the node, which is required for preferential Si allocation to the panicles (Yamaji et al. 2009). Likewise, Lsi2 and Lsi3 (a newly discovered efflux Si transporter) are also involved in Si transfer to the panicles in rice (Yamaji et al. 2013). However, no further information is yet available concerning the function of Lsi3 in plants.

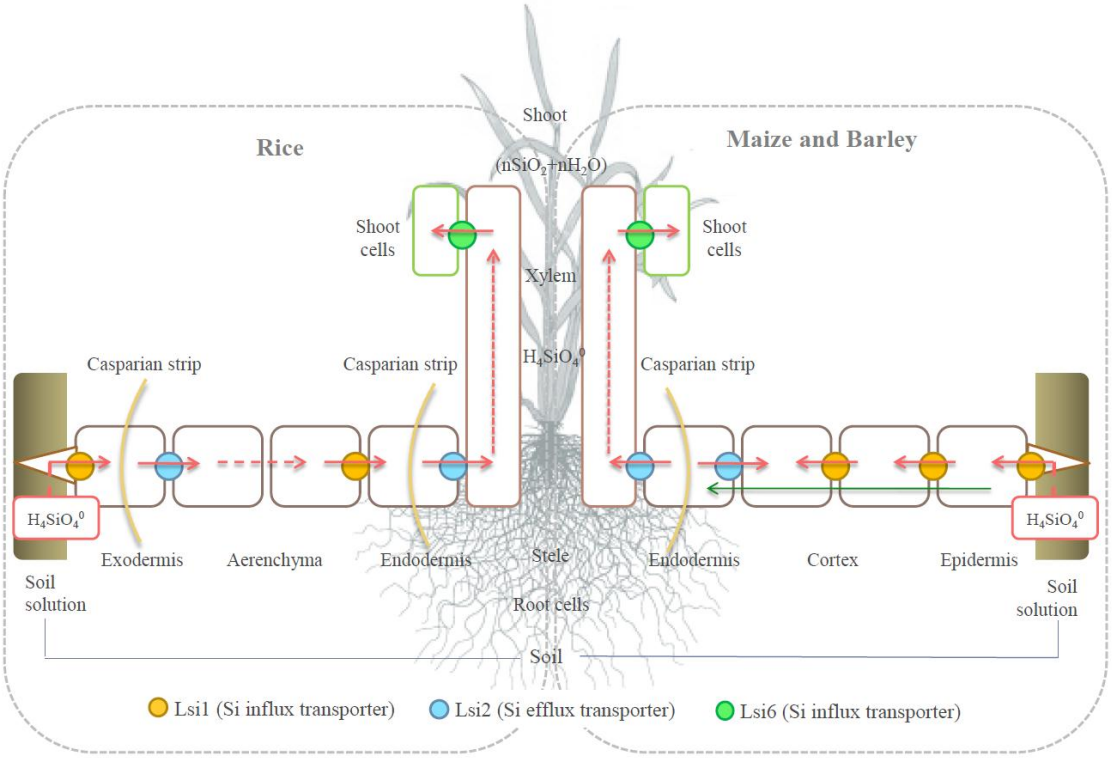

Figure 1. Model of Si uptake and transport mediated by Si transporters in different gramineous species. Rice, maize and barley are Si-accumulator plant species that exhibit distinctive Si uptake systems. In rice, Si (as monosilicic acid) is taken up from external solution to the exodermis by the influx transporter OsLsi1 and it is then released by an efflux transporter OsLsi2 to diffuse through the apoplast of the aerenchyma. Successively, both OsLsi1 and OsLsi2 transfer Si from endodermis into the stele. In barley and maize, Si can be taken up from external solution by epidermal and cortical cells by influx transporters HvLsi1 and ZmLsi1. Thereafter, Si is transported by the symplastic pathway to the endodermis (green arrow) and successively released to the stele by the efflux transporter ZmLsi2 (maize) and HvLsi2 (barley). In all three plant species, Si is translocated by the transpiration stream to the shoots via the xylem and unloaded into the symplast of xylem parenchyma cells by another influx transporter, Lsi6. In shoots, silicic acid is transformed to amorphous

silica (SiO_2–nH_2O), which is mainly accumulated in the cell walls of plant tissues. Differences of root structures among species are also shown. Taken from Ma et al. (2011) with the permission of Proceedings of the Japan Academy, Series B.

Gene expression analyses have shown that *OsLsi1* and *OsLsi2* genes are mainly expressed in the roots of rice and their suppression caused a significant decrease in Si uptake by rice roots (Table 1). Correspondingly, *OsLsi1* and *OsLsi2* have been shown to be transcriptionally down-regulated by Si supply (Ma et al. 2002; 2006; 2007b); however, an increase of expression level of *OsLsi1* in Si treated plants was found by Kim et al. (2014). Furthermore, genotypic differences in the Si accumulation resulted from the difference in the expression levels of *OsLsi1* and *OsLsi2* genes in rice roots (Ma et al. 2007b).

OsLsi6 is expressed in leaf sheaths, blades and root tips as well as and in nodes during the reproductive growth stage (Yamaji et al. 2009). Like *OsLsi1* and *OsLsi2*, *OsLsi6* gene expression was also down-regulated by Si supply in the roots and leaf blades but not in the leaf sheath (Yamaji et al. 2008). Furthermore, knockout of *OsLsi6* does not affect the uptake, but alters Si deposition in leaves (Yamaji et al. 2008).

Table 1 also shows the cellular localization of Si transporters. OsLsi1 and OsLsi2 are polarly localized in the plasma membrane of the exodermal and endodermal cells, where the Casparian strips are formed. Whereas OsLsi1 is localized to the distal side of these two cell layers, OsLsi2 is localized on the proximal side (Ma et al. 2006; 2007b; Yamaji and Ma 2007; 2011). By contrast, OsLsi6 is mainly localized in the adaxial side of the xylem parenchyma cells of leaves as well as at the transfer cells with polarity facing the vessel in node I, which is below the panicle (Yamaji et al. 2008; 2009).

Table 1. Characteristics of Si transporters in different plant species.

Transporter	Plant species	Gene	Expression pattern	Cellular localization	Function	Reference
Lsi1	*Oryza sativa*	*OsLsi1*	Roots Down-regulated by Si supply	Plasma membrane with polar localization on the distal side of both exodermis and endodermis of the root cells.	Silicon transport from the external solution into root cortical cells	Ma et al. 2006a
	Hordeum vulgare	*HvLsi1*	Roots Unaffected by Si supply	Plasma membrane with polar localization on the distal side of epidermal, hypodermal, and cortical cells.		Chiba et al. 2009
	Zea mays	*ZmLsi1*	Roots Unaffected by Si supply	Plasma membrane with polar localization on the distal side of epidermal, hypodermal, and cortical cells.		Mitani et al. 2009a
	Triticum aestivum	*TaLsi1*	Roots Unaffected by Si supply	Plasma membrane.		Montpetit et al. 2012
	Cucurbita moschata	*CmLsi1*	Roots /Shoots	Plasma membrane of all root cells, without polar localization.		Mitani et al. 2011a
	Glycine max	*GmLsi1 (GmNIP2;1)*	Roots /shoots Down-regulated by Si supply	Plasma membrane.		Deshmukh et al. 2013
Lsi2	*Oryza sativa*	*OsLsi2*	Roots Down-regulated by Si supply	Plasma membrane with polar localization at proximal side of both exodermis and endodermis of the root cells.	Silicon transport out of the root cells towards the stele.	Ma et al. 2007b
	Hordeum vulgare	*HvLsi2*	Roots Down-regulated by Si supply	Plasma membrane of endodermis root cells, without polar localization.		Mitani et al. 2009b
	Zea mays	*ZmLsi2*	Roots Down-regulated by Si supply	Plasma membrane of endodermis root cells, without polar localization.		Mitani et al. 2009b
	Cucurbita moschata	*CmLsi2*	Roots /shoots	Not determined.		Mitani et al. 2011b

Table 1. Characteristics of Si transporters in different plant species. (Continued)

Transporter	Plant species	Gene	Expression pattern	Cellular localization	Function	Reference
Lsi6	*Oryza sativa*	*OsLsi6*	Roots / Shoots/ Node I Down-regulated by Si supply	Plasma membrane with polar localization on the distal side of root cells. Parenchyma cells with polar localization on the side adjacent to xylem vessels in leaves. Xylem transfer cells with polarity facing toward the xylem vessel.	Silicon transport out of the xylem into the leaf tissues Inter-vascular transfer of Si at the node I, from the enlarged vascular bundles coming from the roots to the diffuse vascular bundles connected to the panicle in rice and barley.	Yamaji et al. 2008 Yamaji et al. 2009
	Hordeum vulgare	*HvLsi6*	Roots / Shoots/ Node I Unaffected by Si supply	Plasma membrane with polar localization on the distal side of root cells. Parenchyma cells with polar localization on the side adjacent to xylem vessels in leaves and also in the outer parenchyma cells surrounding the phloem. Xylem transfer cells with polarity facing toward the xylem vessel.		Yamaji et al. 2012
	Zea mays	*ZmLsi6*	Roots / Shoots Unaffected by Si supply	Xylem parenchyma cells with polar localization on the side adjacent to vessels in leaves. Without polar localization in roots.		Mitani et al. 2009a
	Glycine max	*GmLsi6 (GmNIP2;2)*	Roots / Shoots Unaffected by Si supply	Plasma membrane.		Deshmukh et al. 2013
Horstail Major intrinsic protein (MIP) family	*Equisetum arvense*	Multigene family	Roots / Shoots, depending on the gene	Not determined.	Not determined	Grégoire et al. 2012

Homologs of rice Si transporters have also been identified in others plant species. They differ in cellular localization and expression pattern, denoting that Si homeostasis is differentially regulated. Lsi1 has been also isolated and characterized in maize (ZmLsi1) (Mitani et al. 2009a), barley (HvLsi1) (Chiba et al. 2009) and wheat (TaLsi1) (Montpetit et al. 2012) as well as in two dicot species: pumpkin (CmLsi1) (Mitani et al. 2011a) and soybean (Deshmukh, et al. 2013). Unlike OsLsi1, both ZmLsi1 and HvLsi1 showed polar localization at the distal side of epidermal, hypodermal and cortical cells (Chiba et al. 2009; Mitani et al. 2009a) (Table 1). Conversely, the pumpkin transport CmLsi1 is localized at all root cells with no polarity. However, when the corresponding gene *CmLsi1* was expressed in rice, it showed polar localization at the distal side of plasma membrane of both the exodermis and endodermis similar to OsLsi1 (Mitani et al. 2011a). Like *OsLsi1*, expression analysis showed that *HvLsi1*, *ZmLsi1* and *TaLsi1* were mainly detected in roots but their expression level was not affected by Si supply (Chiba et al. 2009; Mitani et al. 2009a; Montpetit et al. 2012). Nevertheless, Bokor et al. (2014) recently found that *ZmLsi1* was down-regulated by Si application. *GmNIP2;1* was expressed in both roots and shoots of soybean and it was down-regulated by Si (Deshmukh et al. 2013). Likewise, *CmLsi1* was expressed in both roots and shoots of pumpkin and its functional characterization showed that a mutation in only one amino acid residue of the Lsi1 is probably the cause of the difference in Si uptake between two pumpkin cultivars (Mitani et al. 2011a).

Additionally, a multigene family of aquaporin Si transporters has been identified in the horsetail (*Equisetum arvense*), which is one of the major Si accumulator in the plant kingdom and requires Si for survival (Hodson et al. 2005; Grégoire et al. 2012). A comparison of functional domains and phylogenetic analysis of sequences revealed that the horsetail proteins belong to a different group than Si transporters of vascular plants.

Interestingly, some of these horsetail Si transporters exhibit a higher Si transport activity than those of rice (Grégoire et al. 2012). However, neither the specific function of each gene or their localization at the cellular level has been investigated yet.

Following identification of homologs of Lsi1, homologs of Si efflux transporters Lsi2 have also been found in maize (ZmLsi2) (Mitani et al. 2009b), barley (HvLsi2) (Mitani et al. 2009b) and pumpkin (CmLsi2) (Mitani et al. 2011b). It has been reported that unlike OsLsi2, maize and barley Lsi2 are only present in the endodermis of roots without polarity (Table 1). The encoding genes *ZmLsi2* and *HvLsi2* are mainly expressed in the roots. In these plant species the expression of *Lsi2* was down-regulated in response to Si supply, in contrast to *ZmLsi1* and *HvLsi1* (Mitani et al. 2009b; Bokor et al. 2014) (Table 1). Moreover, *Lsi2* is expressed in both roots and shoots of pumpkin (Mitani et al. 2011b), which is different from the pattern expression of monocots species. In addition, a study revealed that the genotypic variation in Si uptake among barley cultivars is a consequence of the difference in the expression level of only *HvLsi2*; which differs from that reported in rice (Mitani et al. 2009b).

The gene encoding the Lsi6 transporter has also been characterized in barley (*HvLsi6*) (Yamaji et al. 2012), maize (*ZmLsi6*) (Mitani et al. 2009a) and soybean (*GmNIP2;2*) (Deshmukh et al. 2013). Similarly to *Lsi6* of rice, *HvLsi6, ZmLsi6* and *GmNIP2;2* were also expressed in both roots and shoots (Table 1). As in rice, *GmNIP2;2* was down-regulated by Si supply, but the expression pattern of *HvLsi6* and *ZmLsi6* was unaffected (Mitani et al. 2009a ; Yamaji et al. 2012; Deshmukh et al. 2013) (Table 1). Likewise, Bokor et al. (2014) found that Si addition did not affect the expression level of *ZmLsi6* in the first leaf of maize plants but was up-regulated in the second leaf. Additionally, ZmLsi6 and HvLsi6 transporters showed polar localization in the parenchyma cells adjacent to xylem vessels in leaves, similar to OsLsi6 (Mitani et al. 2009a; Yamaji et al.

2012). Nevertheless, only HvLsi6 was detected in the outer parenchyma cells surrounding the phloem area (Yamaji et al. 2012). Similarly to OsLsi6, HvLsi6 is also localized at the xylem transfer cells, indicating its involvement in the intervascular transfer of Si (Yamaji et al. 2012).

The effect of abiotic stress on gene expression of Si transporters has also been studied. Accordingly, expression of *OsLsi1* and *OsLsi2* is rapidly decreased by both dehydration stress and exogenous ABA treatment (Yamaji and Ma 2007; 2011). On the other hand, suppression and overexpression of *OsLsi1* induced differential expression of genes associated with tolerance to UV-B radiation (Fang et al. 2011). Recently, it has been found that Si supply increase the expression of *OsLsi1* and *OsLsi2* genes under cadmium (Cd) and copper (Cu) toxicities in rice plants (Kim et al. 2014). By contrast, the expression level of both *ZmLsi1* and *ZmLsi2* was down-regulated in maize roots subjected to Zn and Si supply, while an increase of *ZmLsi6* expression level was observed in shoots (Bokor et al. 2014).

Even though these findings have contributed substantially to understanding the uptake and transport of Si by plants, only four Si transporters have been fully characterized in a small number of plant species. Moreover, there is little information available about the response of these transporters in plants subjected to stress. Variations in localization, expression or activity of Si transporters could explain the dissimilar Si concentrations among plant species, and consequently differences in Si-induced responses to cope with abiotic and biotic forms of stress.

2.4. Silicon and mineral stress under acidic conditions

Uptake of mineral nutrients by plants depends not only on the presence or amounts of soil nutrients but also on the forms in which these nutrients occur in the soil and their accessibility to plant roots (Marschner 1997; Mengel and Kirkby 2001). Many factors can influence nutrient uptake by plants, e.g. root development, external pH, nutrient interaction with soil components (e.g. adsorption), as well as specific interactions between one nutrient and another (Robson and Pitman 1983). These interactions which are nutrient specific can affect uptake, distribution and function. They can induce both deficiencies and toxicities but on the other hand have a synergistic effect on plant growth (Marschner 1997). In this respect Si is able to enhance the availability of several mineral elements in the soil-plant system through complex interactions which in turn control plant growth and development. Most of the beneficial effects of Si on vascular plants have been attributed to a higher accumulation of this element in different tissues. We believe that a study of the mechanisms underlying Si uptake and transport in plants is a crucial area of research that could lead to elucidating the role of Si in mitigating mineral stress under conditions of soil acidity.

Soil acidity is one of the main problems that limits agricultural production on a global scale (Kochian et al. 2004). Acidification is a natural process in soils, mainly caused by excessive rainfall, which results in the leaching of basic cations held on exchange sites in the soil. These bases can also be released by exchange with H^+ excreted from plant roots during nutrient uptake (Mora et al. 2006). The acidification process can be accelerated by some agricultural practices e.g. by the excessive use of acidifying fertilizers (Mora et al. 1999) and use of legumes as a source of N (Bolan et al. 1991). Under acid soil conditions, large and toxic concentrations of Al and Mn can become plant available and a decrease in availability of some essential nutrients such as P, Ca,

Mg and K can occur (Mora et al. 1999; Bolan et al. 2003; Mora et al. 2006). As reported below, it has been demonstrated that Si can ameliorate the deleterious impacts of P deficiency, as well as the negative effects of metal toxicity derived from Mn and Al by either internal (plant related) or external (soil related) mechanisms. As a consequence, the productivity of key crops growing in acidic soils might thus be improved, and Si transporters could be used as a strategic tool to enhance plant tolerance to mineral stress and to stimulate further research. Here it would be necessary to: (i) identify and characterize Si transporters in other plants than those already studied; (ii) identify and characterize novel Si transporters, (iii) investigate the mechanisms involved in the regulation of sensing, signal transduction and gene expression of these transporters, and (iv) establish possible, if any, relationship between Si transporters and their influence on plant mineral stress (Fig. 2).

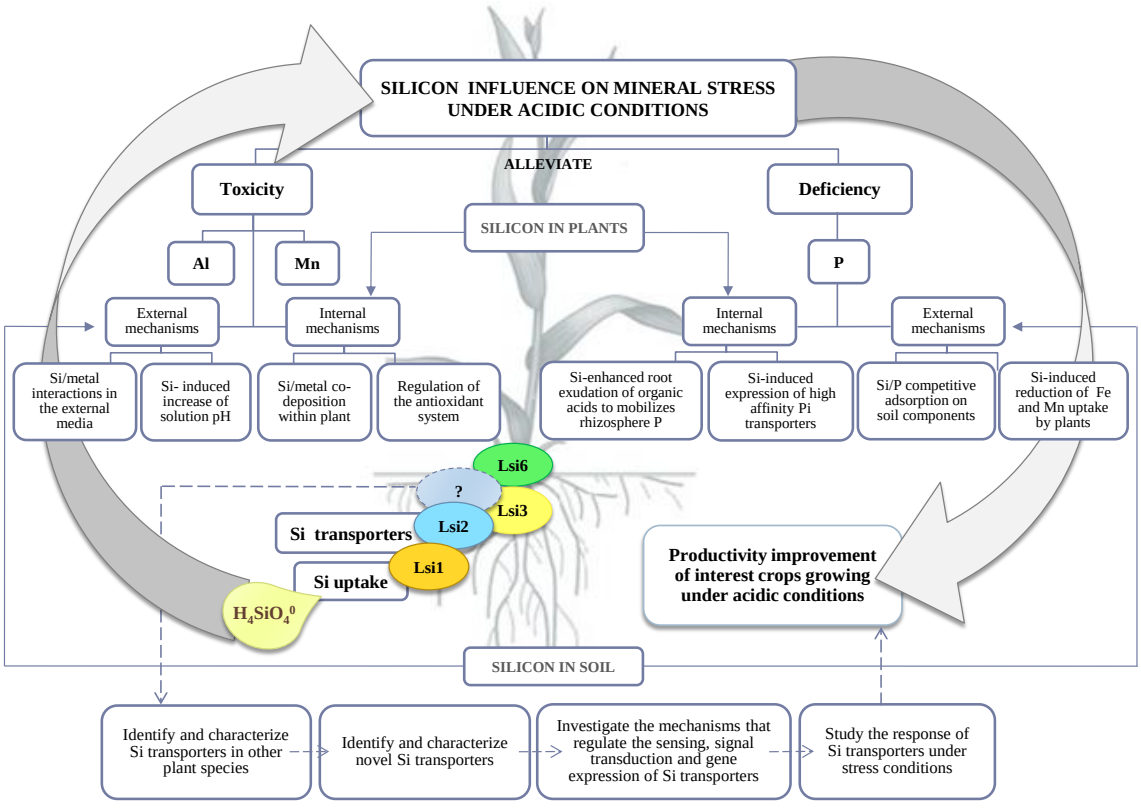

Figure 2. Overview of Si uptake and its role in plants under acidic conditions. Silicon transporters are shown in colored circles; dashed arrows indicate research areas that still remain to be investigated.

2.4.1. Phosphorus deficiency

Phosphorus is an important nutrient required by plants because it is a key component of molecules such as nucleic acids, phospholipids and ATP. Thus, P plays important roles in many plant processes such as energy metabolism, photosynthesis, respiration, enzyme reactions and in the regulation of metabolic pathways (Schachtman et al. 1998).

In acid soils, phosphate ions may be specifically adsorbed on the surfaces of clay minerals and iron (Fe) or Al oxides (Hingston and Raupach 1967; Ryden et al. 1977). This sorption is also controlled by inorganic anions and organic matter in soils (Parfitt 1978). As a consequence of these adsorption reactions, P availability to plants is decreased (Vance et al. 2003). Interactions between Si and P in soil have been studied as silicate application to soil reduces phosphate adsorption thereby increasing its availability which has been attributed mainly to competition between silicate and phosphate ions for the adsorption sites of various soil constituents (Obihara and Russell 1972; Pardo and Guadalix 1990; Lee and Kim 2007).

In contrast to these findings some studies have demonstrated that P availability in soil is not increased by Si addition (Ma and Takahashi 1990a; 1991). Different experimental conditions could be an important reason for such varied and even contradictory conclusions. Nevertheless, under acidic soil conditions, an indirect effect of raising P availability and utilization by Si supply could be expected due to lower solubility and uptake of metals (e.g. Mn, Fe, Al and Cd) (Ma and Takahashi 1990a; Liang et al. 2005).

The ability of silicate to compete with phosphate is highly pH-dependent because silicic acid is weakly dissociated below pH 9.0 (Dietzel 2000), which limits its effectiveness as competitor in the pH-range of most soils. Therefore, P adsorption would be expected to be much greater than that of Si in acid soils as the pK_a value of orthosilicic acid is much higher than that of orthophosphoric acid (9.8 and 2.1, respectively). This statement is supported by the results of Lee and Kim (2007), who showed that with increasing pH, phosphate adsorption decreased and silicic acid adsorption increased. In addition, Lee et al. (2004) found that increasing silicate concentrations augmented phosphate desorption in two soils, but at pH values ranging from 7 to 9. However, Owino-Gerroh and Gascho (2004) demonstrated that application of sodium silicate to acid soils reduced phosphate adsorption as a result of an increase in soil pH. Consequently, an enhancement of soil P availability was triggered by Si, which further improved the plant growth probably by increasing P uptake when Si concentration was high in solution. De and Datta (2007) have also demonstrated that Si can reduce P adsorption in acid soils, and Hartono (2008) showed that the application of calcium silicate to Andisols could increase P availability in the soil.

The first evidence of the beneficial effect of Si fertilization on P status in plants came from a 14-year field experiment conducted at the Rothamsted Experimental Station; when P fertilizers were not applied, yield of barley from a field fertilized with Si was higher than in a field without Si amendment (Fisher 1929). The direct (*in planta*) effect of Si under P deficiency was earlier attributed to enhanced plant P utilization by increased phosphorylation and distribution of phosphate esters (Cheong and Chan 1973). Very recently, increased exudation capacity of citrate and malate for P mobilization in the rhizosphere, along with an increased expression of transcripts (*TaMATE1* and

TaALMT1) related to the organic anion efflux transports, has been reported in the roots of Si-treated wheat plants grown under low P conditions (Kostic-Kravljanac, 2015).

2.4.2. Aluminium and manganese toxicities

Aluminium (Al) and manganese (Mn) are considered the most important factors that limit plant growth in acid soils (Foy 1984; Rengel 2000; Kochian et al. 2004). Aluminium is not a plant nutrient nor does it have any known function in metabolism (Arunakumara et al. 2013). For a few plant species, however, it has been shown to have a beneficial effect on growth at low concentration (Broadley et al. 2012). By far the predominant effect of Al is its toxic effect in soils of low pH. By contrast, Mn is an essential micronutrient that plays an important role in metabolic processes such as photosynthesis, respiration, and biosynthesis of proteins and carbohydrates in vascular plants (El-Jaoual et al.1998).

Aluminium occurs in different forms in soil depending on pH. Similarly, Mn speciation is not only affected by pH, but also by redox conditions (Kochian et al. 2004). Aluminium mostly forms insoluble oxides and complex aluminosilicates (AS) at pH values greater than 5.0. At lower pH values, Al is solubilized to the monomeric form of Al^{3+}, which is highly toxic to plants (Kochian et al. 2004; Ryan and Delhaize, 2010). Likewise, in acid soils, an excess of Mn^{2+} in solution is to be expected (Rengel 2000). Aluminium toxicity gives rise to a rapid inhibition of root growth as well as disruption of structure and/or functions of the cell wall (Horst et al. 2010), plasma membrane (Yamamoto et al. 2001), signal transduction pathways (Goodwin and Sutter 2009), and nutrient homeostasis (Delhaize and Ryan 1995). Manganese toxicity may depress photosynthesis as well as the uptake, translocation and utilization of other mineral

elements (Ryan and Delhaize 2010). In addition, numerous studies have shown that Al and Mn toxicities can also induce the formation of reactive oxygen species (ROS), and thus trigger oxidative stress in plants (Darkó et al. 2004; Mora et al. 2009; Cartes et al. 2012; Ribera et al. 2013).

Beneficial effects of Si on Al toxicity have been found in soybean (Baylis et al. 1994), barley (Hammond et al. 1995), sorghum (Hodson et al. 1993) maize (Corrales et al. 1997), wheat (Zsoldos et al. 2003), rice (Singh et al. 2011), and also in some conifers (Hodson et al. 1999). Different mechanisms have been hypothesized to be responsible for lower Al uptake by roots as a consequence of Si addition. In a comprehensive review on this subject, Cocker et al. (1998a) suggested that Si can decrease the toxic effects of Al by three possible mechanisms that implicate: (i) an increase in pH solution induced by Si-sources, (ii) the reduction of Al availability or (iii) an internal plant detoxification. Decrease of Al uptake due to Si application has been related to the formation of complexes of hydroxyaluminosilicates (HAS) in the external solution. Barceló et al. (1993) suggested that Si could alleviate the toxic effects of Al in maize, probably by significantly decreasing the Al^{3+} concentration in the growth media. Similarly, Ma et al. (1997) observed that the concentration of Al^{+3} in culture solution was strongly reduced when Si was applied. Moreover, many studies propose that Al-Si interactions within plants could also play an important role in the amelioration of Al toxicity. Although Cocker et al. (1998b) found that Si significantly diminished Al toxicity in two wheat cultivars, Si neither reduced the toxic levels of Al species in the external medium, nor the amount of Al taken up by roots. They therefore suggested that a plant component may be involved in the mechanism that underlies such amelioration. This component appears to be mainly related to the formation of AS or HAS into the cell walls. In this regard, Corrales et al. (1997) found that maize plants pretreated with

Si showed a lower Al uptake as well as Al exclusion from the roots. In this study the observed decrease in Al toxicity was not a consequence of a reduction of Al availability in the solution. Likewise, Si treatment did not affect Al concentration in the nutrient solution, but led to the formation of HAS in the root apoplast, alleviating Al toxicity in maize (Wang et al. 2004). In addition, Zsoldos et al. (2003) showed that Al-Si interactions within the roots enabled wheat plants to overcome Al toxicity. Prabagar et al. (2011), using suspension cultures of Norway spruce, found a lower concentration of free Al in the cell wall, which was mainly attributed to the formation of AS complexes. It has also been reported that Si addition increased chlorophyll and carotenoid contents in leaves, thus reducing Al toxicity symptoms in rice (Singh et al. 2011).

Cocker et al. (1998a) also mentioned in their review that malate or other organic compounds secreted into the bulk and the cell walls of the roots could promote the formation of AS and HAS. However, further studies have evaluated the role of root exudation of organic compounds as a mechanism of Si-induced alleviation of Al toxicity. For instance, Si has been found to enhance the root concentration of succinate and both root and shoot concentrations of malate in Al-exposed maize plants (Barceló et al. 1993), thereby suggesting that Al chelation by malate is one of the mechanisms for the Si amelioration effects. By contrast, Si did not affect the exudation of low molecular weight organic acids by roots of wheat (Cocker et al. 1998b) and maize (Kidd et al. 2001; Wang et al. 2004); however, an increased exudation of phenolic compounds was observed leading to Al detoxification (Cocker et al. 1998b; Kidd et al. 2001). Likewise, Si counteracted the negative impacts of Al in borago (*Borago officinalis*) by enhancing phenolic compounds and decreasing lipid peroxidation (Shahnaz et al. 2011).

Silicon has been reported to increase the tolerance to Mn toxicity in some plant species by different mechanisms, including the interaction between Si and Mn in the cell walls,

as well as by the stimulation of the antioxidant system. For instance, in rice (Horiguchi 1988; Ma and Takahashi 1990a) and sorghum (Galvez et al. 1989) Si supply decreased the uptake of Mn. However, in barley (Horiguchi 1987), rice (Horiguchi 1988) and bean (Horst and Marschner 1978) Si did not substantially affect Mn uptake, but prevented the uneven distribution of Mn in the tissues, thus ameliorating the symptoms of Mn toxicity in leaves. Moreover, Horst et al. (1999) demonstrated that Si application reduced apoplastic Mn concentrations due to Si-induced alterations of Mn-binding properties of the cell wall. Similarly, Iwasaki et al. (2002) showed that Si enhanced Mn tolerance by reducing Mn concentration in the leaf apoplast of cowpea. Based on Mn concentration in both symplast and apoplast of cucumber leaves, Rogalla and Römheld (2002) reported that Si-mediated tolerance of Mn as a consequence of Mn-Si interactions in the apoplast. Thus, both a stronger binding of Mn to cell walls was generated and a lower Mn concentration within the symplast was found. However, the Si-mediated strong binding of Mn to the cell walls was only detectable in the plants grown with a simultaneous supply of Si and high Mn concentrations in the nutrient solution (Rogalla and Römheld 2002), whereas the cation exchange capacity of the leaf cell wall material obtained from the high Mn-treated cucumber plants was not affected by supply of Si to roots (Dragisic Maksimovic et al. 2012). Moreover, studies in cowpea suggest that the alleviation of Mn toxicity could not be attributable only to a decrease in free leaf apoplastic Mn through its enhanced cell-wall binding capacity in Si-treated plants (Iwasaki et al. 2002; Führs et al. 2009). Additionally, it has been shown that Si was able to ameliorate Mn toxicity in pumpkin through the binding of Mn to Si around the base of trichomes on the leaf surface (Iwasaki et al. 1999). Sequestration of Mn into the vacuoles might play an important role in Si-mediated Mn tolerance in some plant

species (e.g., bean) (Horst and Marschner 1978), but again this mechanism has not been observed in others (e.g., cowpea) (Horst et al. 1999).

More recently, it has been demonstrated that Si also plays an important role on plant antioxidant system, especially under stress conditions. Silicon-mediated alleviation of Mn toxicity decreased the oxidative damage of biological membranes by regulating the activities of antioxidant enzymes and/or the concentration of antioxidants compounds in plants such as cucumber (Shi et al. 2005; Feng et al. 2009; Dragisic Maksimovic et al. 2012) and rice (Li et al. 2012). The addition of Si indirectly decreased the accumulation of hydroxyl radicals in the leaf apoplast of cucumber with excess Mn by decreasing the free apoplastic Mn^{2+} (a Fenton catalyst), while adding monosilicic acid to the Mn^{2+}/H_2O_2 reaction mixture did not directly affect the Fenton reaction *in vitro* (Dragisic Maksimovic et al. 2012).

Dragisic Maksimovic et al. (2007) showed that the concentrations of phenolic compounds, such as coniferyl alcohol and coumaric and ferulic acids, in the leaf extracts tended to be lower in Si-treated plants at high Mn supply. On the other hand, application of Si induced a significant increase in the concentrations of chlorogenic and caffeic acids in the leaf extracts of high-Mn-treated plants. Peroxidase (POD) and polyphenoloxidase (PPO) activities were enhanced by the high Mn supply in both root and leaf extracts, while the root application of Si decreased POD and PPO activities in both roots and leaves. The results of Dragišic Maksimovic et al. (2007) in cucumber and Führs et al. (2009) in cowpea suggested that Si nutrition modulated the metabolism and utilization of phenols mainly at the leaf level by stimulating the formation of Si-polyphenol complexes. Concomitantly, lower concentrations of phenolic compounds available to act as substrates for PPO and POD in Si-treated Mn-stressed plants may thus be responsible for depressing the generation of ROS.

2.5. Conclusion and perspectives

Current knowledge of the uptake, transport and accumulation of Si has increased understanding of the beneficial effects of this element in vascular plants. Accordingly, it has been demonstrated that Si acquisition by plants growing in soil is attributed to an effective uptake system mediated not only by diffusion of Si from the bulk soil to the root surface but also by transporters within the plant. Such transporters coordinate Si transport from soil to roots as well as Si distribution within the plant. To date only four Si transporters have been identified in a few plant species, and little is known about the response of Si transporters under stress conditions. Further work should therefore be focused on the cloning of genes involved in Si uptake and transport from other plant species as well as on the identification and characterization of novel Si transporters. Furthermore, more studies are needed to elucidate the mechanisms involved in the regulation of sensing and signal transduction pathways and gene expression of these transporters.

A comprehensive understanding of Si uptake and transport provides an attractive opportunity to optimize the Si uptake system by either plant breeding or agronomic management, with consequent enhancement of productivity of key crops growing under mineral stress. Thus, the incorporation of Si into acid soils and its subsequent uptake could be envisaged as a potential strategy to overcome the negative effects produced by either Al and/or Mn excess as well as by P deficiency, which commonly coexist in acid soils limiting agricultural production on a global scale.

Acknowledgements. This work was supported by the FONDECYT project 1120901, CONICYT Doctoral Scholarship 21120704 and MEC project 80130066. Part work cited in this paper was supported by the Serbian Ministry of Education, Science and Technological Development Grant ON-173028 to M. N. We thank Dr. Ernest A. Kirkby (Faculty of Biology, University of Leeds, UK) for valuable comments and improvement of the English text.

CHAPTER III

Silicon uptake by ryegrass: Molecular cloning and gene expression analysis of Si transporters

MANUSCRIPT IN PREPARATION

Silicon uptake by ryegrass: Molecular cloning and gene expression analysis of Si transporters

Sofía Pontigo[1,2], Leyla Parra[1,2], María de la Luz Mora[2,4], Paula Cartes[2,4*]

[1]Programa de Doctorado en Ciencias de Recursos Naturales, Universidad de La Frontera, Avenida Francisco Salazar 01145, P.O. Box 54-D, Temuco, Chile.

[2]Center of Plant-Soil Interaction and Natural Resources Biotechnology, Scientific and Technological Bioresource Nucleus (BIOREN-UFRO), Universidad de La Frontera, Avenida Francisco Salazar 01145, P.O. Box 54-D, Temuco, Chile.

[4]Departamento de Ciencias Químicas y Recursos Naturales, Facultad de Ingeniería y Ciencias, Universidad de La Frontera, Avenida Francisco Salazar 01145, P.O. Box 54-D, Temuco, Chile.

*Corresponding author: paula.cartes@ufrontera.cl

Abstract

Silicon (Si) is recognized as a beneficial element for vascular plants that increases the plant stress tolerance. Ryegrass, a forage species of great interest in Southern Chile, is able to accumulate relatively high Si concentrations. However, neither the uptake mechanisms have been characterized nor the transporters that mediate these processes have been identifying yet. In this study, we first analyzed the concentration- and time-dependent kinetics of Si uptake in two ryegrass (Jumbo and Nui.) cultivars. To examine the concentration-dependent kinetics, plants were cultivated at five Si doses (0, 0.5, 1.0, 2.0 or 4.0 mM Si), and harvest was performed at 24 hours and 21 days after the

initiation of treatments. To evaluate the time-dependent kinetics, plants were grown under 0, 0.5 or 2 mM Si doses during 0, 3, 6, 9, 12 and 24 hours. Both kinetics experiments showed differences in Si accumulation between cultivars; however, these variations were only observed at the short-term. Lineweaver-Burk linearization showed differences in K_m values between Jumbo and Nui, but similar values of V_{max}. Subsequently, based on sequence homology approach, two putative Si transporter genes, *LpLsi1* and *LpLsi2*, were identified. These genes shared high sequence similarity with their homologues in vascular plants. Conserved domains characterizing Si transport activity including the aromatic/arginine (Ar/R) selectivity filter, two Asn-Pro-Ala (NPA) motifs and the spacing between NPA domains, were found in the deduced amino acid sequence of LpLsi1. In addition, a gene expression analysis showed that both *LpLsi1* and *LpLsi2* were only expressed in roots, and the expression level was down-regulated by Si supply. Our findings confirm the presence of putative Si transporters in ryegrass. The assessment of Si uptake kinetics and the identification of these Si transporters genes provide new evidence about the Si accumulation ability of ryegrass.

Keywords: Silicon uptake; kinetics; Lsi-genes; gene expression; *Lolium perenne* L.

3.1 Introduction

Silicon (Si) is an abundant and differentially distributed element in soils that is proven to have multiple biological functions in plants as response to several environmental stressors. Accordingly, Si is regarded as a beneficial element that improves plant tolerance to biotic stresses such as pathogen infections and insect pests (Fauteux et al. 2005; Romero et al. 2011), as well as abiotic detrimental conditions including drought

(Hattori et al. 2005; Gong et al. 2005; Hosseini et al. 2017), UV-B radiation, high and low temperatures (Ma 2004; Liang et al. 2015), salinity (Ahmad et al. 1992; Zhu et al. 2004; Liang et al. 2007; Rios et al. 2017), metal toxicity and nutrient imbalance (Wu et al. 2013; Adrees et al., 2015; Liang et al., 2015; Pontigo et al. 2015; Tripathi et al., 2015, 2016; Pontigo et al. 2017; Ribera et al. 2018)

The positive effects of Si have been mostly associated to a high Si accumulation capacity by plants. However, the benefits of Si and its essentiality continue under debated because of there is an uneven distribution of this element within the plant kingdom (Hodson et al. 2005). Silicon is taken up in the form of uncharged molecule of silicic acid (H_4SiO4^0), and then it is accumulated in a range from 0.1 % to 10 % of the dry mass depending on the plant species/ genotype (Epstein 1994; Ma and Takahashi, 2002). The species with high Si concentrations in their tissues are mostly monocots, which accumulate up to 10% on dry weight basis, whereas dicots usually accumulate less than 0.1%.

A better understanding of the mechanisms mediating Si uptake, transport and accumulation in plants was achieved after Si transporters were discovered in rice (Ma et al. 2006, 2007b). Current evidence shows that Si accumulation results from an efficient uptake system mediated by transporter proteins that exert coordinated functions for effective Si transport from soil to roots as well as its distribution within the plant (Ma and Yamaji 2015; Pontigo et al 2015; Guo-chao et al. 2018). Four genes encoding Si transporters have been identified in vascular plants, known as *Lsi1* (or *NIP2;1*), *Lsi2*, *Lsi6* (or *NIP2;2*) and *Lsi3* (Ma et al., 2011; Yamaji et al., 2013). Both Lsi1 and Lsi6 function as Si influx transporters and belong to the Nodulin 26-like major intrinsic proteins (NIP III), a subgroup of aquaporins (Gomes et al., 2009; Deshmukh and Bélanger, 2015). The membrane channel Lsi1 facilitates the passive transport of Si from

the external solution to the root cells, whereas Lsi6 has been implicated in unloading Si from the xylem to the shoots and in the inter-vascular transfer of Si (Ma et al., 2006; Yamaji et al., 2009). On the other hand, Lsi2 and Lsi3 are active efflux Si transporters energetically driven by a proton gradient that belong to the less-studied family of putative anion transporters (Ma et al., 2007b; Yamaji et al., 2011). Lsi2 releases Si into the xylem (Ma et al., 2007b), and recently it has been found that both Lsi2 and Lsi3 are also involved in Si transfer to the panicles in rice (Yamaji et al., 2015).

To date, Si transporters have been identified in rice (OsLsi1; OsLsi2: OsLsi3; OsLsi6), maize (ZmLsi1; ZmLsi2; ZmLsi6), barley (HvLsi1; HvLsi2; HvLsi6) and wheat (TaLsi1) (Chiba et al. 2009; Grégoire et al. 2012; Ma et al. 2006a; 2007b; Mitani et al. 2009a; 2009b; Montpetit et al. 2012; Yamaji et al. 2008; 2009; 2012; 2013). Among dicots, pumpkin (CmLsi1; CmLsi2), soybean (GmLsi1; GmLsi6), cucumber (CsLsi1; CsLsi2; CsLsi6), potato (StLsi1; StLsi2) and strawberry (FaLsi1; FaLsi2) have also been found to have *Lsi*-like genes (Mitani et al. 2011a; 2011b; Deshmukh et al. 2013; Wang et al. 2014; Vulavala et al. 2015; Ouellette et al. 2017; Sun et al. 2018). Although all these transporters appear to be keys for plants to get benefit from Si nutrition, little is known about the response of Si transporters under stress conditions, and the mechanisms responsible for Si uptake and transport in other species remain poorly understood.

Ryegrass (*Lolium perenne* L.), a dominant forage grass species in Southern Chile, belongs to the Si-accumulating group species (Jarvis, 1987; Nanayakkara et al., 2008). Even though our previous studies have demonstrated that Si uptake reduced the deleterious effect of Al toxicity as well as soil acidity in ryegrass (Pontigo et al, 2017; Ribera-Fonseca et al. 2018), little is known about the mechanisms implicated in the uptake and transport of Si in this crop. Moreover, the transporters involved in such

processes in ryegrass have not been characterized yet. Thus, this study aimed to characterize the Si uptake system of ryegrass through the molecular cloning and gene expression pattern of Si transporters genes.

3.2 Material and Methods

3.2.1 Plant materials and growth conditions

Ryegrass seeds were soaked with 2% v/v sodium hypochlorite for 10 minutes, washed repeatedly with distilled water and then germinated in plates on moist filter paper in a growth chamber at 21°C. After 10 days, seedlings were transferred into 9-L plastic pots filled with a continuously aerated basal nutrient solution as described by Taylor and Foy (1985). Plants were grown in a greenhouse under controlled conditions (16 h/8 h (l/d) photoperiod at 20 °C and 70-80% relative humidity). During the growth period, the pH of the solution was adjusted daily to 4.5 with dilute HCl and/or NaOH.

3.2.2 Kinetics of Si uptake

Two Si uptake hydroponic assays were performed by using ryegrass cultivars (Jumbo and Nui). In both experiments, Si treatments were initiated by adding sodium silicate (Na_2SiO_3) as Si source 10 days after plants grown in nutrient solution.

To examine the concentration-dependent kinetics of Si uptake, plants were cultivated with five Si doses (0, 0.5, 1.0, 2.0 and 4.0 mM Si). Plant samples were harvested 24 hours and 21 days after the initiation of treatments.

To evaluate the time-dependent kinetics of Si uptake, plants were grown under 0, 0.5 or 2 mM Si doses during 0, 3, 6, 9, 12 and 24 hours.

The experimental design of both assays considered three replicates per treatment in a completely randomized design. At the end of the experiments, shoots and roots harvested and subsamples of fresh material were dried at 65°C for 48 h to determine dry weight (DW) and the mineral concentration of Si. In addition, plant samples were collected from the time-dependent kinetics assay for molecular analyses as detailed below.

3.2.3 Plant Si concentration

Analysis of Si was performed as described by Pavlovic et al. (2013) with slight modifications. Briefly, dried plant samples (0.1 g) were digested with 5 mL concentrated nitric acid (HNO_3) on a hot plate at 70°C for ~5 h. Samples were diluted with 10mL distilled water, transferred into 25-mL plastic flasks to which 1mL hydrofluoric acid (HF, 40%) was added, and left overnight. At the next day, 5 mL 2% (w/v) boric acid (H_3BO_3) was added to eliminate HF excess, and the flask volume was adjusted to 25 mL with distilled water. Concentration of Si was determined by flame atomic absorption spectrophotometry (FAAS) at 251.6 nm. Two reference samples were included in each analytical run.

3.2.4 Isolation and characterization of Si transporters genes from ryegrass

Total RNA was extracted from roots using the NucleoSpin® RNA Plant Kit (Macherey-Nagel GmbH and Co. KG, Düren, Germany). First strand cDNA was synthesized from 1 µg of total RNA using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, California, USA) following the manufacturer's recommendations. Multiple sequence alignment between the *Lsi1* gene (*OsLsi1*, accession number

AK069842) and the other members of NIPIII group allowed to detect conserved regions which were used to design primers potentially able to anneal with a putative ryegrass homologue. Similarly, primers for *Lsi2* gene were obtained by analyzing a sequence alignment between (*OsLsi2*, accession number AB222273.1) and *Lsi2* from other plants species. Primers for each gene were designed using Primer3 version 0.4.0 program and primer BLAST tool (Table 1).

Each PCR reaction was performed using cDNA of ryegrass roots as follows: 2 min at 95°C of initial denaturation, 30 cycles of 30 s at 95°C of denaturation, 30 s at 53-60 °C of annealing and 1 min at 72 °C of extension, followed by 5 min at 72 °C for a final extension. The purification and sequencing of PCR products were carried out through Macrogen Inc. sequencing Service (Korean Biotechnology Company). A 556 bp and 579 bp amplicons corresponding to the internal position of putative ryegrass Si transporters genes *Lsi1* and *Lsi2*, respectively, were obtained.

In order to obtain the complete reading frame (ORF) of *LpLsi1* and *LpLsi2* genes, both 3'- and 5'- rapid amplification of cDNA ends (RACE) was conducted using the SMARTer RACE 5'/3' Kit (Clontech® Laboratories, Inc). RACE-PCR reactions were performed using 3'- and 5'-RACE-Ready cDNA according to the following thermal cycling: 30 cycles of 30 s at 95°C of denaturation, 30 s at 68°C of annealing and 3 min at 72ºC of extension. Several control reactions were included in the RACE-PCR reactions such as: i) a negative control using the universal primer mix (UPM) alone to amplify the cDNA, ii) a negative control using each GSP by itself, iii) a positive control using the 5'- and 3'-RACE-Ready cDNA made from mouse heart total RNA, and iv) a negative control of PCR reaction. Amplified fragments were purified and cloned into the linearized pRACE vector with In-Fusion HD Cloning kit (Clontech Laboratories, Inc.). The ligated plasmids were transformed into Stellar Competent Cells (Clontech

Laboratories, Inc.), and the transformants were selected on Luria-Bertrani (LB) agar plates containing 100 µg mL^{-1} of ampicillin. Independent clones were designated, and plasmid DNA was isolated using UltraClean Standard Mini Plasmid Prep Kit (MoBio Laboratories, Inc.). All sequences were confirmed by sequencing.

Table 1. List of primers used for molecular cloning and characterization of putative *LpLsi1* and *LpLsi2.*

Name	Sequence 5'→ 3'
Homolog screening	
1-Fw	AACAAACTCCAGGGCGAACTAC
1-Rv	AAATTGCCCCTGCGAAGATGGA
2-Fw	AACCTGGTCATCGCCTTCAATAGC
2-Rv	GTACGTTTGATGCGAGGTTGGAGA
5'/3' RACE procedure	
GSP-A5'RACE	GATTACGCCAAGCTTGGAGCACTGCCTTGAGCACGAAGGAC
GSP-A3'RACE	GATTACGCCAAGCTTTCGCCGTCTTCCGGCATTTCCCA
GSP-C5'RACE	GATTACGCCAAGCTTAGACGAGCAGCGAGTACGACACCTTCAC
GSP-C3'RACE	GATTACGCCAAGCTTGCGTCCCTCAAGAGCCCGCTCAA
PCR full-length cDNA	
Lsi1v-Fw	CTAATACGACTCACTGTGTGCAAGTTCGTGGTCGGAAATG
Lsi1v-Rv	GATTACGCCAAGCTTACAGGCCAAGAGAGCGAGAGCAATC
Ls2v-Fw	CTAATACGACTCACTTGGCCTTCGCGGTGTTCTGGATG
Lsi2v-Rv	GATTACGCCAAGCTTATGCCTACGGCGGTGACGATGA
Expression analyses	
LpLsi1-Fw	ACGCCCAGCATGTACTACAAC
LpLsi1-Rv	TCATGAACACCAGCAGGAAC
LpLsi2-Fw	CTCTGCATGTACTGGAAGGAC
LpLsi2-Rv	GTTGAGAGGGTTGAGAGTGTG
LpeEF1A-Fw	GGCTGATTGTGCTGTGCTTA
LpeEF1A-Rv	CTCACTCCAAGGGTGAAAGC
LpActin-Fw	CCTTTTCCAGCCATCTTTCA
LpActin-Fw	GAGGTCCTTCCTGATGTCCA

3.2.5 Bioinformatics analysis and phylogenetic tree construction

DNA sequences were assembled into a single consensus sequence using Geneious v.3.6.1 software tool and analyzed with NCBI-BLAST (the National Center for Biotechnology Information, USA, www.ncbi.nlm.nih.gov). Phylogenetic trees for Lsi1 were constructed using MEGA v.6 software tool (Tamura et al. 2013). Protein and nucleotide sequences were aligned by ClustalW and subjected to construct phylogenetic tree using maximum likelihood method with 1000 bootstrap iterations. Functional annotation of Lsi1 was performed with Conserved Domain Database (CDD, https://www.ncbi.nlm.nih.gov/cdd/). The transmembrane domain profile was obtained using TMHMM Server v. 2.0.

3.2.6 Gene expression analyses

The expression pattern of both putative Si transporters, *LpLsi1* and *LpLsi2*, in ryegrass tissues was examined by semi-quantitative RT-PCR. PCR reactions were carried out as described above considering 60°C of annealing. *LpeEF1A* was used as reference gene. The resulting PCR products were checked by electrophoresis on 1.2% agarose/EtBr gels. In addition, quantitative real-time reverse transcription polymerase chain (qRT-PCR) reactions were conducted to assess the expression pattern of *LpLsi1* and *LpLsi2* genes in roots samples of ryegrass cv. Nui supplied with 0.5 and 2 mM Si during 3, 6, 9, 12 and 24 hours. All qRT-PCR reactions were performed using Brilliant II SYBR Green qPCR Master mix (Stratagene, Cedar Creek, TX, USA) in an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, California, USA). Cycling conditions were 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s. The specific primers used in this analysis are shown in Table 1. The primer sets used for *LpLsi1* and *LpLsi2* were designed using the Primer3 (v. 0.4.0). Housekeeping

genes, *LpActin* or *LpeEF1A (m)*, were used as internal controls. All the experiments were performed using three biological replicates, each with three analytical replicates. Threshold values (Ct) were employed to quantify relative gene expression using the comparative method described by Pfaffl (2001).

3.2.7 Data analysis

Experimental data were analyzed using an analysis of variance (ANOVA) following normality and homoscedasticity tests. Differences among means were separated using the Tukey test at the 0.05 probability level. In addition, the relationship between two response variables was investigated by Pearson correlation.

3.3 Results

3.3.1 Silicon uptake in two ryegrass cultivars

The concentration-dependent kinetics showed that in both ryegrass cultivars Si concentration augmented in shoots and roots at increasing Si doses after 24 h and 21 d (Fig. 1A-D). At both 24 h and 21 d after Si supply, similar root Si concentration was observed between ryegrass cultivars (Fig. 1 A-C). However, shoot Si concentration in Jumbo was 1.4 and 1.6- fold higher than Nui at 24 h of exposition to 2 mM and 4 mM Si, respectively (Fig. 1 B). Both cultivars preferentially accumulated Si in the shoots irrespective of the added Si dose. Accordingly, over 70% and 90% of the total Si taken up by roots was accumulated in shoots after the exposure to all Si doses tested for 24 h and 21 d, respectively.

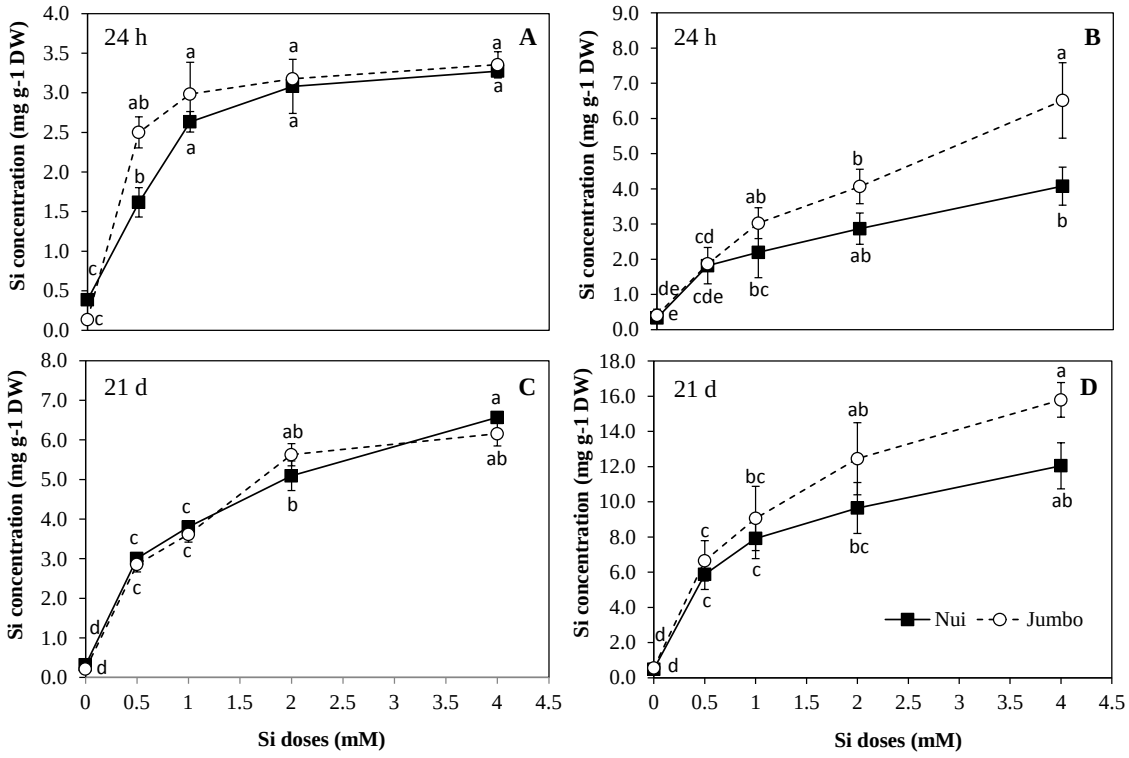

Figure 1. Silicon concentration in roots (A-C) and shoots (B-D) of ryegrass cv. Jumbo and cv. Nui grown in a nutrient solution supplemented with different Si doses during 24 h and 21 d. Data are means of three replicates ± standard error.

Figure 2 shows that Si uptake was saturated at about 2 mM Si in both ryegrass cultivars. Values of K_m and V_{max} were estimated by Lineweaver-Burk linearization (1/v against 1/s, where v is net uptake and s is substrate concentration). V_{max} value was estimated to be 0.14 and 0.12 mg g^{-1} DW h^{-1} for Jumbo and Nui, respectively. The K_m values were 0.17 mM for Jumbo and 0.06 mM for Nui.

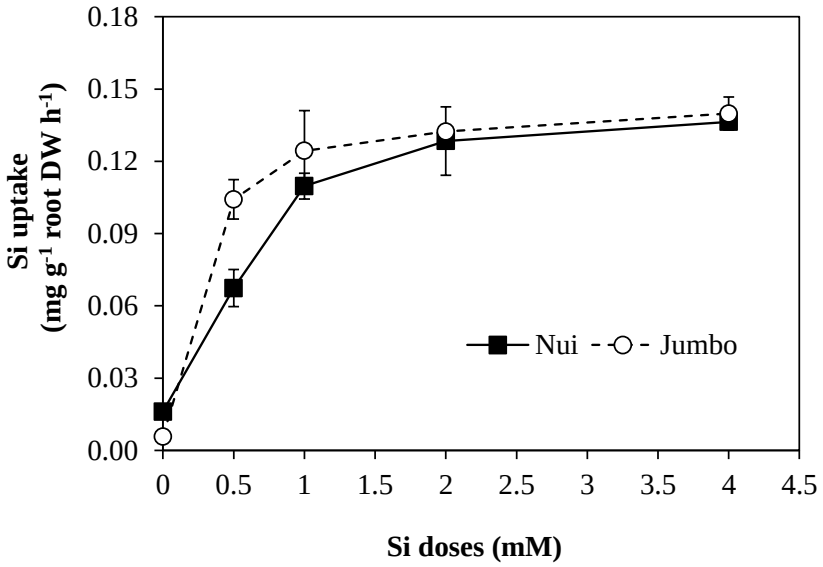

Figure 2. Silicon uptake by roots of ryegrass cv. Jumbo and cv. Nui grown in a nutrient solution supplemented with different Si doses during 24 h. Data are means of three replicates ± standard error.

The time-dependent kinetics assay showed that Si concentration in the roots and shoots of both ryegrass cultivars increased with the time (Fig. 3 A-D). During the time-course of the experiment, root Si concentration increased from 0.98 ± 0.34 mg g^{-1} DW to 2.69 ± 0.43 mg g^{-1} DW in plants of cv. Jumbo supplied with 0.5 mM Si, whereas Si concentration in roots of cv. Nui ranged from 0.11 ± 0.03 mg g^{-1} DW to 1.30 ± 0.04 mg g^{-1} DW (Fig. 3 A). At 2 mM Si added, root Si concentration augmented from 1.59 ± 0.19 mg g^{-1} DW to 3.33 ± 0.50 mg g^{-1} DW (cv. Jumbo) and from 0.37 ± 0.03 mg g^{-1} DW to 2.90 ± 0.04 mg g^{-1} DW (cv. Nui) as shown in Figure 3 C. Apparent differences in Si uptake were observed between Jumbo and Nui exposed to either 0.5 mM or 2 mM Si (Fig. 3 A-C). Consequently, Si concentration in roots was up to 9.0 and 4.3-fold greater in Jumbo than Nui when plants were supplied with 0.5 and 2 mM Si, respectively (Fig. 3 A-C). Nevertheless, both ryegrass cultivars did not show differences

in shoot Si concentration at 0.5 mM Si, and only slight variations were observed when 2 mM Si was added (Figure 3 B-D).

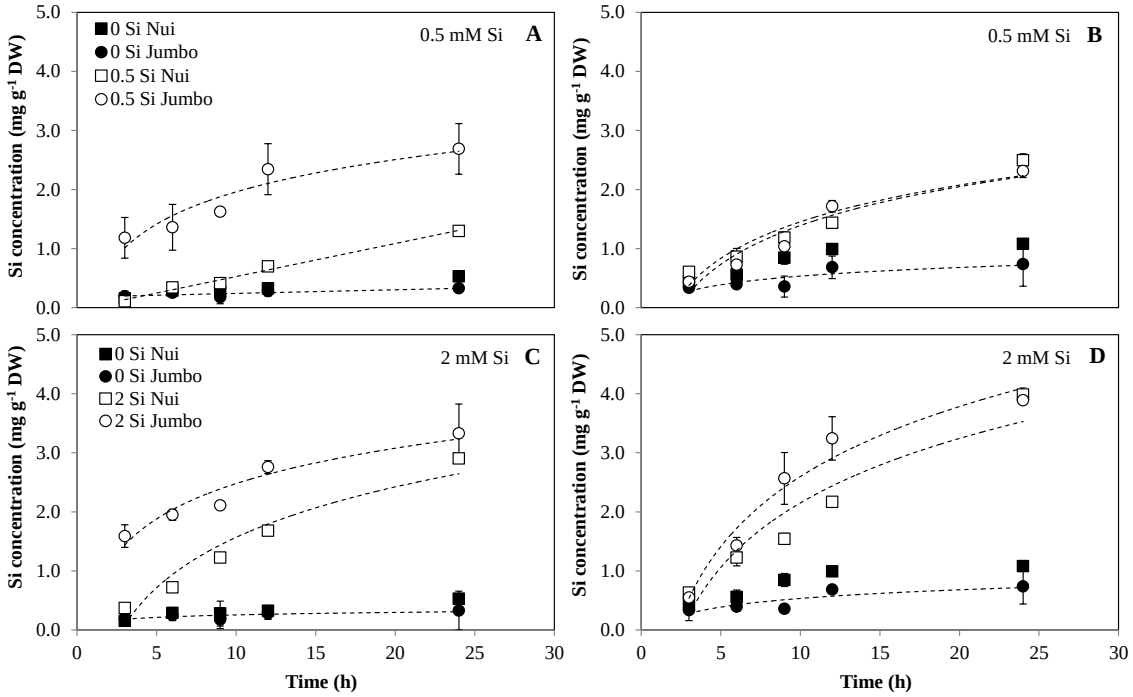

Figure 3. Silicon concentration in roots (A-C) and shoots (B-D) of ryegrass cv. Jumbo and cv. Nui grown in nutrient solutions supplemented with or without 0.5 or 2 mM Si at different times (h). Data are means of three replicates ± standard error.

3.3.2 Isolation of two putative Si transporters from ryegrass

On the basis of the known sequences of Si transporters from rice and other plant species in GenBank, two putative sequences codifying for Lsi1 and Lsi2 were identified from ryegrass. A full-length cDNA encoding to a putative ryegrass influx Si transporter was isolated and named as *LpLsi1* (Accession number KY315994). As shown in Figure 4, the full length cDNA of *LpLsi1* was 1254 bp in size with an 888 bp open reading frame (ORF). The sequence was predicted to encode to a protein of 295 amino acids with a

molecular weight of the deduced protein of 31.7835 kDa, using the software available at http://web.expasy.org/protparam/. BLAST analysis showed that the amino acid sequence of LpLsi1 shared 88% identity with a homologous sequence from *Hordeum vulgare* (HvLsi1; accession number: BAH24163.1) and *Triticum urartu* (TuLsi1; accession number: ADM47602.1). Likewise, close similarity was found between LpLsi1 and either Aquaporin NIP2-1 from *Zea mays* (accession number: NP_001105637.1) or Aquaporin NIP2-1 from *Oryza sativa* (accession number: XP_015626173.1).

In addition, phylogenetic analysis clustered LpLsi1 along with influx Si transporters from other monocot species including barley, wheat, maize and rice (Figure 5). Similarly, protein sequence alignment of LpLsi1 with known influx Si transporters reported in other plant species showed conserved aromatic/arginine (Ar/R) selectivity filter, two Asn-Pro- Ala (NPA) motifs and the spacing between NPA domains (Figure 6). A single efflux (Lsi2) Si-transporter was also obtained from ryegrass. However, it was only partially recovered (accession number KY315995). This partial coding sequence comprised 951 bp. Despite it was not possible to deduce the amino acid sequence of this clone, BLASTx search indicated that it shared 90% and 88% of identity with Lsi2 efflux transporters of *Hordeum vulgare* (HvLsi2; accession number BAH84976.1.1) and *Brachypodium distachyon* (BdLsi2; accession number XP_003559015.1) respectively. In addition, *LpLsi2* displayed 81% and 82% of identity with Lsi2 from *Oryza sativa* (OsLsi2; accession number: CCH63884.1) and *Zea mays* (ZmLsi2; accession number: NP_001183945.1)

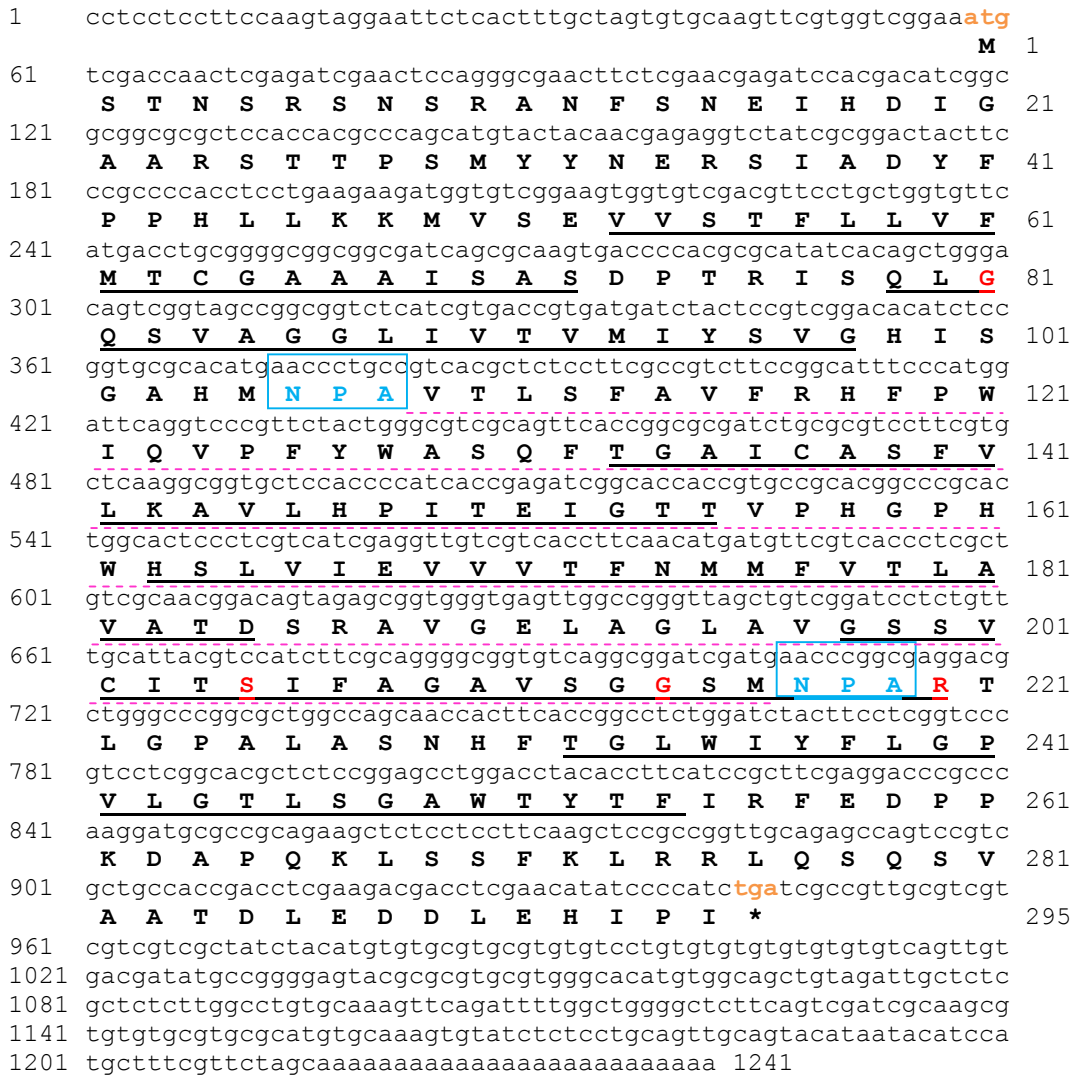

Figure 4. Full-length cDNA of *LpLsi1* and its predicted amino acid sequence. Full-length cDNA of *LpLsi1* is characterized by a total length of 1254 bp, including a 57 bp untranslated 5' region, an open reading frame (ORF) of 888 bp, a 296 bp untranslated 3' region with the TGA stop codon and poly (A) tail of 26 bp. The ORF of *LpLsi1* encodes to a deduced amino acid sequence of 295 residues. Conserved NPA domains and the Ar/R selectivity filter were indicated in blue and red letters, respectively. Predicted transmembrane domains (TM) and the conserved 108 amino acid distance between NPA domains were underlined in black and pink, respectively. The stop codon was marked by an asterisk.

3.3.3 Gene expression analyses of ryegrass Si transporters

A semi-quantitative expression analysis showed that both *LpLsi1* and *LpLsi2* were expressed specifically in roots tissues (Figure 7). Additionally, the expression of these genes was examined by qPCR in response to Si supply during the time-course (Figure 8). As result, continuous Si supply up to 24 h decreased the relative expression of both *LpLsi1* and *LpLsi2* genes. The expression level of *LpLsi1* and *LpLsi2* was down-regulated up to 4.4- and 2.6-fold, respectively, in response to 0.5 mM Si added. A similar expression pattern was observed when plants were supplied with 2 mM Si, thus reducing the transcript level of *LpLsi1* and *LpLsi2* by about 6.1- and 1.7-fold, respectively. A negative correlation between shoot Si concentration and the expression levels of either Lsi1 (r = -0.817, $p \leq 0.01$) or Lsi2 (r = -0.654, $p \leq 0.01$) was also found.

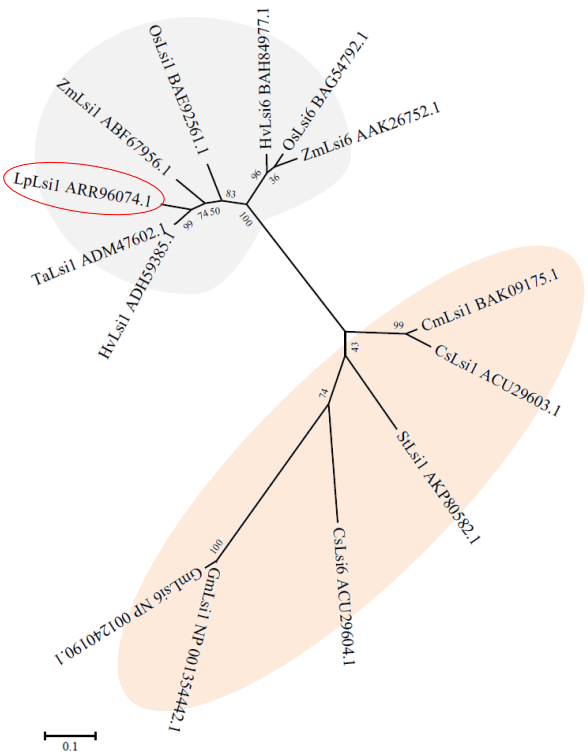

Figure 5. Phylogenetic relationship of LpLsi1 and influx Si transporters reported in other plant species. Amino acid sequences from ryegrass, barley, wheat, maize, rice,

pumpkin, cucumber, potato and soybean were indicated with the prefixes Lp, Hv, Ta, Zm, Os, Cm, Cs, St, and Gm, respectively. The bar represents 0.1 estimated amino acid changes per sequence position.

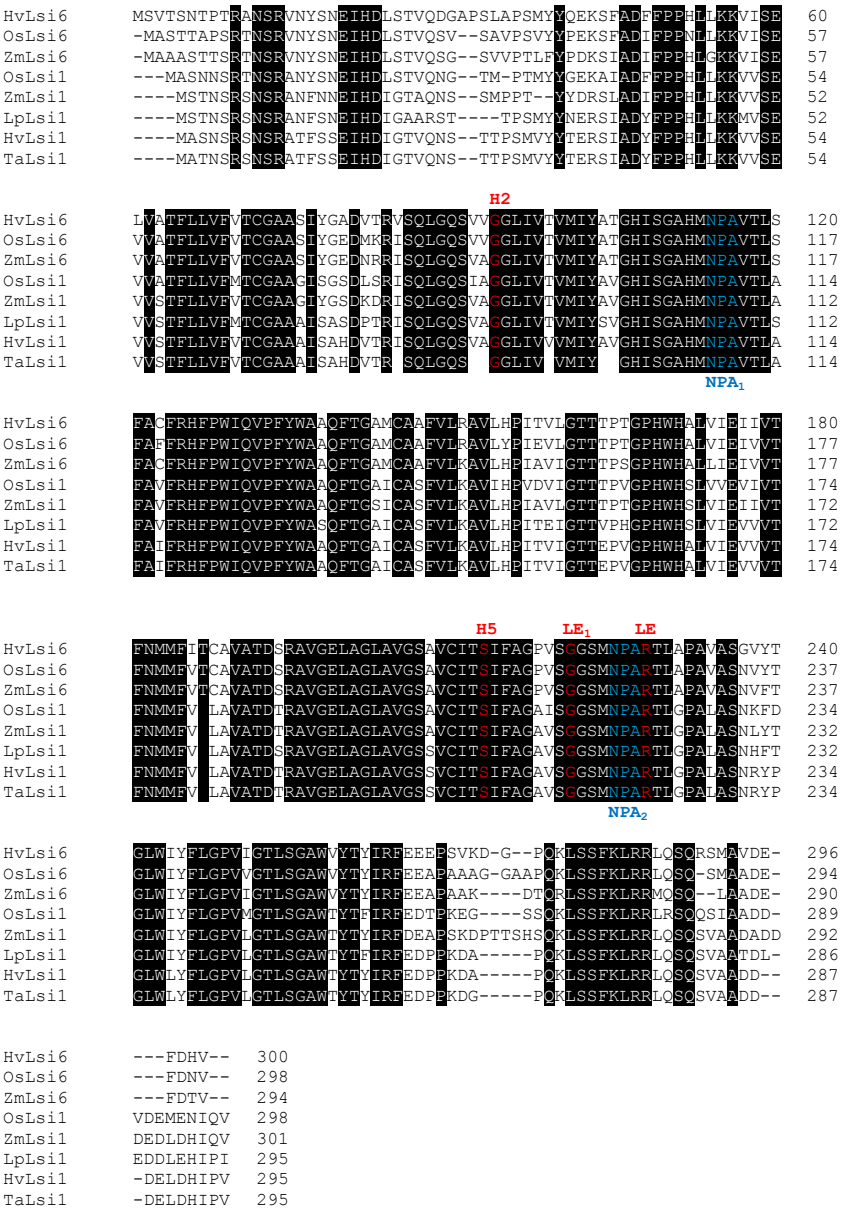

Figure 6. Sequence alignment of LpLsi1 and influx Si transporters reported in other plant species. Identical amino acid residues were shaded in black. Conserved NPA domains and the Ar/R selectivity filter formed from helix 2 (H_2), helix 5 (H_5), and loop E ($LE_1$and LE_2) domains were indicated in blue and red letters, respectively.

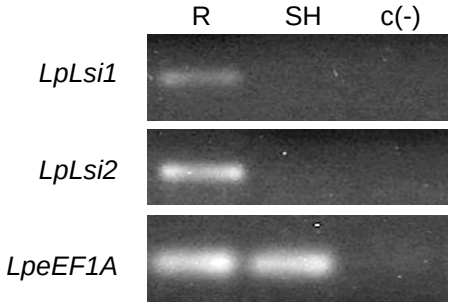

Figure 7. Gene expression patterns of *LpLsi1* an *LpLsi2* in roots (R) and shoots (SH) tissues of ryegrass determined by RT-PCR. *LpeEF1A* was used as reference gene.

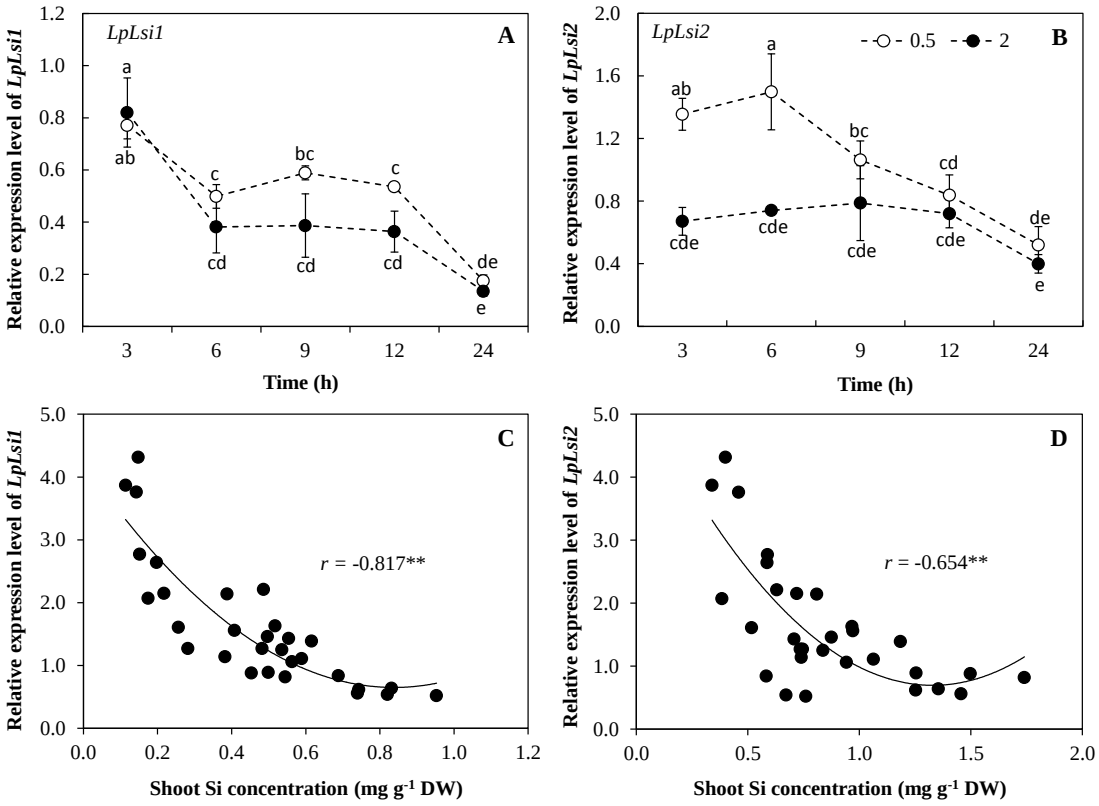

Figure 8. Expression pattern of *LpLsi1* (A) and *LpLsi2* (B) genes in roots of ryegrass cv. Nui continuously supplied with either 0.5 mM (open circles) or 2 mM (filled circles) Si for up to 24 h and its relationship with shoot Si accumulation (C-D). The expression levels were normalized in relation to *Actin* or *eEF1A(m)* gene expression. Data are means of three replicates ± standard error.

3.4 Discussion

Since the beneficial effects provided by Si are closely related with Si accumulation in plants, the elucidation of the possible mechanisms of Si uptake and transport in plants is crucial for exploiting the Si-induced beneficial effects on agricultural systems. Early kinetics studies of Si uptake in rice (Tamai and Ma 2003; Ma et al. 2004) provided the first evidence of the presence of Si transporters in plants. Hence, it was suggested that Si uptake in rice was mediated by a kind of protein transporter showing low affinity by silicic acid (Tamai and Ma 2003). In this study, we assessed the Si uptake system by means of kinetic studies in two ryegrass cultivars (Figs. 1 and 2). The concentration-dependent kinetics showed that in both ryegrass cultivars the Si concentration in roots was steadily increased as Si dose rose (Fig. 1 and 2). However, Si uptake was saturated at about 2 mM Si added (Fig. 2). This finding agrees with the saturation of Si uptake previously reported by Tamai and Ma (2003) and Mitani and Ma (2005) for rice. Based on classical enzyme kinetics (Epstein 1976), V_{max} and K_m values were estimated. The estimated K_m values were 0.17 mM for cv. Jumbo and 0.06 mM for cv. Nui. Ma et al. (2004) and Mitani and Ma (2005) reported similar K_m values for rice (0.16 mM), cucumber (0.15 mM) and tomato (0.16 mM). Although both ryegrass cultivars showed similar V_{max} , different values of K_m suggest that Jumbo and Nui may have different affinities for silicic acid. In contrast, Ma et al. (2007a) reported similar K_m values between cultivars Nipponbare (0.33 mM) and Kasalath (0.34 mM) indicating the involvement of specific transporters in rice roots of genotypes. In addition, dissimilarities in V_{max} values were related with a higher abundance of Si transporters in cv. Nipponbare than cv. Kasalath (Ma et al. 2007a). For ryegrass, differences in K_m values between cultivars might involve either the differential contribution of known

proteins responsible for Si uptake and transport or the involvement of undiscovered Si transporters. Further studies are needed to support this assumption.

On the other hand, significant dissimilarities in shoot Si concentration were observed between cv. Jumbo and cv. Nui exposed to a solution containing 2 mM or 4 mM Si (Fig. 1 B-D). However, these variations in shoot Si concentration were only detected at the short-term. Indeed, the time kinetics experiment showed differences in Si uptake between ryegrass cultivars as early as 3 h after the roots were exposed to the nutrient solution containing Si (Fig. 3 A-C). Consequently, Si concentrations in roots were higher in cv. Jumbo than cv. Nui at either 0.5 mM or 2 mM Si supplied (Fig. 3 A-C). Genotypic variations in Si accumulation have been reported in species such as rice (Deren 2001; Ma et al., 2007a), sugarcane (Deren, 2001) and barley (Ma et al., 2003). In fact, differential gene expression patterns of Si transporters have also been found between genotypes of rice. Nevertheless the mechanisms responsible for such responses remains to be elucidated (Ma et al., 2007a).

The recent discovery of specific Si transporters has improved the understanding of the molecular mechanisms controlling Si uptake in vascular plants. Two different types of transporters, including Si permeable channel and efflux transporter, have been implicated in the Si uptake process (Ma and Yamaji 2015). To further analyze the Si uptake system of ryegrass, two cDNA sequences potentially belonging to influx (Lsi1) and efflux (Lsi2) Si transporters were isolated (Fig. 4 and 5). Close similarity (88%) and phylogenetic relationship were found between LpLsi1 and homologous of Si influx transporters reported in other plant species (Fig. 5). In addition, the predicted amino acid sequence indicated that LpLsi1 is a membrane protein possessing high conservation of Si transport domains determining the functional selectivity for silicic acid (Fig. 6). Lsi1 is a NIP (nodulin-26-like proteins) member in plant aquaporins that

facilitate the passive transport of water and/or small uncharged solutes such as glycerol, ammonia, boric acid and silicic acid (Deshmukh and Belanger 2015; Vatansever et al. 2017). The predicted amino acid sequence of LpLsi1 presented six transmembrane domains, which is also well conserved in typical aquaporins (Fig.4). Silicic acid selectivity in Lsi1 has been associated with two highly conserved Asn-Pro-Ala (NPA) motifs and a Gly-Ser-Gly-Arg (GSGR) ar/R filter. Juxtaposition of asparagines in two NPA motifs forms the NPA region. The Asn residues make hydrogen bond with the transport molecules and may function in the proton exclusion (Forrest and Bhave 2007). Moreover, Deshmukh et al. (2015) showed that a precise distance of 108 amino acids between the NPA domains is also essential for Si permeability in plants. On the other hand, the ar/R region is formed by four residues from the helix 2 (H2), helix 5 (H5), loop E1 (LE1) and loop E2 (LE2) (Wu and Beitz 2007; Liu and Zhu 2010; Mitani et al. 2011c; Deshmukh and Bélanger, 2015). It is referred as ar/R region because of the conserved arginine residue in the loop E and abundance of aromatic residues at H2 (Mitani et al. 2011c). This region functions as a selectivity filter for the substrates that acts a barrier, determines the transport rate, and makes hydrogen and van der Waals bounds (Sui et al. 2001). In ar/R filter for silicic acid transport, only GSGR residues were reported to have specificity for silicic acid (Mitani et al. 2011c). GSGR ar/R filter comprise small amino acids, forming a pore with a sufficiently large diameter to allow the passage of silicic acid (Mitani et al. 2011c).

New insights into the prediction of the activity and relative capacity of Si transport in plants have been proposed. Based on sequence homology and availability of GSGR filter, a phylogenetic analysis performed by Vatansever et al. (2017) showed that Lsi1s formed three clusters as low, moderate and high Si accumulators. Likewise, protein modeling of Lsi1 transporters in 17 plant species showed that Asn residues of two NPA

motifs were available in the predicted binding sites of all species, while ar/R selectivity filter residues such as GSGR or ASGR were found in binding sites of the high and moderate Si accumulators (Vatansever et al. 2017). This outcomes support the fact that two Asn residues in two NPA motifs, and GSGR or ASGR ar/R selectivity filters are of crucial importance for the silicic acid transport in plants.

Thus, our findings indicate that LpLsi1 encode a membrane protein belonging to NIP III subgroup of aquaporins according to the definition proposed by Liu et al. (2009). Moreover, based on the reported by Vatansever et al. (2017), the conserved sequences identified in LpLsi1 confirm the high Si accumulation capacity of ryegrass as previously suggested by Jarvis (1987) and Nanayakkara et al. (2008).

On the other hand, identified Lsi2 genes in vascular plants encoded a membrane protein of 472-547 amino acid residues with conserved 10-11 putative transmembrane domains (Ma et al. 2007; Mitani et al 2009a; Vatansever et al. 2017). Different to Lsi1, Lsi2 transporter homologs in different plant species showed sequences highly conserved even in low Si-accumulators (Vatansever et al. 2017). We also found a partial coding sequence (*LpLsi2*) showing high identity with known Lsi2 efflux transporters *ZmLsi2*, *HvLsi2* and *OsLsi2*. However, an exhaustive analysis of *LpLsi*2 sequence was not possible since the full length cDNA was not satisfactorily achieved.

Consistent with previous reports performed on monocot species (Ma et al, 2006; 2007b; Mitani et al,2009a; 2009b; Chiba et al, 2009; Montpetit et al, 2012), we found that *LpLsi1* and *LpLsi2* were only expressed in roots (Fig. 7). By contrast, the expression of these Si transporters in dicot species have been detected in shoot and root tissues (Mitani et al, 2011a; 2011b; Deshmukh et al, 2013; Wang et al, 2014; Vulavala et al, 2015). The effect of Si supply on the expression of *LpLsi1* and *LpLsi2* was also

investigated. As result, the expression level of both *LpLsi1* and *LpLsi2* was down-regulated in response to continuous Si supply for 24 h (Fig. 8 A-B). These findings were in concordance with our previous report for ryegrass (Pontigo et al. 2017). The regulation of Si transporter expression differs with plant species. For example, the expression of *Lsi1* in rice was down-regulated by Si, but unaffected in barley, maize and wheat (Chiba et al. 2009; Mitani et al. 2009b; Montpetit et al. 2012), whereas Si decreased the expression of *Lsi2* in rice, barley and maize (Ma et al., 2007; Mitani et al., 2009a). A recent study has proposed that the Si-induced down-regulation of Si transporter genes is controlled by Si accumulation in the shoots of rice (Mitani et al, 2016). Similarly, Hosseini et al. (2017) reported that barley Si transporters *HvLsi1* and *HvLsi2* correlated negatively with the accumulation of Si in the shoots of plants grown with sufficient potassium (K) supply, whereas under a K deficiency conditions this relation was positive. In agreement with these researches, we also found a negative correlation between shoot Si concentration and the expression of both *LpLsi1* and *LpLsi2* (Fig. 6 C-D) indicating that the expression of these ryegrass Si transporters was negatively regulated by Si accumulation in the shoots. A recent study has identified a candidate region of a cis-acting element in the upstream region of OsLsi1, which is probably involved in the Si response of OsLsi1 expression in rice roots (Mitani et al. 2016). However, more studies are needed to advance in the understanding of the mechanisms controlling the expression of Si transporters genes in plants.

Overall, our findings confirm the existence of putative influx (LpLsi1) and efflux (LpLsi2) Si transporters in ryegrass with characteristics highly conserved among Si transporters from different plant species. The assessment of Si uptake kinetics and the identification of these Si transporters genes provide new evidence about the Si accumulation ability of ryegrass.

Acknowledgments

This work was supported by the FONDECYT projects 1120901 and 1161326, and CONICYT Doctoral Scholarship 21120704.

CHAPTER IV

Silicon-mediated alleviation of aluminum toxicity by modulation of Al/Si uptake and antioxidant performance in ryegrass plants

PUBLISHED

Pontigo, S., K. Godoy, H. Jiménez, A. Gutiérrez-Moraga, M.L. Mora & P. Cartes. 2017. Silicon-mediated alleviation of aluminum toxicity by modulation of Al/Si uptake and antioxidant performance in ryegrass plants. Frontiers in Plant Science 8: 642. doi:10.3389/fpls.2017.00642-

Silicon-mediated alleviation of aluminum toxicity by modulation of Al/Si uptake and antioxidant performance in ryegrass plants

Sofía Pontigo[1,2], Karina Godoy[2], Héctor Jiménez[1], Ana Gutiérrez-Moraga[2,3], María de la Luz Mora[2,4], Paula Cartes[2,4*]

[1]Programa de Doctorado en Ciencias de Recursos Naturales, Universidad de La Frontera, Temuco, Chile.

[2]Center of Plant-Soil Interaction and Natural Resources Biotechnology, Scientific and Technological Bioresource Nucleus (BIOREN-UFRO), Universidad de La Frontera, Temuco, Chile.

[3]Departamento de Producción Agropecuaria, Facultad de Ciencias Agropecuarias y Forestales Universidad de La Frontera, Temuco, Chile.

[4]Departamento de Ciencias Químicas y Recursos Naturales, Facultad de Ingeniería y Ciencias, Universidad de La Frontera, Temuco, Chile.

* Correspondence: paula.cartes@ufrontera.cl

Abstract

Silicon (Si) has been well documented to alleviate aluminum (Al) toxicity in vascular plants. However, the mechanisms underlying these responses remain poorly understood. Here, we assessed the effect of Si on the modulation of Si/Al uptake and the antioxidant performance of ryegrass plants hydroponically cultivated with Al (0 and 0.2 mM) in combination with Si (0, 0.5, and 2.0 mM). Exposure to Al significantly increased Al

concentration, mainly in the roots, with a consequent reduction in root growth. However, Si applied to the culture media steadily diminished the Al concentration in ryegrass, which was accompanied by an enhancement in root dry matter production. A reduced concentration of Si in plant tissues was also observed when plants were simultaneously supplied with Al and Si. Interestingly, Si transporter genes (*Lsi1* and *Lsi2*) were down-regulated in roots after Si or Al was applied alone; however, both *Lsi1* and *Lsi2* were up-regulated as a consequence of Si application to Al-treated plants, denoting that there is an increase in Si requirement in order to cope with Al stress in ryegrass. Whereas Al addition triggered lipid peroxidation, Si contributed to an attenuation of Al-induced oxidative stress by increasing phenols concentration and modulating the activities of superoxide dismutase (SOD), catalase, peroxidase, and ascorbate peroxidase antioxidant enzymes. Differential changes in gene expression of SOD isoforms (Mn-SOD, Cu/Zn-SOD and Fe-SOD) and the profile of peroxide (H_2O_2) generation were also induced by Si in Al-stressed plants. This, to the best of our knowledge, is the first study to present biochemical and molecular evidence supporting the effect of Si on the alleviation of Al toxicity in ryegrass plants.

Keywords: silicon, aluminum, Si transporter genes, phenols, antioxidant enzymes, SOD isoforms genes.

4.1 Introduction

Aluminum (Al) toxicity represents one of the main yield-limiting factors for crops in acid soils (von Uexkull and Mutert, 1995). Under acidic conditions, large and toxic amounts of Al^{3+} become available to plants, thereby affecting a wide range of physical, cellular, and molecular processes, with a consequent reduction in plant growth (Kochian

et al. 2005; Mora et al. 2006; Ryan and Delhaize, 2010; Cartes et al. 2010; 2012; Singh et al. 2017). Alterations in the structure and/or functions of cell wall components (Horst et al. 2010), plasma membrane properties (Yamamoto et al. 2001), nutrient homeostasis (Delhaize and Ryan, 1995; Gupta et al. 2013; Singh et al. 2017), and signal transduction pathways (Matsumoto, 2000; Ma et al. 2002; Sivaguru et al. 2003; Goodwin and Sutter, 2009) can be induced as a consequence of Al binding to numerous cell sites. In most plant species, reactive oxygen species (ROS) production can also be induced by Al toxicity (Kochian et al. 2005), leading to oxidative damage of biomolecules and biological membranes (Yamamoto et al. 2001; 2002; 2003; Singh et al. 2017).

To cope with the deleterious effects of Al, plant species have developed diverse mechanisms, which are generally associated with Al exclusion (also referred to as avoidance or resistance) and/or internal tolerance mechanisms (e.g., Barcelo and Poschenrieder, 2002; Kochian et al. 2005; Poschenrieder et al. 2008). Briefly, exclusion mechanisms involve the root exudation of organic acid anions and/or phenolic compounds, which bind Al^{3+} and limit its uptake into the cytosol. Tolerance mechanisms comprise internal detoxification by forming Al complexes with organic substances in the cytosol, compartmentalization in the vacuole, and enhanced scavenging of ROS (e.g., Barcelo and Poschenrieder 2002; Kochian et al. 2005; Poschenrieder et al. 2008). Molecular approaches have revealed that Al resistance in several plant species is regulated by genes encoding membrane transporter proteins involved in the efflux of organic acid anions, including members of the ALMT (aluminum-activated malate transporters) and MATE (multidrug and toxic compound extrusion) families (Sasaki et al. 2004; Furukawa et al. 2007; Ryan et al. 2011). In addition, a bacterial-type ATP binding cassette (ABC) transporter (Huang et al. 2009) and antioxidant defense genes (e.g., Milla et al. 2002; Goodwin and Sutter, 2009; Du et

al. 2010; Panda and Matsumoto, 2010) have also been implicated in Al tolerance in plants.

Over the last decades, silicon (Si) has become a focus of increasing interest in plant science, since it is considered as a beneficial element for plant growth, particularly under conditions of biotic and abiotic stress (Ma, 2004; Liang et al. 2007; Guntzer et al. 2012; Ma and Yamaji 2015). To date, several pieces of evidence have indicated that most of the beneficial effects of Si depend on the differential ability of plants to take up Si. Recently, it has been reported that Si accumulation is ascribed to an efficient uptake system mediated by both channel-type and efflux transporters, which perform coordinated functions for effective Si transport from soil to roots and its subsequent distribution within the plants (e.g. Ma et al. 2006; 2007; Mitani et al. 2009a; 2009b; Chiba et al. 2009; Mitani et al. 2011a; 2011b; Yamaji et al. 2008; 2009; 2012; Grégoire et al. 2012; Montpetit et al. 2012; Deshmukh et al. 2013; Ma and Yamaji 2015). Overall, these transporters appear to be keys features that enable plants to gain an advantage from Si uptake. Nevertheless, the regulation of Si transporters under stress conditions remains poorly understood.

The significant role of Si in the toxicity associated with metals, including manganese (Mn), iron (Fe), cadmium (Cd), arsenic (As), chromium (Cr), copper (Cu), lead (Pb), zinc (Zn), and Al, has been widely reported (Li et al. 2012; Vaculík et al. 2012; Adrees et al. 2015; Pontigo et al. 2015; Liang et al. 2015; Tripathi et al. 2015; 2016). On the basis of the current evidence, Si can regulate plant resistance and/or tolerance to metal toxicity by either external (*ex planta*) or internal (*in planta*) mechanisms (Cocker et al. 1998a; Adrees et al. 2015; Pontigo et al. 2015; Liang et al. 2015; Tripathi et al. 2016). In this regard, it has been proposed that the alleviation of Al stress by Si in plants can mainly be explained by the following events: (i) Si-induced increase in solution pH (Li

et al. 1996; Cocker et al.1998a), (ii) formation of Al-Si complexes in the growth media (Barcelo et al. 1993; Baylis et al. 1994; Ma et al. 1997; Cocker et al. 1998a) or/and within the plant (Corrales et al. 1997; Cocker et al. 1998b; Britez et al. 2002; Zsoldos et al. 2003; Wang et al. 2004; Prabagar et al. 2011), (iii) exudation of organic acid anions and phenolic compounds (Barcelo et al. 1993; Cocker et al. 1998b; Kidd et al. 2001), and (iv) increase in the chlorophyll and carotenoid contents of leaves (Singh et al. 2011). Activation of the plant antioxidant system has also been reported in response to Si supply under Al stress (Shahnaz et al. 2011; Shen et al. 2014; Tripathi et al. 2016). However, to our knowledge, there is a dearth of reports regarding the molecular aspects of the effect of Si on the genes involved in antioxidant defense.

Perennial ryegrass (*Lolium perenne* L.) is a temperate pasture species supporting forage-based intensive dairy and beef production systems in many parts of the world. Due to elevated yields and high nutritional value, ryegrass has become one of the most commonly cultivated species in the permanent pastures of Southern Chile. Nevertheless, large areas of these pastures are sown on acidic soils, which exhibit elevated availability of toxic Al^{+3}, thereby limiting their yield and quality (Mora et al. 2006). Furthermore, our previous studies have demonstrated that toxic levels of Al induced oxidative damage and activated antioxidant enzymes in ryegrass roots, including peroxidase (POD), ascorbate peroxidase (APX), and superoxide dismutase (SOD) (Cartes et al. 2010; 2012). In an attempt to identify new alternatives to alleviate the deleterious effects produced by Al on ryegrass, we aimed in this study to investigate the effect of Si on the modulation of Si/Al uptake and the antioxidant performance of ryegrass plants subjected to Al toxicity.

4.2 Material and Methods

4.2.1 Plant material and growth conditions

Seeds of ryegrass (*Lolium perenne* L. cultivar Nui) were soaked with 2% v/v sodium hypochlorite for 10 min, washed repeatedly with distilled water, and then germinated on moist filter paper in a growth chamber at 21°C. After 10 d, seedlings were transferred to 12-L plastic pots containing a continuously aerated basal nutrient solution described by Taylor and Foy (1985). After 10 d in nutrient solution, ryegrass plants were treated with Al and Si. Aluminum (as $AlCl_3$, Merck reagent) was added to the solution at doses of 0 and 0.2 mM. The activity of free Al^{3+} in the nutrient solution, calculated by Geochem-EZ (Shaff et al. 2010), corresponded to 85 µM. Aluminum doses were added in combination with 0, 0.5, and 2 mM Si (as Na_2SiO_3, Merck reagent) in a completely randomized factorial design with three replicates per treatment. During the growth period, the pH of the solution was adjusted daily to 4.5 using dilute HCl or NaOH, and the nutrient solution was changed every 7 days. Plants were cultured in a greenhouse under controlled growth conditions as follows: 25/20°C day/night temperature, a 16/8 h (light/dark) photoperiod, 350 µmol m^{-2} s^{-1} photosynthetic photon flux (PPF) and 70%-80% relative humidity. Plants were harvested 10 days after the initiation of treatments, and shoot and root samples were stored at -20°C or -80°C for subsequent evaluation of biochemical and molecular parameters. In addition, subsamples of fresh material were dried at 65ºC for 48 h in order to determinate the dry weight as well as Si and Al concentrations.

4.2.2 Determination of the mineral concentration of Al and Si in plant tissues

Aluminum analysis was performed on dried roots and shoots. Plant samples were ashed at 500°C for 8 h and treated with 2 M HCl. After filtration of the resulting solution, the

total amount of Al was quantified by flame atomic absorption spectrophotometry (FAAS) at 324.7 nm, as described by Sadzawka et al. (2007). Silicon concentration was assayed as described by Pavlovic et al. (2013) with modifications. Dry plant samples were digested with 5 mL concentrated HNO_3 on a hot plate at 70ºC for approximately 5 h. Samples were diluted with 10 mL of deionized water, followed by the addition of 1 mL HF (40%), and left overnight. The following day, 5 mL 2% (w/v) H_3BO_3 was added to eliminate excess HF and the volume of the solution was adjusted to 25 mL with deionized water. The Si concentration in the digested samples was determined by FAAS at 251.6 nm. For each chemical analysis, two reference samples were included in each analytical run.

4.2.3 Biochemical analyses

4.2.3.1 Lipid peroxidation assay

Lipid peroxidation was analyzed using the thiobarbituric acid reactive substances (TBARS) assay, according to the modified method of Du and Bramlage (1992). The absorbance of the samples was measured at 532, 600, and 440 nm in order to correct for interference generated by TBARS-sugar complexes.

4.2.3.2 Determination of total phenols

Total soluble phenols were spectrophotometrically assayed at 765 nm using Folin-Ciocalteu reagent according to the method described by Slinkard and Singleton (1977) with minor modifications (Ribera et al, 2013). Total phenol concentration was calculated using chlorogenic acid as a phenolic compound standard.

4.2.3.3 Antioxidant enzyme assays

Superoxide dismutase (SOD; EC. 1.15.1.1), catalase (CAT; EC. 1.11.1.6), peroxidase (POD; EC. 1.11.1.7), and ascorbate peroxidase (APX; EC. 1.11.1.11) enzyme activities were evaluated from frozen samples stored at -80ºC. Plant material was ground in liquid nitrogen and macerated in 50 mM potassium phosphate buffer (K_2HPO_4–KH_2PO_4; pH 7.0). The homogenate was centrifuged at 11,000 × g for 15 min at 4°C, and the supernatant was used for assay of enzyme activities. SOD, CAT, APX, and POD activities were calculated on a protein basis. The protein content in the extracts was measured spectrophotometrically using the method described by Bradford (1976), with bovine serum albumin (BSA) used as a standard.

Superoxide dismutase activity was analyzed by measuring inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture contained 400 µL of 0.1 M potassium phosphate buffer pH 7.0, 10 µL of 10 mM ethylenediaminetetraacetic acid (EDTA), 50 µL of 260 mM methionine, 80 µL of 4.2 mM NBT, 170 µL of 130 µM riboflavin, and 300 µL of enzyme extract. The reaction tubes were illuminated for 15 min and the absorbance of samples was measured at 560 nm. Non-illuminated and illuminated reactions without enzyme extract were used as controls. One SOD unit was defined as the amount of enzyme corresponding to 50% inhibition of NBT reduction (Donahue et al. 1997).

Catalase (CAT; EC. 1.11.1.6) activity was measured by monitoring the decomposition of hydrogen peroxide (H_2O_2) at 240 nm for 120 s. A 10-µL aliquot of enzyme extract was added to a reaction mixture containing 1 mL of extraction buffer and 3 µL of H_2O_2 (30% v/v). The enzyme activity was calculated using a molar extinction coefficient of 39.4 mM-1 cm-1 (Pinhero et al. 1997).

Peroxidase (POD; EC. 1.11.1.7) activity was determined by estimating the formation of tetraguaiacol at 470 nm during 1 min. A 15-µL volume of enzyme extract was added to a reaction mixture containing 1 mL of extraction buffer, 5 µL of H_2O_2 (30% v/v), and 5 µL of guaiacol. A molar extinction coefficient of 26.6 mM^{-1} cm^{-1} was used to calculate the enzymatic activity (Pinhero et al. 1997).

Ascorbate peroxidase (APX; EC. 1.11.1.11) activity was assayed according to the method described by Nakano and Asada (1981), by measuring ascorbate decomposition at 290 nm for 1 min. The coarse extract (40 µL) was diluted in a reaction mixture containing 1 mL of extraction buffer, 5 µL of H_2O_2 (30% v/v), and 40 µL of 10 mM ascorbic acid. Enzyme activity was calculated using a molar extinction coefficient of 2.8 mM^{-1} cm^{-1}.

4.2.4 Gene expression analyses

Ryegrass tissues were subjected to RNA extraction using a NucleoSpin® RNA Plant Kit (Macherey-Nagel GmbH and Co., KG, Düren, Germany). First-strand cDNA was synthesized from 1 µg of total RNA using an AffinityScript qPCR cDNA Synthesis Kit (Stratagene, Cedar Creek, TX, USA) following the manufacturer's recommendations. Quantitative real-time polymerase chain (qRT-PCR) reactions were conducted in order to determinate the expression patterns of Si transporter genes (*Lsi1* and *Lsi2*) in roots, as well as those of three SOD isoform genes (*Cu/ZnSOD*, *Fe-SOD*, and *Mn-SOD*) in shoots and roots. All qRT-PCR reactions were performed using Brilliant II SYBR Green qPCR Master mix (Stratagene, Cedar Creek, TX, USA) in an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, California, USA). Cycling conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 30 s, 60°C for 1 min, and 72°C for 30 s. The specific primers used in this study are shown in Table 1.

The primer sets used for *LpLsi1* (GenBank accession number KY315994) and *LpLsi2* (GenBank accession number KY315995) were designed using the Primer3 (v. 0.4.0) and primer BLAST tools. Primers sequences for *LpCu/ZnSOD*, *LpFe-SOD*, and *LpMn-SOD* were obtained from Ribera et al. (2013). Housekeeping genes, *LpActin* or *LpeEF1A (m)*, were used as internal controls (Ribera et al. 2013). All the experiments were performed using three biological replicates, each with three technical replicates.

Table 1. List of primers sequences used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis of Si transporters and SOD isoforms genes.

Gene name*	Forward primer (5'-> 3')	Reverse primer (5'-> 3')
Lsi1	ACGCCCAGCATGTACTACAAC	TCATGAACACCAGCAGGAAC
Lsi2	CTCTGCATGTACTGGAAGGAC	GTTGAGAGGGTTGAGAGTGTG
Fe-SOD	GTTGCCAAGGGAAATCCTGAACCA	AACCCCAGCCGTTTATCTTCAAGC
Cu/Zn-SOD	GTGTTGCTCCCATCAATGTTGT	CCTGCCAAGATCATCAGCATC
Mn-SOD	AATACGAAAATGTGGCTGTGTG	AAAATCTGCATTGTGCATTACG
Actin	CCTTTTCCAGCCATCTTTCA	GAGGTCCTTCCTGATGTCCA
eEF1A (m)	GGCTGATTGTGCTGTGCTTA	CTCACTCCAAGGGTGAAAGC

*Gene name: *Lsi1*, Low Si transporter 1; *Lsi2*, Low Si transporter 2; *Fe-SOD*, iron superoxide dismutase; *Cu/Zn-SOD*, copper/zinc superoxide dismutase; *Mn-SOD*, manganese superoxide dismutase; *Actin*, Actin; *eEF1A(m)*, Eukaryotic elongation factor 1 alpha. *Actin* or *eEF1A(m)* were used as housekeeping genes.

4.2.5 Detection of H_2O_2 production by flow cytometry

Suspensions of shoot protoplasts were obtained using the method described by Okuno and Furusawa (1977). The protoplasts were centrifuged at 2,500 × g for 5 min at 4°C and incubated with the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) to detect intracellular H_2O_2 using the method described by Maxwell et al.

(1999) with modifications. H_2O_2 production was analyzed using flow cytometry (BD FACS Canto IISN: V96101286; Becton Dickinson, USA). All measurements were performed using an Ar ion laser excited at 488 nm and emitting at 530 nm. The images were processed through the BD FACSDivaTM, v 6.0 program. A positive control (intact protoplasts plus 100 μM H2O2) and negative control (suspension of intact protoplasts without H_2O_2) were used.

4.2.6 Confocal microscopy

A profile of H_2O_2 generation in protoplast extracts was also examined by Confocal Laser Scanning Microscopy (CLSM). H2DCFDA fluorescence emission was recorded at excitation/emission of 488/530 nm, and chlorophyll autofluorescence was measured at 633 nm laser excitation and emission of 750 nm. The images were processed using Image Processing software (software FV10-ASW v.0.2c; Arquimed).

4.2.7 Statistical analysis

Experimental data were analyzed using an analysis of variance (ANOVA) following normality and homoscedasticity tests. Differences among means were separated using the Tukey test at the 0.05 probability level. In addition, the relationship between two response variables was investigated by Pearson correlation.

4.3 Results

4.3.1 Concentrations of Al and Si in plants and dry matter production

Aluminum treatment mostly increased Al concentration in roots, whereas significantly lower amounts of Al accumulated in the shoots (Table 2). However, increasing Si doses gradually decreased shoot and root Al concentrations by up to 49% and 56%, respectively, in Al-treated plants (Table 2). Interestingly, a negative correlation between

Si concentration and Al concentration was observed in shoots (r = 0.927, $p \leq 0.01$) and roots (r = 0.935, $p \leq 0.01$) of ryegrass grown with Al and Si (Table 3). In addition, the Si concentration of ryegrass tissues steadily increased with an increase in Si dose, but this increment was less noticeable when plants were simultaneously supplied with Al and Si (Table 2). Of the total amount of Si taken up by plants, over 80% accumulated in the shoots.

No changes in shoot growth were observed in plants treated with Al alone, whereas root dry matter production was reduced by approximately 28.5%. Silicon treatments did not affect ryegrass growth when Si was applied to plants cultivated without Al (Table 2). However, root yield was improved by at least 51% when Si was applied to Al-treated plants. Moreover, a positive correlation (r = 0.823, $p \leq 0.01$) between Si concentration and dry weight was observed for the roots of Al-treated plants supplied with increasing concentrations of Si (Table 3).

4.3.2 Analysis of Si transporter gene expression in response to Al toxicity

The relative expression of two putative Si transporter genes (*LpLsi1* and *LpLsi2*) in roots was assessed in ryegrass subjected to different Al and Si supplementation. In plants grown without Al, the expression level of *LpLsi1* and *LpLsi2* was down-regulated by approximately 4.2- and 2.8-fold, respectively, in response to Si addition to the growth media (Figures 1A, B). A similar expression pattern was observed when Al was applied alone, with the expression levels of *LpLsi1* and *LpLsi2* being reduced by approximately 7.1- and 2.9-fold, respectively (Figures 1A, B). However, when Al was added in combination with Si, the expression level of these Si transporters was significantly enhanced (Figures 1A, B). The highest Si dose applied to Al-treated plants increased the expression level of LpLsi1 by approximately 5.4-fold (Figure 1A),

whereas that of *LpLsi2* was up-regulated by at least 2.5-fold irrespective of Si dosage (Figure 1B).

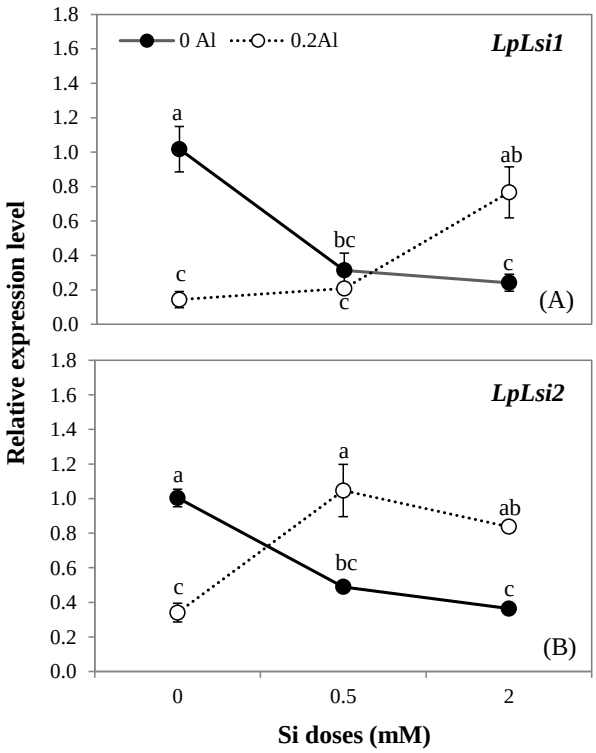

Figure 1. Expression analysis of *LpLsi1* (A) and *LpLsi2* (B) genes determined by qRT-PCR in roots of ryegrass hydroponically cultivated under Al and Si treatments. The expression levels were normalized in relation to *Actin* or *eEF1A(m)* gene expression. Data are means of three replicates ± standard error. Different letters indicate statistically significant differences ($p \leq 0.05$) among treatments.

Table 2. Concentration of Al and Si, and dry matter production of ryegrass plants hydroponically cultivated under different Al and Si treatments.

Values are means ± standard error of three replicates. Different letters indicate statistically significant differences ($p \leq 0.05$) among treatments.

Treatment (mM)	Al concentration (g kg^{-1} DW)		Si concentration (g kg^{-1} DW)		Dry weight (g)	
	Shoots	*Roots*	*Shoots*	*Roots*	*Shoots*	*Roots*
0 Al – 0 Si	0.02 ± 0.00 cd	0.16 ± 0.02 d	0.31 ± 0.09 e	0.33 ± 0.03 e	6.53 ± 0.29 bc	1.37 ± 0.06 ab
0 Al – 0.5 Si	0.01 ± 0.00 d	0.15 ± 0.00 d	5.85 ± 0.44 c	6.42 ± 0.20 c	7.04 ± 0.29 abc	1.37 ± 0.10 ab
0 Al – 2 Si	0.01 ± 0.00 d	0.13 ± 0.01 d	13.78 ± 0.26 a	13.47 ± 0.09 a	6.69 ± 0.22 abc	1.39 ± 0.13 a
0.2 Al – 0 Si	0.07 ± 0.00 a	3.84 ± 0.24 a	0.21 ± 0.03 e	0.38 ± 0.10 e	6.07 ± 0.42 c	0.98 ± 0.06 b
0.2 Al – 0.5 Si	0.04 ± 0.00 b	2.68 ± 0.10 b	4.40 ± 0.13 d	4.30 ± 0.15 d	7.95 ± 0.42 ab	1.48 ± 0.08 a
0.2 Al – 2 Si	0.03 ± 0.00 bc	1.69 ± 0.11 c	10.29 ± 0.19 b	11.88 ± 0.20 b	8.09 ± 0.32 a	1.61 ± 0.06 a

Table 3. Pearson's correlation among plant growth, chemical and biochemical parameters of ryegrass hydroponically cultivated under different Al and Si treatments.

	Al	Si	Dry weight	TBARS	Total phenols	SOD	CAT	POD	APX
					Shoots				
Al	1.00								
Si	-0.927**	1.00							
Dry weight	-0.849**	0.721*	1.00						
TBARS	0.946**	-0.947**	-0.757*	1.00					
Total phenols	-0.904**	0.859**	0.756*	-0.813**	1.00				
SOD	0.693*	-0.827**	-0.432	0.646	-0.721*	1.00			
CAT	-0.099	0.076	-0.118	-0.023	0.418	-0.110	1.00		
POD	0.863**	-0.776*	-0.781*	0.715*	-0.932**	0.666	-0.275	1.00	
APX	0.823**	-0.599	-0.745*	0.657	-0.744*	0.489	-0.073	0.836**	1.00
					Roots				
Al	1.00								
Si	-0.935**	1.00							
Dry weight	-0.876**	0.823**	1.00						
TBARS	0.740*	-0.734*	-0.800**	1.00					
Total phenols	-0.825**	0.741*	0.706*	-0.523	1.00				
SOD	0.883**	-0.961**	-0.778*	0.787*	-0.731*	1.00			
CAT	-0.691*	0.838**	0.524	-0.399	0.738*	-0.795*	1.00		
POD	-0.796*	0.925**	0.666	-0.509	0.690*	-0.858**	0.956**	1.00	
APX	-0.925**	0.980**	0.806**	-0.666	0.800**	-0.930**	0.894**	0.962**	1.00

Asterisks indicate significance as follows: ** $p \leq 0.01$,* $p \leq 0.05$.

4.3.3 Lipid peroxidation

The addition of 0.2 mM Al increased root lipid peroxidation by approximately 29% (Figure 2B); however, no differences in oxidative damage were observed in shoots as a consequence of Al supply (Figure 2A). Likewise, no significant changes in TBARS accumulation were observed among plants grown with only Si (Figures 2A, B). However, Si at the highest concentration supplied diminished lipid peroxidation in Al-treated plants by approximately 32.6% and 27.7% in shoots and roots, respectively (Figures 2A, B). Consequently, lipid peroxidation was negatively correlated with Si concentration in shoots (r = -0.947, $p \leq 0.01$) and roots (r = -0.734, $p \leq 0.05$), as shown in Table 3.

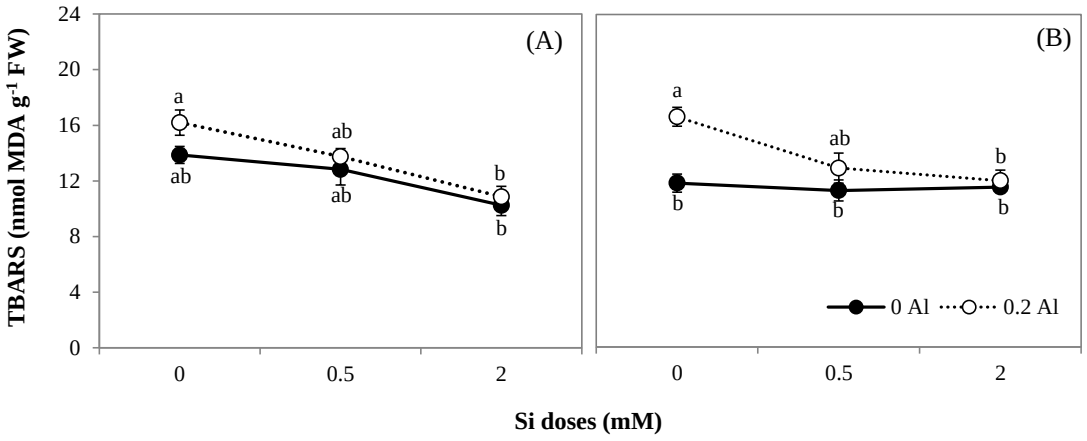

Figure 2. Lipid peroxidation in shoot (A) and root (B) of ryegrass hydroponically cultivated under Al and Si treatments. Data are means of three replicates ± standard error. Different letters indicate statistically significant differences ($p \leq 0.05$) among treatments.

4.3.4 Plant antioxidant responses

Plants treated with Al showed an evident increment in total phenols (Figures 3A, B). A significant increase in total phenol concentration was also observed in the shoots and roots of ryegrass treated with the highest Si dose, with a further increase being observed in plants treated with both Al and Si (Figures 3A, B).

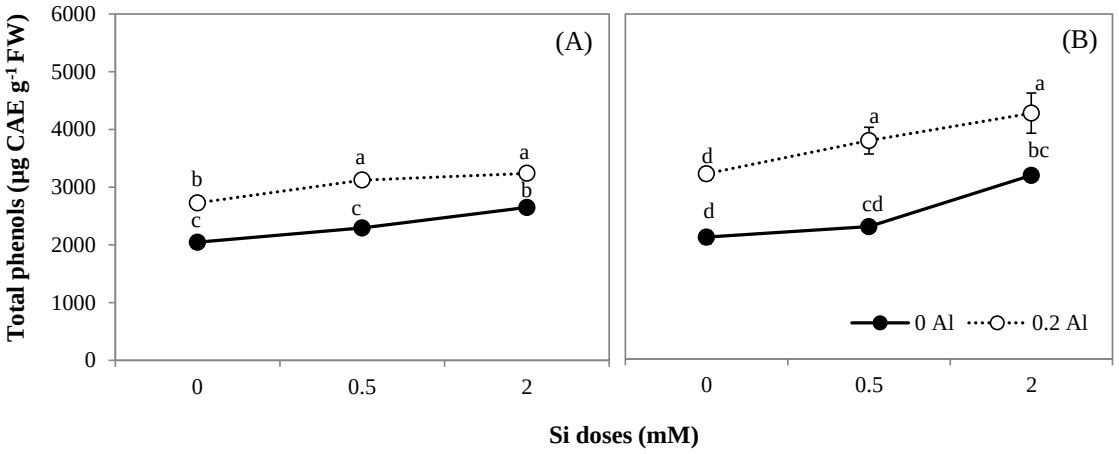

Figure 3. Total phenol concentration in shoot (A) and root (B) of ryegrass hydroponically cultivated under Al and Si treatments. Data are means of three replicates ± standard error. Different letters indicate statistically significant differences ($p \leq 0.05$) among treatments.

In order to investigate the effect of Si on the ROS scavenging enzyme system under Al stress conditions, the activities of SOD, CAT, POD, and APX enzymes were evaluated (Figures 4A-H). Aluminum supplied alone significantly increased SOD activity by approximately 37.2% in shoots and 27.5% in roots (Figure 4A, B). Likewise, the highest Si dose activated SOD enzyme in non-Al-treated plants (Figures 4A, B). However, when Al and Si were simultaneously applied, SOD activity was significantly reduced by 20.08% and 43.8% in shoots and roots, respectively (Figures 4A, B).

The application of Al alone increased CAT activity in shoots and roots by at least 4.2- and 4.7-fold, respectively (Figures 4C, D). In plants grown in the absence of Al, Si enhanced CAT activity by approximately 3.0-fold (shoots) and 5.8-fold (roots) (Figures 4C, D). Plants supplied with Al + Si did not show significant differences in CAT activity compared with those supplied with Al alone, the exception being in the roots of plants supplied with the highest Si dose, which exhibited an approximate 60% increase (Figures 4C, D).

Shoot POD activity increased by approximately 30% in Al- treated plants compared with non-treated plants, although no significant changes were observed in roots (Figures 4E, F). The addition of Si augmented POD activity in plants grown without Al (Figures 4E, F). This effect was most evident in roots, in which the activity of this enzyme was increased by 2.1-fold at the highest Si supply (Figure 4F). Likewise, root POD was activated by approximately 1.7-fold under combined Al and Si treatments (Figure 4F), whereas in shoots the enzyme activity was diminished (Figure 4E).

Addition of Al to the growth media considerably increased APX activity by approximately 2.7-fold and 1.8-fold in shoots and roots, respectively (Figures 4G, H). Similarly, Si application elevated APX activity in ryegrass (Figures 4G, H), and this effect was enhanced by 2.2-fold in the roots of plants receiving the combined Al-Si treatments (Figure 4H). Conversely, Si supply decreased shoot APX activity by approximately 25.9% in Al-treated plants (Figure 4G).

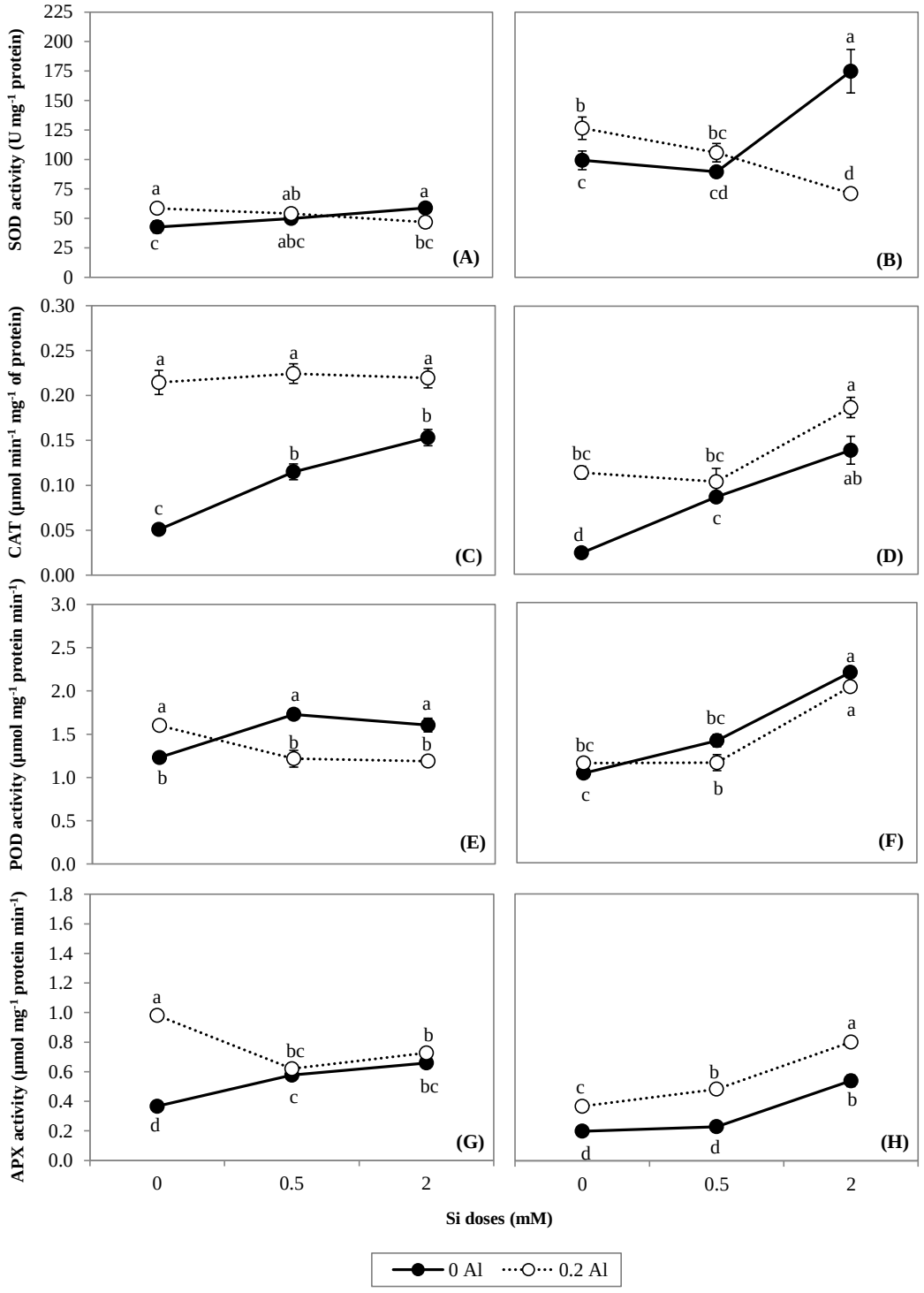

Figure 4. The activity of antioxidant enzyme SOD (A, B), CAT (C, D), POD (E, F) and APX (G, H) in shoots and roots of ryegrass hydroponically cultivated under Al and Si treatments. Data are means of three replicates ± standard error. Different letters indicate statistically significant differences ($p \leq 0.05$) among treatments.

The changes in antioxidant responses of Al-stressed plants as a consequence of Si uptake were additionally examined by means of Pearson correlation as shown in the Table 3. Briefly, we found a negative correlation between Si concentration and SOD activity in shoots (r = -0.827, $p \leq 0.01$) and roots (r = -0.961, $p \leq 0.01$). Conversely, for roots, we observed positive relationships between Si concentration and either total phenols (r = 0.741, $p \leq 0.05$) or the antioxidant enzymes of the second line of defense (CAT, r = 0.838, $p \leq 0.01$; POD, r = 0.925, $p \leq 0.01$; APX, r = 0.980, $p \leq 0.01$).

4.3.5 Analysis of SOD isoform gene expression in response to Al and Si treatments

Genes of SOD isoforms (*Fe-SOD*, *Cu/Zn-SOD*, and *Mn-SOD*) were differentially expressed as a consequence of Si and Al supply (Figures 5A-F). Aluminium supplied alone reduced the gene expression of *Fe-SOD* and *Cu/Zn-SOD* in shoots (Figures 5A, C), whereas no changes in the expression pattern of these genes was detected in the roots (Figures 5B, D). In addition, expression of the *Mn-SOD* gene was up-regulated by approximately 1.7-fold in shoots and roots exposed to Al (Figures 5E, F). Increasing Si doses lowered the gene expression of *Fe-SOD* by up to 1.9-fold in the shoots and 2.2-fold in the roots of plants cultivated without Al (Figures 5A, B), whereas the transcript levels of *Mn-SOD* were enhanced in shoots by approximately 1.7-fold by Si addition (Figure 5E). In contrast, in plants receiving Si alone, there was no significant changes in the expression level of either shoot *Cu/Zn-SOD* or root *Mn-SOD* genes (Figures 5C, F). However, in roots, Cu/Zn-SOD was down-regulated by at least 1.8-fold as a consequence of Si supply (Figure 5D). In plants simultaneously exposed to Al and Si, the addition of Si did not induce significant changes in the expression level of *Fe-SOD* in shoots and roots (Figures 5A, B). Although a similar expression pattern of *Cu/Zn-SOD* was observed in the shoots of Al-treated plants under the different Si treatments

(Figure 5C), the gene expression of this enzyme was down-regulated by up to 1.9-fold in roots (Figure 5D). Likewise, Si application to Al-treated plants significantly reduced the transcript level of *Mn-SOD* by at least 2.2- and 3.8-fold in shoots and roots, respectively (Figures 5E, F).

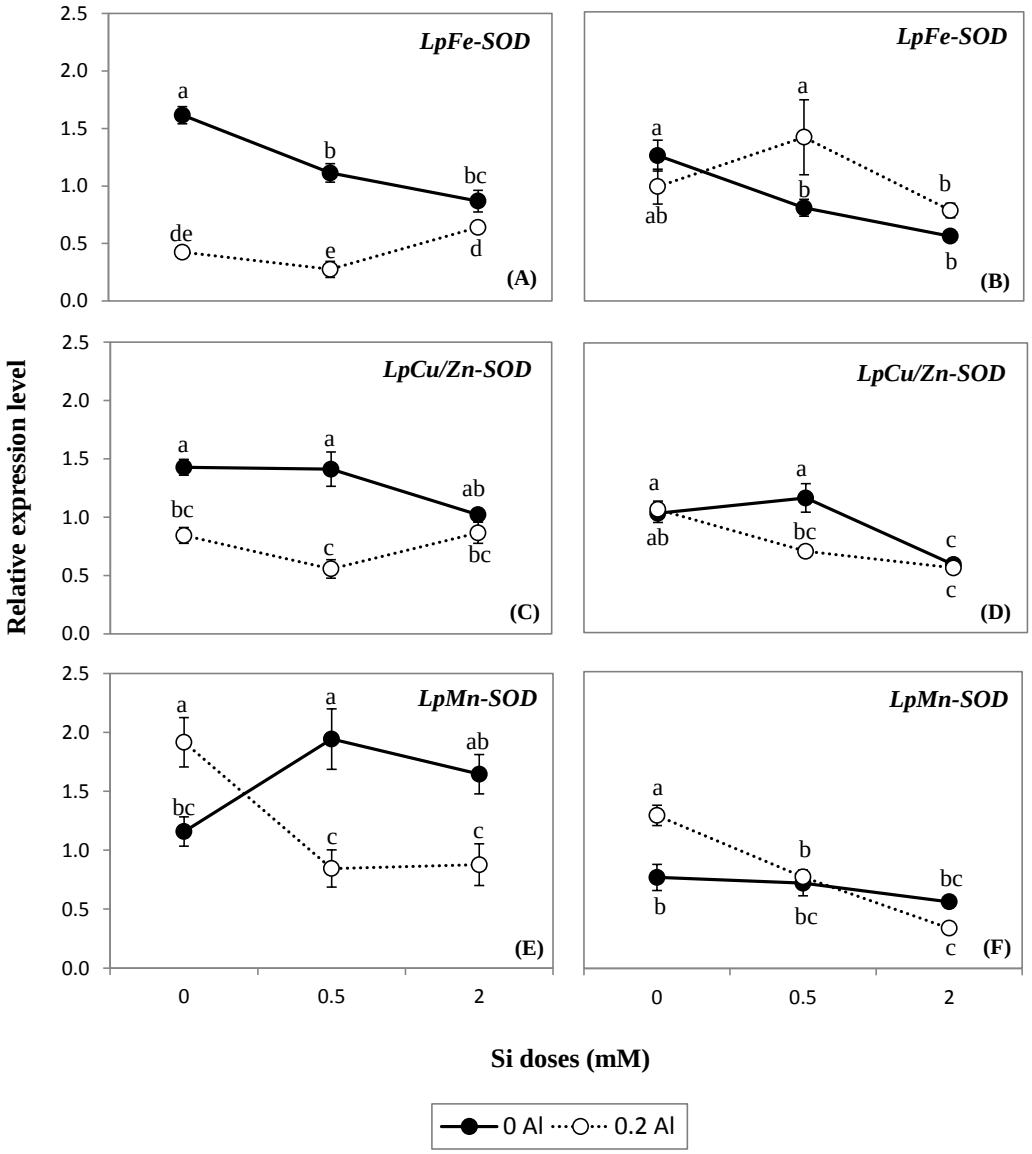

Figure 5. Expression analysis of SOD isoform genes *LpFe-SOD* (A, B), *Cu/Zn-SOD* (C, D) and *Mn-SOD* (E, F) determined by qRT-PCR in shoots and roots of ryegrass hydroponically cultivated under Al and Si treatments. The expression levels were normalized in relation to *Actin* or *eEF1A(m)* gene expression. Data are means of three

replicates ± standard error. Different letters indicate statistically significant differences ($p \leq 0.05$) among treatments.

4.3.6 Hydrogen peroxide production in shoot protoplasts exposed to Al and Si

Aluminum treatment augmented H_2O_2 generation by approximately 38% in shoot protoplasts (Figure 6A). A progressive increase in H_2O_2 production was also observed when Si was added alone, and the accumulation of H_2O_2 was enhanced to an even greater extent in plants simultaneously supplied with Si and Al (Figure 6A). This pattern was consistent with the observations made by confocal microscopy analysis (Figure 6B), which revealed a progressive increase in the fluorescence of an H_2DCFDA probe generated by Si and Al application.

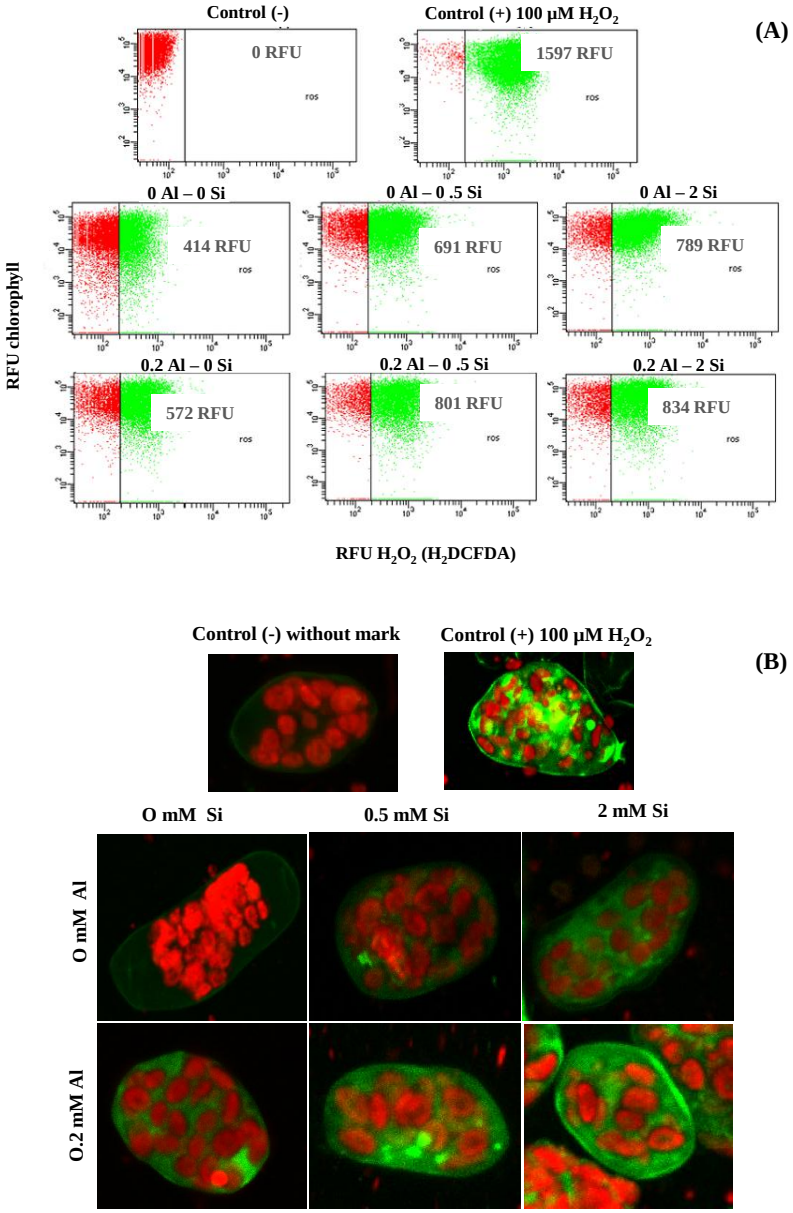

Figure 6. Hydrogen peroxide (H_2O_2) production in shoot protoplasts of ryegrass hydroponically cultivated under Al and Si treatments. (A) Dot plot representation of flow cytometry data. For the positive control, 100 µM H_2O_2 was used. The detection of the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) was expressed as Relative Fluorescence Unit (RFU). (B) Confocal projection images showing the increase of H_2O_2 concentration. Hydrogen peroxide fluorescence (green color) was collected by excitation / emission wave lengths 488nm/530nm by Confocal Laser Scanning Microscope.

4.4 Discussion

Although several previous studies have reported that Si provide beneficial effects on plants subjected to Al stress, the mechanisms underlying these responses have remained poorly understood. Moreover, only a few studies have examined the effect of Si-mediated amelioration of Al toxicity in terms of the regulation of Al and Si uptake systems (e.g., Britez et al. 2002; Wang et al. 2004; Dorneles et al. 2016) and plant antioxidant performance (e.g., Shahnaz et al. 2011; Shen et al. 2014). Likewise, to date, the effect of Si on Al stress in ryegrass, a forage species belonging to Si-accumulator plants (Jarvis, 1987; Nanayakkara et al. 2008), has yet to be addressed.

The high level of toxic Al in acid soils is an important limiting factor for plant production (Mora et al. 2006). In our study, the exposure of plants to 0.2 mM Al significantly increased Al accumulation, mainly in the roots (Table 2), with a consequent reduction of approximately 28.5% in root dry matter production (Table 2). These results are consistent with our previous findings for ryegrass (Cartes et al. 2010), since it is well known that Al toxicity involves the rapid inhibition of root growth (e.g., Matsumoto, 2000; Kochian et al. 2005; Horst et al. 2010; Singh et al. 2017). The role played by Si in promoting plant growth under Al toxicity has been widely accepted (e.g., Hara et al. 1999; Singh et al. 2011; Shen et al. 2014; Tripathi et al. 2016). Correspondingly, Si application to Al-treated plants significantly reduced the Al concentration in ryegrass (Table 2) and improved root dry weight by at least 51% (Table 2). A slight reduction in Si concentration in plant tissues was also found when plants were simultaneously supplied with Al and Si (Table 2). Moreover, our results revealed a negative correlation between Si and Al uptake in plants treated with Al and Si, whereas Si concentration and dry matter production were positively related (Table 3). The reduction in Al and Si uptake might be attributed to the formation of biologically

inactive aluminosilcate (Al-Si) complexes in the growth media, thus lowering Al availability (Barcelo et al. 1993; Baylis et al. 1994; Ma et al. 1997; Cocker et al. 1998a), with the consequent enhancement of root growth. Nevertheless, the formation of Al-Si inside plant tissues could also be involved in the growth-promoting effect of Si under Al stress (Hodson and Sangster, 1993; Cocker et al. 1998b; Wang et al. 2004). Indeed, it has been demonstrated that Al toxicity may be decreased by co-deposition of Al and Si in the root epidermal walls of sorghum (Hodson and Sangster, 1993). Similarly, Cocker et al. (1998b) and Wang et al. (2004) have also suggested that formation of Al-Si complexes in the root apoplast of wheat and maize is a possible mechanism for Al detoxification in plants.

Although all plants contain Si in their tissues, the concentration of this element varies greatly among species, in a range from 0.1% to 10% on a dry weight basis (Epstein 1999; Ma and Takahashi, 2002), which is indicative of the fact that the benefits of Si to plants grown under stress can also be highly variable. Recent studies have shown that Si accumulation in plants is controlled by influx and efflux Si transporters that could be involved in the differential Si-induced responses to cope with different plant stress (e.g. Ma et al. 2006; 2007; Mitani et al. 2009a; 2009b; Chiba et al. 2009; Mitani et al. 2011a; 2011b; Yamaji et al. 2008; 2009; 2012; Grégoire et al. 2012; Montpetit et al. 2012; Deshmukh et al. 2013; Ma and Yamaji 2015). To further investigate the effect of Si uptake on ryegrass subjected to Al stress, we assessed the gene expression of two Si transporters (*Lsi1* and *Lsi2*) in plants with different Al and Si supply (Figures 1A, B). Lsi1 is a channel-type transporter belonging to aquaporin Nodulin26-like intrinsic protein (NIP) III subfamily (Ma et al. 2006), whereas Lsi2 is an Si efflux transporter belonging to the family of putative anion transporters (Ma et al. 2007). Efficient coupling of Lsi1 with Lsi2 controls the uptake of Si in species such as rice, barley, and

maize (Ma et al., 2006; 2007; Mitani et al., 2009a; 2009b; Chiba et al., 2009). Our study showed that in plants cultivated without Al, the mRNA expression levels of both *LpLsi1* and *LpLsi2* were down-regulated in plants supplied with Si (Figure 1A, B). Some studies have shown that the accumulation of *Lsi1* mRNA in maize (*ZmLsi1*), barley (*HvLsi1*), and wheat (*TaLsi1*) is not affected by the addition of Si (Chiba et al. 2009; Mitani et al. 2009a; Montpetit et al. 2012). Nevertheless, Ma et al. (2006; 2007) found that the gene expression of both *OsLsi1* and *OsLsi2* was decreased by approximately 25% in rice, as a consequence of continuous Si application. A similar expression pattern has been detected for *Lsi1* in maize (*ZmLsi1*) (Bokor et al. 2014) as well as for *Lsi2* in barley (*HvLsi2*) (Mitani et al. 2009b) and maize (Bokor et al. 2014). Moreover, a recent study has stated that the Si-induced down-regulation of Si transporter genes is controlled by Si accumulation in the shoots of rice (Mitani et al. 2016).

At present, there is little information on the effect of any plant stress on the transcriptional regulation of Si transporters genes. Bokor et al. (2014) observed that Si supply down-regulated the expression of *ZmLsi1* and *ZmLsi2* in the roots of maize subjected to excess zinc (Zn). By contrast, it has been reported that Si increased the expression level of *OsLsi1* and *OsLsi2* under conditions of cadmium (Cd) and copper (Cu) toxicity in rice plants (Kim et al. 2014). Likewise, Vulavala et al. (2016) found that a putative Si transporter in potato (*StLsi1*) was up-regulated in response to Si and drought stress. Interestingly, we found that the transcript levels of both *LpLsi1* and *LpLsi2* were significantly down-regulated by Al supply, but up-regulated by 5.4-fold (*LpLsi1*) and 2.5-fold (*LpLsi2*) when Al was added in combination with Si (Figures 1A, B). Compared with plants cultivated with Si alone, the reduction in Si concentration in plants simultaneously supplied with Al and Si (Table 2), could be responsible for the up-regulation of *LpLsi1* and *LpLsi2* (Figures 1A, B). This behavior might indicate an

increased requirement for Si in ryegrass in order to cope with Al-induced toxicity. Further studies are needed to confirm this assumption.

As a possible alternative mechanism of Si-mediated Al detoxification in plants, enhancement of the antioxidant defense system has also been proposed (Shahnaz et al. 2011; Shen et al. 2014; Liang et al. 2015; Tripathi et al. 2016). As stated above, Al toxicity can lead to the generation of reactive oxygen species (ROS), such as superoxide radicals ($O_2^{\cdot -}$), hydroxyl radicals (•OH), and hydrogen peroxide (H_2O_2) molecules, which cause oxidative damage to plant cells (e.g., Yamamoto et al. 2001; 2002; 2003; Kochian et al. 2005; Singh et al. 2017). In agreement with previous reports (Cartes et al. 2010; 2012), our results show that 0.2 mM Al increased lipid peroxidation in ryegrass (Figures 2A, B), confirming that oxidative stress occurs under Al supply. Nevertheless, 2 mM Si significantly diminished Al-induced lipid peroxidation by approximately 32% and 28% in shoots and roots, respectively (Figures 2A, B). Moreover, a negative correlation between Si concentration and lipid peroxidation was detected in Al-treated plants (Table 3). Consistent with our findings, Shen et al. (2014) observed a noticeable decrease in lipid peroxidation attributable to Si in peanut grown under Al excess. Similarly, there is increasing evidence showing that oxidative damage to biological membranes decreases as a consequence of Si application to plants subjected to different environmental stresses (e.g. Liang et al. 2003; Zhu et al. 2004; Shi et al. 2005; Gunes et al. 2007; 2008; Li et al. 2012; Khoshgoftarmanesh et al. 2014; Kim et al. 2014; Habibi, 2015; Zia-ur-Rehman et al. 2016).

Whereas Al toxicity enhanced plant phenols concentration (Figures 3A, B) and augmented the activities of antioxidant enzymes (Figures 4A-H), Si application induced differential responses in the antioxidant system of Al-stressed plants (Figures 3A, B and Figures 4A-H). It has been suggested that Si may enhance Al tolerance by increasing

the production of phenolic compounds with Al-chelating ability (Kidd et al. 2001; Shahnaz et al. 2011). Furthermore, it has been reported that Si uptake by plants subjected to certain stresses can lead to increased production of phenolics with antioxidant and/or structural function (Fleck et al. 2010; 2015; Song et al. 2016). Likewise, enzymes and genes involved in the biosynthesis of either soluble phenolics (e.g., flavonoids) or structural polyphenols (e.g., lignin) have also been shown to be induced by Si (Liang et al. 2007; Shetty et al. 2011; Zhang et al. 2013; Song et al. 2016). Here, we found that Si addition (mainly at the highest dose) increased the total phenol concentration in plants treated with Al and Si (Figures 3A, B), and that there was a negative relationship between phenols concentration and lipid peroxidation (Table 3). Thus, the enhanced phenols accumulation triggered by Si may have contributed to the amelioration of Al-induced oxidative stress in ryegrass.

Differential changes in the activity of antioxidant enzymes, as a consequence of Al and Si treatments, were also observed. Superoxide dismutase constitutes the first line of defense in the enzymatic antioxidant responses by catalyzing the dismutation of $O_2^{\cdot -}$ to H_2O_2 and O_2 (Takahashi and Asada, 1983; Alscher et al., 2002). Our results indicate that the highest Si dose decreased SOD activity in plants subjected to Al stress (Figures 4A, B), as supported by the negative correlation between SOD activity and Si concentration (Table 3). In agreement with SOD activity, Si supply significantly reduced the gene expression level of both *LpCu/Zn-SOD* (1.9-fold) and *LpMn-SOD* (3.8-fold) in roots of plants exposed to Al toxicity (Figure 5D, F). Similarly, the transcript level of *LpMn-SOD* was decreased by at least 2.2- fold in shoots of plants receiving Al in combination with Si (Figures 5E), whereas no changes in the expression of *Fe-SOD* were detected (Figures 5A, B). Consequently, our results indicated that *LpCu/Zn-SOD* and *LpMn-SOD* mainly contributed to the total SOD activity. The

decrease in both the total SOD activity (Figure 4A, B) and the gene expression pattern of *LpCu/Zn-SOD* and *LpMn-SOD* isoforms (Figures 5 D, E, F) coincided with a significant reduction in lipid peroxidation at the highest Si dose (Figures 2A, B), denoting that 2 mM Si can diminish the requirement for SOD enzyme in Al-treated plants.

It is noteworthy that the activity of antioxidant enzymes responsible for H_2O_2 scavenging (CAT, POD, and APX) was activated by Si in the roots of Al-stressed plants (Figures 4D, F, H). Moreover, a direct correlation between Si concentration and the activities of CAT, POD, and APX was found in the roots of plants treated with Al and Si (Table 3). The activation of these enzymes was accompanied by a noticeable decrease in lipid peroxidation (Figures 2A, B), with a consequent reduction in the oxidative damage of biological membranes induced by Al.

We also detected an apparent increase in intracellular H_2O_2 production in shoot protoplasts of plants simultaneously supplied with Al and Si (Figure 6A, B). It is remarkable that there is so little information available regarding the role of Si in H_2O_2 generation under either biotic or abiotic stress conditions. In this context, the only study that has examined the relationship between Si and H_2O_2 production in plants subjected to Al toxicity (Lima et al. 2016) showed an opposite trend when compared with our results. Nevertheless, under freezing stress, Habibi (2015) detected an increase in H_2O_2 levels induced by Si in pistachio plants, which is consistent with the findings of the present study. This significant increase in H_2O_2 production might be related to the reduction in POD activity observed in the shoots of plants simultaneously treated with Al and Si (Figure 4E). Indeed, H_2O_2 plays a dual role in vascular plants by either inducing oxidative damage or acting as signaling molecule in several physiological processes, including senescence (Peng et al., 2005), photorespiration and photosynthesis

(Noctor and Foyer, 1998), and growth and development (Foreman et al. 2003). H_2O_2 also functions as a second messenger that modulates the expression of antioxidant enzymes and stress responses (Apel and Hirt, 2004). Accordingly, further work should focus on the mechanisms underlying the Si modulation of H_2O_2 production under Al stress.

Finally, taken together, our findings provide the first biochemical and molecular evidence that Si counteracts the negative effects of Al by modulating Al and Si uptake as well as enzymatic and non-enzymatic antioxidant responses in ryegrass plants.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

SP and PC conceived the idea and wrote the manuscript. SP performed all the experiments and PC supervised the research. AGM and HJ contributed to evaluation and discussion regarding aspects of the study related to gene expression analyses. KG assisted with management and analysis of the flow cytometry and laser scanning confocal microscopy data. MM contributed to discussion on aspects associated with the influence of Si on plants subjected to Al toxicity. All authors contributed to the discussion and approved the final manuscript.

Funding

This work was supported by the FONDECYT projects 1120901 and 1161326, and CONICYT Doctoral Scholarship 21120704.

Acknowledgments

The authors are grateful to Scientific and Technological Bioresource Nucleus of Universidad de La Frontera (BIOREN-UFRO) for providing access to specialized equipment for use in qRT-PCR, flow cytometry, and laser scanning confocal microscopy analysis.

CHAPTER V

General discussion, concluding remarks and future directions

5. General discussion, concluding remarks and future directions

5.1 General discussion

Current scientist evidence demonstrates the benefits of silicon (Si) for vascular plants, particularly in alleviating biotic and abiotic stresses. As mentioned in the previous Chapters, the beneficial role of Si is closely related with the ability of the plant species to take up this element from soil. Hence, some plant species does not respond to Si supply, and this behavior often can be interpreted as a failure by Si to confer protection rather than a biological limitation. This situation has created a lot of ambiguities in the literature and confusion among scientists. Nevertheless, the recent molecular advances associated with the identification and characterization of Si transporters in various plant species have been useful to improve the understanding of benefits that plants can derive from Si uptake.

Silicon is currently viewed as a sustainable alternative to provide tolerance to Al stress, which represents one of the major constraints for crop production in Southern Chile. In an attempt to search new insights to alleviate the negative effects derived from Al in ryegrass, firstly we studied the Si uptake system and then the impact of Si supply on plants grown under Al toxicity. Influx transporters (Lsi1 and Lsi6) belonging to the NIP III aquaporins subgroup and efflux transporters (Lsi2 and Lsi3), known as anion transporters, have been reported to be responsible for the uptake and transport of Si in vascular plants (Ma and Yamaji 2015; Deshmukh and Bélanger 2016). Based on the sequence homology approach, two putative Si transporter genes, *LpLsi1* and *LpLsi2*, were cloned and characterized from ryegrass (Chapter III; supporting information of Annex 1). BLAST analysis showed that the amino acid sequence of LpLsi1 shared high identity (88%) with a homologous sequence from barley and wheat (Table S1.1 of

Annex 1). The available information about amino acid sequences and protein models of influx Si transporters have revealed unique conserved attributes that determine the selectivity for silicic acid (Mitani et al. 2011c; Liu and Zhu 2010; Deshmukh and Bélanger 2015; Deshmukh et al. 2015; Vatansever et al. 2017) as shown in Figure S1.2 of Annex 1. Accordingly, it has been reported that two pore-forming regions in the central channel (two highly conserved NPA (Asn-Pro-Ala) motifs and an aromatic/arginine (ar/R) filter) are associated to substrate selectivity of NIP members (Wu and Beitz 2007). The NPA region is formed by juxtaposition of asparangines (Asn), which make hydrogen bond with the transport molecules and may function in the proton exclusion (Forrest and Bhave 2007). Besides, ar/R region functions as a selectivity filter for substrates: acting a barrier, determining the transport rate, and making H and van der Waals bounds with substrates (Sui et al. 2001). In ar/R filter for silicic acid transport, only Gly-Ser-Gly-Arg (GSGR) residues confer specificity for silicic acid (Mitani et al. 2011c). Thus, conserved domains characterizing both the typical aquaporins and the Si transport activity (Wu and Beitz 2007; Maurel et al. 2008; Mitani et al. 2011c; Deshmukh and Bélanger 2015; Deshmukh et al. 2015; Vatansever et al. 2017) were found in the predicted amino acid sequence of LpLsi1. Based on sequence homology and availability of ar/R selectivity filter, a phylogenetic analysis performed by Vatansever et al. (2017) showed that three clusters associated to Si accumulation ability were formed from Lsi1 transporters. Consequently, protein modeling of Lsi1 in different plant species showed that ar/R selectivity filter containing GSGR residues were found in binding sites of the high Si accumulators (Vatansever et al. 2017). Based in this premise, the conserved amino acid residues identified in the protein sequence of LpLsi1 confirm the high Si accumulation capacity of ryegrass as previously suggested by (Jarvis 1987; Nanayakkara et al. 2008). Phylogenetic analyses

also support that LpLsi1 belongs to a well-defined aquaporins family of previously identified Lsi1 genes (Chapter III). On the other hand, identified Lsi2 genes in plants encoded a polypeptide of 472-547 amino acid residues having conserved 10-11 putative transmembrane domains (Ma et al. 2007; Mitani et al 2009a; Vatansever et al. 2017). Different to Lsi1, Lsi2 transporter homologs in 17 plant species showed highly preserved sequences even in low Si-accumulators (Vatansever et al. 2017). A partial coding sequence of *LpLsi2* showing high identity with known Lsi2 efflux transporters *HvLsi2* (90%), *OsLsi2* (82%), and *ZmLsi2* (81%) was also isolated from ryegrass (Table S1.2 and Figure S1.1 of Annex 1). However, an exhaustive analysis of *LpLsi*2 sequence was not perfomed since the full length cDNA was not satisfactorily achieved.

A gene expression analysis showed that *LpLsi1* and *LpLsi2* were only expressed in roots. In addition, the expression level of both *LpLsi1* and *LpLsi2* was down-regulated by Si supply suggesting that theses transporters can be silicic acid inducible (Chapter III and IV). These findings agree with the expression pattern reported by Ma et al. (2006; 2007b) and Mitani et al. (2009a) for both *Lsi1* and *Lsi2* in other plant species. However, it has been found that Si did not affect the transcript level of Lsi1 in barley, maize and wheat (Chiba et al. 2009; Mitani et al. 2009b; Montpetit et al. 2012).

In order to assess the response to Si supply in two ryegrass cultivars with contrasting Al- tolerance (Al-sensitive, Jumbo; Al-semi-tolerant, Nui) two kinetics experiments of Si uptake were performed (Chapter III). In general, both the concentration- and the time -dependent Si uptake experiments showed that Si accumulation in cv. Jumbo was higher than cv. Nui. However, cultivar variation for Si concentration was mainly observed at the short-term (Chapter III). Genotypic variations in Si accumulation in rice roots have been associated with differences in abundance of Si transporters (Ma et al, 2007a). However, the concentration-dependent kinetics assay in ryegrass showed similar values

of V_{max} between cultivars, whereas variations in K_m values could suggest either the differential contribution of the known proteins responsible for uptake and transport of Si or the involvement of undiscovered Si transporters (Chapter III). Another factor that could determine the differences in Si accumulation between genotypes is the production of root biomass. It is expected that high root growth improves Si uptake. Such effect was not visualized in our study since the experiment was performed at the short-term; thus, ryegrass cultivars did not show any difference in root dry matter production (Figure S2.1 of Annex 2). Likewise, it has been suggested that dissimilarities in Si uptake ability between rice genotypes (Japonica and Indica) could result from the difference in the expression of Si transporter genes (Ma et al., 2007a). Thus, our outcomes about the identification and characterization of genes encoding for Si transporters provide the basis to improve the understanding of Si uptake mechanisms in ryegrass and the benefits that this forage species can arise from Si nutrition.

The role of Si for ryegrass cv. Nui subjected to Al toxicity was reviewed in the Chapter IV and published in Frontiers in Plant Science 2017, 8:642. In order to extend our knowledge about Si/Al uptake under Al stress, studies involving plant growth, Si and Al concentration, gene expression of Si transporters and lipid peroxidation were also performed in ryegrass cv. Jumbo supplied with Al (0 or 0.2 mM) in combination with Si (0, 0.5 or 2 mM Si) doses at 21 days (Annex 3). Our outcomes demonstrated that the application of 0.2 mM Al significantly increased Al accumulation and lipid peroxidation in roots of both ryegrass cultivars, thus reducing the root growth by about 28% and 48% in cv. Nui and cv. Jumbo, respectively (Chapter IV and Figures S3.1, S3.2 and S3.3 of Annex 3). Nevertheless, Si applied to Al-stressed plants decreased Al concentration by up to 56% in cv. Nui and 20% in cv. Jumbo. Moreover, Si supply diminished Al-induced lipid peroxidation by about 28% and 41% in roots of cv. Nui and cv. Jumbo,

respectively (Chapter IV and Figures S3.1 and S3.2 of Annex 3). Consequently, improvement of root dry weight was observed in plants of both cv. Nui (51%) and cv. Jumbo (118%) receiving the combined Al-Si treatments (Chapter IV and Figure S3.3 of Annex 3). Our results also revealed a negative correlation between Si and either Al concentration and lipid peroxidation in both ryegrass cultivars treated with Al and Si, whereas Si concentration and dry matter production were positively related (Table S3.1 of Annex 3).

Differences in root Si concentration between ryegrass cultivars were also observed when plants were simultaneously supplied with Al and Si (Figure S3.4 of Annex 3). Compared with plants cultivated with Si alone, a slight reduction of root Si concentration was found in cv. Nui treated with Al and Si, whereas Si concentration in the roots of cv. Jumbo was increased. Interestingly, ryegrass cultivars with contrasting Al-tolerance also exhibited differential gene expression profiles of Si transporters under Al stress (Figure S3.5 of Annex 3). Since our results showed that the expression level of *LpLsi1* and *LpLsi2* was decreased by Si application in both cultivars (Chapter III and IV), the up-regulation of *LpLsi1* and *LpLsi2* in the Al-semi-tolerant cv. Nui simultaneously supplied with Al and Si was consistent with the reduction of Si concentration at the same condition. In contrast, a down-regulation of *LpLsi1* in the Al-sensitive cv. Jumbo coincided with the increase of Si concentrations in roots when Al and Si were applied in combination. These facts might denote differential requirements of Si between ryegrass cultivars aimed to cope with Al toxicity. Moreover, differential response among cultivars might result from either the involvement of unidentified Si transporters or the existence of external signals, still unknown, regulating the expression and/or activity of Si transporters. Recent findings showing that shoot Si accumulation induces a down-regulation of Si transporters genes, and the presence of a cis-acting

element in the Lsi1 promoter regulating the OsLsi1 expression in rice (Mitani et al. 2016) support our premise. Further studies are needed to advance in the understanding of the mechanisms controlling the expression of Si transporters genes in plants.

Despite both the root Si concentration and the expression level of *LpLsi1* and *LpLsi2* were dissimilar between ryegrass cultivars under Al stress, a reduction of Al concentration in plant tissues of cv. Nui and cv. Jumbo was observed as a consequence of Si application to nutrient solution (Figure S3.1 of Annex 3). Decrease of Al accumulation induced by Si has been also found by Dorneles et al. (2016) and Vega et al. (2019; submitted). Consequently, Si addition in presence of Al could be generating a reduction of the Al accumulation as result of the formation of Al-Si complexes in the solution, thus lowering Al availability and improving root biomass production (Barcelo et al, 1993; Baylis et al, 1994; Ma et al, 1997; Cocker et al, 1998a).

As an alternative mechanism involved on the Si-mediated Al detoxification in plants, the enhancement of the antioxidant defense system has also been proposed (Shahnaz et al., 2011; Shen et al., 2014; Liang et al., 2015; Tripathi et al., 2016). In this regard, this study showed molecular and biochemical evidence supporting the role of Si on the improvement of the antioxidant performance of ryegrass cv. Nui subjected to Al toxicity (Chapter IV). Consistent with our findings, there is increasing evidence showing that oxidative stress decreases as a consequence of Si supply to plants subjected to either Al excess or other environmental stresses (e.g., Liang et al., 2003; Zhu et al., 2004; Shi et al., 2005; Gunes et al., 2007, 2008; Li et al., 2012; Khoshgoftarmanesh et al., 2014; Kim et al., 2014; Habibi, 2015; Zia-ur-Rehman et al., 2016). Interestingly, Si uptake by ryegrass enhanced total phenols concentration in plants grown under Al exposure

(Chapter IV). It has been proposed that Si influences phenolics metabolism and utilization by Si-polyphenol complexation (Dragišic Maksimovic et al. 2007). In addition, it has also been reported that Si may enhance Al tolerance by increasing the production of phenolic compounds with antioxidant and/or structural function (Fleck et al., 2010, 2015; Song et al., 2016; Ribera et al. 2018). Moreover, the activities and/or gene-expression profiles of key enzymes involved in the biosynthesis of either soluble phenolics (e.g., flavonoids) or structural polyphenols (e.g., lignin) have also been shown to be induced by Si in plants subjected to various stresses (Liang et al., 2007; Shetty et al., 2011; Zhang et al., 2013; Song et al., 2016). On the other hand, under Al stress, the reduction of lipid peroxidation triggered by Si also coincided with the decrease of both the total SOD activity and the gene expression pattern of *LpCu/Zn-SOD* and *LpMn-SOD* isoforms (Chapter IV), suggesting that Si supply can diminish the requirement for SOD enzyme in Al-treated plants. Likewise, enzymes belonging to the second defense line against oxidative stress (CAT, POD, and APX) were activated as a response to Si supply (Chapter IV), in agreement with the earlier reports on vascular plants subjected to Al (Ribera et al. 2018; Shen et al. 2014;), manganese (Shi et al. 2005), salt (Hashemi et al. 2010) and boron (Inal et al. 2009) toxicity.

Finally, the main results of this research and the aspects that still remain to be investigated are summarized in Figure 1.

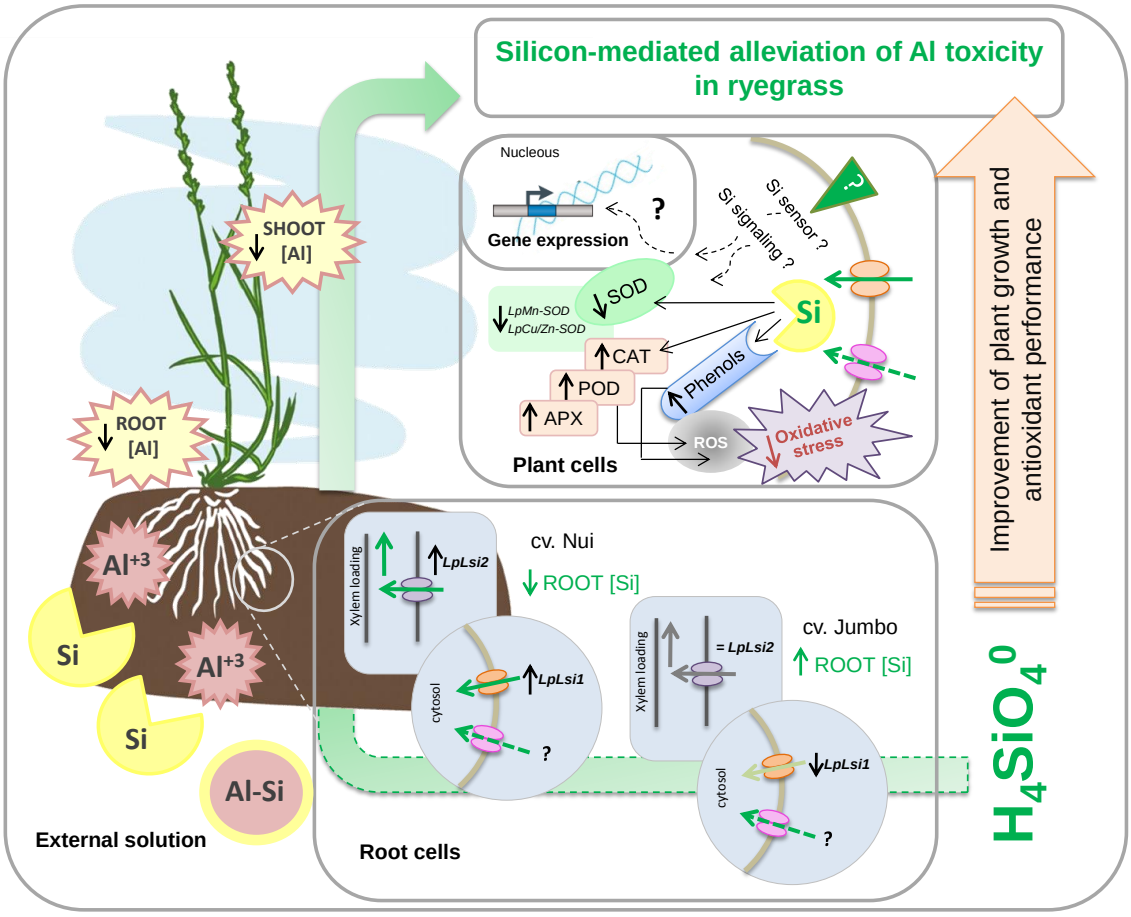

Figure 1. Overview of Si uptake and its role in ryegrass under Al toxicity. Dashed arrows indicate the aspects that still remain to be investigated.

5.2 Concluding remarks and future directions

- Our findings confirm the presence of putative influx (LpLsi1) and efflux (LpLsi2) Si transporters in ryegrass with characteristics highly conserved among Si transporters from different gramineous species. The identification of these Si transporters genes provides new evidence about the Si accumulation ability of ryegrass.

- Our research also provides the first evidence that Si alleviates Al toxicity in ryegrass. Silicon counteracts Al stress by modulating Al/Si uptake and by reducing the Al-induced oxidative stress with the consequent improvement of root growth in cultivars with contrasting Al-tolerance. Under Al toxicity, differential Si accumulation and gene expression pattern of Si transporters might denote either a different Si requirement between cultivars to counteract Al stress or the involvement of unknown regulatory element(s) determining the function of Si transporters. This evidence underlines the importance of studying Si uptake mechanisms and its molecular regulation under stress conditions.

This Thesis project not only contributes to extend the understanding of the Si uptake system of ryegrass and the diversity of membrane transporters in vascular plants, but also the impact of Si on the antioxidant performance of ryegrass cultivated under Al toxicity. Future assessments regarding both the functional characterization and the cellular localization of LpLsi1 and LpLsi2 could help to explain the differences in Si uptake and gene expression of these Si transporters between cultivars. Further in-depth studies are needed to elucidate the mechanisms involved in the regulation of sensing and signal transduction pathways controlling the gene expression of Si transporters. The

knowledge of the molecular nature of Lsi genes could be used to optimize the benefits that ryegrass plants can derive from Si uptake under either Al toxicity or another stressors.

References

Adrees M, Ali S, Rizwan M, Zia-ur-Rehman M, Ibrahim M, Abbas F, Farid M, Qayyum FM, Irshad MK (2015) Mechanisms of silicon-mediated alleviation of heavy metal toxicity in plants: a review. Ecotoxicol. Environ. Saf. 119: 186-197.

Ahmad R, Zaheer SH, Ismail S (1992) Role of silicon in salt tolerance of wheat (*Tritium aestivum L.*). Plant Sci 85: 43-50.

Alscher RG, Erturk N, Heath LS (2002) Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J. Exp. Bot. 53: 1331-1341.

Apel K, Hirt H (2004) Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55: 373-399.

Arnon DI, Stout PR (1939) The essentiality of certain elements in minute quantity for plant with special reference to copper. Plant Physiol 14: 371-375.

Arunakumara K, Walpol, B, Yoon, MH (2013) Aluminum toxicity and tolerance mechanism in cereals and legumes - A review. J Korean Soc Appl Bi 56: 1-9.

Barceló J, Guevara P, Poschenrieder Ch (1993) Silicon amelioration of aluminium toxicity in teosinte (*Zea mays L. ssp. mexicana*). Plant Soil 154: 249-255.

Barceló J, Poschenrieder C (2002) Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminium toxicity and resistance: a review. Environ. Exp. Bot. 48:75-92.

Baylis AD, Gragopoulou C, Davidson KJ, Birchall JD (1994) Effects of silicon on the toxicity of aluminum to soybean. Commun Soil Sci Plan 25: 537-546.

Bokor B, Bokorová S, Ondos S, Svubová R, Lukacova Z, Hyblova M, Szemes T, Lux A (2014) Ionome and expression level of Si transporter genes (Lsi1, Lsi2, and Lsi6) affected by Zn and Si interaction in maize. Environ Sci Pollut Res Int. 22:6800-6811.

Bolan N, Adriano D, Curtin D (2003) Soil acidification and liming interactions with nutrient and heavy metal transformation and bioavailability. Adv Agron 1: 215-272.

Bolan NS, Hedley MJ, White RE (1991) Processes of soil acidification during nitrogen cycling with emphasis on legume based pastures. Plant Soil 134: 53-63.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Britez, RM, Watanabe T, Jansen S, Reissmann CB, Osaki M (2002) The relationship between aluminium and silicon accumulation in leaves of *Faramea marginata* (Rubiaceae). New Phytol. 156: 437-444.

Broadley M, Brown P, Cakmak I, Ma JF, Rengel Z, Zhao F (2012) Beneficial Elements. In: Marschner P (ed) Marschner's Mineral Nutrition of Higher Plants, 3rd edition. Elsevier, Amsterdam, pp 249-269.

Cartes P, Jara AA, Pinilla L, Rosas A, Mora ML (2010) Selenium improves the antioxidant ability against aluminium-induced oxidative stress in ryegrass roots. Ann. Appl. Biol. 156: 297-307.

Cartes P, McManus M, Wulff-Zottele C, Gutiérrez A, Mora ML (2012) Describing differential superoxide dismutase expression under aluminium stress in ryegrass cultivars at the short term. Plant Soil 350: 353-363.

Chandler-Ezell K, Pearsall D, Zeidler J (2006) Root and tuber phytoliths and starch grains document manioc (*Manihot esculenta*), arrowroot (*Maranta arundinacea*), and llerén (*Calathea sp*.) at the Real Alto site, Ecuador. Econ Bot 60: 103-120.

Chen CH, Lewin J (1969) Silicon as a nutrient element for *Equisetum arvense*. Can J Botany 47: 125-131.

Cheong YWY, Chan PY (1973) Incorporation of P32 in phosphate esters of the sugar cane plant and the effect of Si and Al on the distribution of these esters. Plant Soil 38: 113-123.

Chiba Y, Mitani N, Yamaji N, Ma JF (2009) HvLsi1 is a silicon influx transporter in barley. Plant J 57: 810-818.

Cocker KM, Evans DE, Hodson MJ (1998a) The amelioration of aluminium toxicity by silicon in higher plants: solution chemistry or an in plants mechanism?. Physiol Plantarum 104: 608-614.

Cocker KM, Evans DE, Hodson, MJ (1998b) The amelioration of aluminium toxicity by silicon in wheat (*Triticum aestivum* L.): malate exudation as evidence for an in planta mechanism. Planta 204: 318-323.

Cornelis JT, Delvaux B, Georg RB, Lucas Y, Ranger J, Opfergelt S (2011) Tracing the origin of dissolved silicon transferred from various soil-plant systems towards rivers: a review. Biogeosciences 8: 89-112.

Corrales I, Poschenrieder C, Barceló J (1997) Influence of silicon pretreatment on aluminium toxicity in maize roots. Plant Soil 190: 203-209.

Coskun D, Deshmukh R, Sonah H, Menzies JG, Reynolds O, Ma JF, Kronzucker HJ, Bélanger R (2019) The controversies of silicon's role in plant biology. New Phytol. 221: 67-85.

Currie HA, Perry CC (2007) Silica in plants: Biological, biochemical and chemical studies. Ann Bot-London 100: 1383-1389.

Darkó E, Ambrusa H, Stefanovits- Banyai E, Fodor J, Bakos F, Barnabás B (2004) Aluminium toxicity, Al tolerance and oxidative stress in an Al- sensitive wheat genotype and in Al- tolerant lines developed by *in vitro* microspore selection. Plant Sci 163: 583:591.

Datnoff LE, Deren CW, Snyder GH (1997) Silicon fertilization for disease management of rice in Florida. Crop Prot 16: 525-531.

De N, Datta SH (2007) Relationship between phosphorus sorption and soil acidity as affected by bicarbonate and silicate ions. Commun Soil Sci Plan 38: 679-694.

Delhaize E, Ryan PR (1995) Aluminium toxicity and tolerance in plants. Plant Physiol 107: 315-321.

Deren CW (2001) Plant genotype, silicon concentration and silicon-related responses. In: Datnoff LE, Snyder GH, Korndorfer GH (eds) Silicon in Agriculture. Elsevier Science BV, Amsterdam, pp 149-158.

Deshmukh R, Bélanger R (2015) Molecular evolution of aquaporins and silicon influx in plants. Functional Ecology 30: 1277-1285.

Deshmukh R, Vivancos J, Guérin V, Sonah H, Labbé C, Belzile F, Bélanger R (2013) Identification and functional characterization of silicon transporters in soybean using

comparative genomics of major intrinsic proteins in Arabidopsis and rice. Plant Mol Biol 83: 303-315.

Dietzel M (2000) Dissolution of silicates and the stability of polysilicic acid. Geochim Cosmochim Ac 64: 3275-3281.

Donahue JL, Okpodu CM, Cramer CL, Grabau EA, Alscher RG (1997) Responses of antioxidants to paraquat in pea leaves. Relationships to resistance. Plant Physiol. 113: 249-257.

Dorneles AOS, Pereira AS, Rossato LV, Possebom G, Sasso VM, Bernardy K, SandriI RQ, NicolosoI FT, Ferreira PAA, TabaldiI LA (2016) Silicon reduces aluminum content in tissues and ameliorates its toxic effects on potato plant growth. Cienc. Rural 46: 506-512.

Dragisic Maksimovic J, Bogdanovic J, Maksimovic V, Nikolic M (2007) Silicon modulates the metabolism and utilization of phenolic compounds in cucumber (*Cucumis sativus* L.) grown at excess manganese. J Plant Nutr Soil Sci 170: 739-744.

Dragisic Maksimovic J, Mojovic M, Maksimovic V, Römheld V, Nikolic M (2012) Silicon ameliorates manganese toxicity in cucumber by decreasing hydroxyl radical accumulation in the leaf apoplast. J Exp Bot 63: 2411-2420.

Du B, Nian H, Zhang Z, Yang C (2010) Effects of aluminum on superoxide dismutase and peroxidase activities, and lipid peroxidation in the roots and calluses of soybeans differing in aluminum tolerance. Acta Physiol. Plant. 32: 883-890.

Du Z, Bramlage WJ (1992) Modified thiobarbaturic acid assay for measuring lipid peroxidation in sugar rich plant tissue extracts. J. Agric. Food Chem. 40: 1566-1570.

El-Jaoual T, Cox D (1998) Manganese toxicity in plants. J Plant Nutr 21: 353-386.

Epstein E (1976) Kinetics of ion transport and the carrier concept In: Lüttge U, Pitman MG (eds) Encyclopedia of plant physiology. New Series, IIB. Springer, Berlin Heidelberg New York, pp 70-94.

Epstein E (1999) Silicon. Annu Rev Plant Phys 50: 641-664.

Fang CX, Wang QS, Yu Y, Li QM, Zhang HL, Wu XC, Chen T, Lin WX (2011) Suppression and overexpression of Lsi1 induce differential gene expression in rice under ultraviolet radiation. Plant Growth Regul 65: 1-10.

Fauteux F, Rémus-Borel W, Menzies J, Bélanger R (2005) Silicon and plant disease resistance against pathogenic fungi. FEMS Microbiol Lett 249: 1-6.

Feng J, Shi Q, Wang, X (2009) Effects of exogenous silicon on photosynthetic capacity and antioxidant enzyme activities in chloroplast of cucumber seedlings under excess manganese. Agric Sci China 8: 40-50.

Fisher RA (1929) A preliminary note on the effect of sodium silicate in increasing the yield of barley. J Agr Sci 19: 132-139.

Fleck AT, Nye T, Repenning C, Stahl F, Zahn M, Schenk MK (2010) Silicon enhances suberization and lignification in roots of rice (*Oryza sativa*). J. Exp. Bot. 62: 2001-2011.

Fleck AT, Schulze S, Hinrichs M, Specht A, Waßmann F, Schreiber L, Schenk MK (2015) Silicon promotes exodermal Casparian band formation in Si-accumulating and Si-excluding species by forming phenol complexes. PLoS One 10: e0138555.

Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jonesk JDG, Davies JM, Dolan L (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 422: 442-446.

Forrest KL, Bhave M (2007) Major intrinsic proteins (MIPs) in plants: a complex gene family with major impacts on plant phenotype. Func. Integr. Genom. 7: 263-289.

Foy CD (1984) Physiological effects of hydrogen, aluminum, and manganese toxicity in acid soils. In: Adams F (ed) Soil Acidity and liming, American Society of Agronomy, Madison, Wisconsin, pp 57-97.

Foy CD (1992) Soil chemical factors limiting plant root growth. Adv Soil Sci 19: 97-149.

Führs H, Götze S, Specht A, Erban A, Gallien S, Heintz D, Van Dorsselaer A, Kopka J, Braun HP, Horst WJ (2009) Characterization of leaf apoplastic peroxidases and metabolites in *Vigna unguiculata* in response to toxic manganese supply and silicon. J Exp Bot 60: 1663-1678.

Furukawa J, Yamaji N, Wang H, Mitani N, Murata Y, Sato K, Katsuhara M, Takeda K, Ma JF (2007) An Aluminum-activated citrate transporter in barley. Plan Cell Physiol. 8; 1081-1091.

Galvez L, Clar RB, Gourley LM, Maranville JW (1989) Effects of silicon on mineral composition of sorghum grown with excess manganese. J Plant Nutr 12: 547-561.

Głazowska S, Baldwin L, Mravec J, Bukh C, Hansen TH, Jensen MM, Fangel JU, Willats W, Glasius M, Felby C, Schjoerring, J. K. (2018). The impact of silicon on cell wall composition and enzymatic saccharification of Brachypodium distachyon. Biotechnol. Biofuels 11:171.

Guerriero G, Hausman JF, Legay S (2016) Silicon and the plant extracellular matrix. Front Plant Sci. 7: 463.

Gomes D, Agasse A, Thiébaud P, Delrot S, Gerós H, Chaumont F (2009) Aquaporins are multifunctional water and solute transporters highly divergent in living organisms. Biochim Biophys Acta.,1788: 1213-1228

Gong H, Zhu X, Chen K, Wang S, Zhang C (2005) Silicon alleviates oxidative damage of wheat plants in pots under drought. Plant Sci 169: 313-321.

Goodwin SB, Sutter TR (2009) Microarray analysis of Arabidopsis genome response to aluminium stress. Biol Plantarum 53: 85-99.

Grégoire C, Rémus-Borel W, Vivancos J, Labbé C, Belzile F, Bélanger RR (2012) Discovery of a multigene family of aquaporin silicon transporters in the primitive plant *Equisetum arvense*. Plant J 72: 320-30.

Gunes A, Pilbeam DJ, Inal A, Bagci EG, Coban S (2007) Influence of silicon on antioxidant mechanisms and lipid peroxidation in chickpea (*Cicer arietinum* L.) cultivars under drought stress. J Plant Interact. 2: 105-113.

Gunes A, Pilbeam DJ, Inal A, Coban S (2008) Influence of silicon on sunflower cultivars under drought stress, I: Growth, antioxidant mechanisms, and lipid peroxidation. Commun. Soil Sci. Plant Anal. 39: 1885-1903.

Guntzer F, Keller C, Meunier JD (2012) Benefits of plant silicon for crops: A review. Agron Sustain Dev 32: 201-213.

Gupta N, Gaurav SS, Kumar A (2013) Molecular basis of aluminium toxicity in plants: A Review. Am. J. Plant Sci. 4: 21-37.

Habibi G (2015) Exogenous silicon leads to increased antioxidant capacity in freezing-stressed pistachio leaves. Acta Agric. Slov. 105: 43-52.

Hashemi A, Abdolzadeh A, Reza Sadeghipour H (2010) Beneficial effects of silicon nutrition in alleviating salinity stress in hydroponically grown canola, *Brassica napus* L. plants. Soil Sci. Plant Nutr .56: 244-253.

Hammond KE, Evans DE, Hodson MJ (1995) Aluminium/silicon interactions in barley (*Hordeum vulgare* L.) seedlings. Plant Soil 173: 89-95.

Hara T, Gu MH, Koyama H (1999) Ameliorative effect of silicon on aluminum injury in the rice plant. Soil Sci. Plant Nutr. 45: 929-936.

Hartono A (2008) The effect of calcium silicate on the phosphorus sorption characteristics of Andisols Lembang West Java. Jurnal Tanah dan Lingkungan 10: 14-19.

Hattori T, Inanaga S, Araki H, An P, Morita S, Luxová M, Lux A (2005) Application of silicon enhanced drought tolerance in *Sorghum bicolor*. Physiol Plantarum 123: 459-466.

Henriet C, Draye X, Oppitz I, Swennen R, Delvaux B (2006) Effects, distribution and uptake of silicon in banana (*Musa* spp.) under controlled conditions. Plant Soil 287: 359-374.

Hernandez-Apaolaza L (2014) Can silicon partially alleviate micronutrient deficiency in plants? A review. Planta 240: 447-458.

Hildebrand M, Dahlin K, Volcani BE (1998) Characterization of a silicon transporter gene family in *Cylindrotheca fusiformis*: sequences, expression analysis, and identification of homologs in other diatoms. Mol Gen Genet 260: 480-486.

Hildebrand M, Volcani BE, Gassmann W, Schroeder JI (1997) A gene family of silicon transporters. Nature 385: 688-689.

Hingston FJ, Raupach M (1967) The reaction between monosilicic acid and aluminium hydroxide. I. Kinetics of adsorption of silicic acid by aluminium hydroxide. Aust J Soil Res 5: 295-309.

Hodson MJ, Sangster AG (1988) Silica deposition in the inflorescence bracts of wheat (*Triticum aestivum* L.) I. Scanning electron microscopy and light microscopy. Can J Bot 66: 829-838.

Hodson MJ, Sangster AG (1989) X-ray microanalysis of the seminal root of *Sorghum bicolor* (L.) Moench. with particular reference to silicon. Ann Bot 64: 659-667.

Hodson MJ, Sangster AG (1993) The interaction between silicon and aluminium in *Sorghum bicolor* (L.) Moench: Growth analysis and X-ray microanalysis. Ann Bot-London 72: 389-400.

Hodson MJ, Sangster AG (1999) Aluminium/silicon interactions in conifers. J Inorg Biochem 76: 89-98.

Hodson MJ, White PJ, Mead A, Broadley MR (2005) Phylogenetic variation in the silicon composition of plants. Ann Bot-London 96: 1027-1046.

Horiguchi T (1988) Mechanism of manganese toxicity and tolerance of plants. IV. Effects of silicon on alleviation of manganese toxicity of rice plants. Soil Sci Plant Nutr 34: 65-73.

Horiguchi T, Morita S (1987) Mechanism of manganese toxicity and tolerance of plants. VI. Effect of silicon on alleviation of manganese toxicity of barley. J Plant Nutr 10: 2299-2310.

Horst WJ, Fecht M, Naumann A, Wissemeier AH, Maier P (1999) Physiology of manganese toxicity and tolerance in *Vignaun guiculata* (L.) Walp. J Plant Nutr Soil Sc 162: 263-274.

Horst WJ, Marschner H (1978) Effect of silicon on manganese tolerance of bean plants (*Phaseolus vulgaris* L.). Plant Soil 50: 287-303.

Horst WJ, Wang Y, Eticha D (2010) The role of the apoplast in Al induced inhibition of root elongation and in Al resistance of plants: a review. Ann Bot-London 106: 185-197.

Hosseini SA, Maillard A, Hajirezaei MR, Ali N, Schwarzenberg A, Jamois F, Yvin, JC (2017) Induction of Barley Silicon Transporter *HvLsi1* and *HvLsi2*, increased silicon concentration in the shoot and regulated Starch and ABA Homeostasis under Osmotic stress and Concomitant Potassium Deficiency. Front Plant Sci. 8: 1359.

Huang CF, Yamaji N, Mitani N, Yano M, Nagamura Y, Ma JF (2009) A bacterial-type ABC transporter is involved in aluminum tolerance in rice. Plant Cell 21: 655-667.

Inal A, Pilbeam DJ, Gunes A (2009) Silicon increases tolerance to boron toxicity and reduces oxidative damage in barley. J. Plant. Nutr. 32: 112-128.

Iwasaki K, Maier P, Fecht M, Horst WJ (2002) Influence of the apoplastic silicon concentration on the manganese tolerance of cowpea (*Vigna unguiculata* L. Walp.). J Plant Physiol 136: 3762-3770.

Iwasaki K, Matsumura A (1999) Effect of silicon on alleviation of manganese toxicity in pumpkin (*Cucurbita moschata* Duch cv. Shintosa). Soil Sci Plant Nutr 45: 909-920.

Jarvis SC (1987) The uptake and transport of silicon by perennial ryegrass and wheat. Plant Soil 97: 429-437.

Khoshgoftarmanesha, AH, Khodarahmia S, Haghighib M (2014) Effect of silicon nutrition on lipid peroxidation and antioxidant response of cucumber plants exposed to salinity stress. Arch. Agron. Soil Sci. 60: 639-653.

Kidd PS, Llugany M, Poschenrider C, Gunse B, Barcelo J (2001) The role of roots exudates in aluminium resistence and silicon- induced amelioration of aluminium toxicity in three varities of maize (*Zea mays* L.). J Exp Bot 52: 1339-1352.

Kim YH, Khan AL, Kim DH, Lee SY (2014) Silicon mitigates heavy metal stress by regulating P-type heavy metal ATPases, *Oryza sativa* low silicon genes, and endogenous phytohormones. BMC Plant Biol 14: 13.

Knight CTG, Kinrade SD (2001) A primer on the aqueous chemistry of silicon. In: Datnoff LE, Snyder GH, Korndorfer GH (eds) Silicon in Agriculture. Elsevier Science BV, Amsterdam, pp 57-84.

Kochian LV, Hoekenga OA, Piñeros MA (2004) How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorous efficiency. Annu Rev Plant Biol 55: 459-493.

Kochian LV, Pineros MA, Hoekenga OA (2005) The physiology, genetics and molecular biology of plant aluminum resistance and toxicity. Plant Soil, 274: 175-195.

Kostic-Kravljanac LM (2015) Modulation of the processes in wheat rhizosphere in responses to the amendments of soils degraded by mining waste. PhD Thesis, University of Belgrade, (in Serbian with an abstract in English).

Lee YB, Hoon C, Hwang JY, Lee IB, Kim PJ (2004) Enhancement of phosphate desorption by silicate in soils with salt accumulation. Soil Sci Plant Nutr 50: 493-499.

Lee YB, Kim PJ (2007) Reduction of phosphate adsorption by ion competition with silicate in soil. Korean J Environ Agric 26: 286- 293.

Li P, Song A, Li Z, Fan F, Liang Y (2012) Silicon ameliorates manganese toxicity by regulating manganese transport and antioxidant reactions in rice (*Oryza sativa* L.). Plant Soil 354: 404-419.

Li YC, Sumner ME, Miller WP, Alva AK (1996) Mechanism of silicon induced alleviation of aluminum phytotoxicity. J. Plant Nutr. 19: 1075-1087.

Liang Y, Chen Q, Liu Q, Zhang W, Ding R (2003) Exogenous silicon (Si) increases antioxidant enzyme activity and reduces lipid peroxidation in roots of salt-stressed barley (*Hordeum vulgare L.*). J. Plant Physiol. 160: 1157-1164.

Liang Y, Hua H, Zhu YG, Zhang J, Cheng C, Römheld V (2006) Importance of plant species and external silicon concentration to active silicon uptake and transport. New Phytol 172: 63-72.

Liang Y, Nikolic M, Bélanger R, Gong H, Song A (2015) Silicon in Agriculture. Springer, Dordrecht.

Liang Y, Si J, Römheld V (2005) Silicon uptake and transport is an active process in *Cucumis sativus* L. New Phytol 167: 797-804.

Liang Y, Sun W, Zhu YG, Christie P (2007) Mechanisms of silicon mediated alleviation of abiotic stresses in higher plants: a review. Environ Pollut 147 : 422-428.

Likhoshway YV, Masyukova YA, Sherbakova TA, Petrova DP, Grachev MA (2006) Detection of the gene responsible for silicic acid transport in *Chrysophycean algae*. Dokl Biol Sci 408: 256-260.

Lima MDR, Barros Jr UO, Barbosa MAM, Segura FR, Silva FF, Batista BL, Lobato AKS (2016) Silicon mitigates oxidative stress and has positive effects in *Eucalyptus platyphylla* under aluminium toxicity. Plant Soil Environ. 62: 164-170.

Liu QP, Wang HS, Zhang ZH, Wu JS, Feng Y (2009) Divergence in function and expression of the NOD26-like intrinsic proteins in plants. BMC Genom. 10:313.

Liu, Q, Zhu, Z (2010) Functional divergence of the NIP III subgroup proteins involved altered selective constraints and positive selection. BMC Plant Biol. 10: 256.

Lux A, Luxová M, Morita S, Abe J, Inanaga S (1999) Endodermal silicification in developing seminal roots of lowland and upland cultivars of rice (*Oryza sativa* L.). Can J Bot 77: 955-960.

Ma JF (2004) Role of silicon in enhancing the resistance of plants to biotic and abiotic stresses. Soil Sci Plant Nutr 50: 11-18.

Ma JF, Higashitani A, Sato K, Takeda K (2003) Genotypic variation in silicon concentration of barley grain. Plant Soil 249: 383-387.

Ma JF, Mitani N, Nagao S, Konishi S, Tamai K, Iwashita T, Yano M. (2004) Characterization of the silicon uptake and molecular mapping of the silicon transporter gene in rice. Plant Physiology 136: 3284-3289.

Ma JF, Sasaki M, Matsumoto H (1997) Al-induced inhibition of root elongation in corn, *Zea mays* L. is overcome by Si addition. Plant Soil 188: 171-176.

Ma JF, Takahashi E (1990a) Effect of silicon on the growth and phosphorus uptake of rice. Plant Soil 126: 115-119.

Ma JF, Takahashi E (1990b) The effect of silicic acid on rice in P-deficient soil. Plant Soil 126: 121-125.

Ma JF, Takahashi E (1991) Effect of silicate on phosphate availability for rice in P-deficient soil. Plant Soil 133: 151-155.

Ma JF, Takahashi E (2002) Soil, fertilizer, and plant silicon research in Japan. Elsevier Science BV, Amsterdam.

Ma JF, Tamai K, Ichii M, Wu K (2002) A rice mutant defective in active Si uptake. Plant Physiol 130: 2111-2117.

Ma JF, Tamai K, Yamaji N, Mitani N, Konishi S, Katsuhara M, Ishiguro M, Murata Y, Yano M (2006) A silicon transporter in rice. Nature 440: 688-691.

Ma JF, Yamaji N (2006) Silicon uptake and accumulation in higher plants. Trends Plant Sci 11: 392-397.

Ma JF, Yamaji N (2015) A cooperative system of silicon transport in plants. Trends Plant Sci. 20: 435-442.

Ma JF, Yamaji N, Mitani N (2011) Transport of silicon from roots to panicles in plants. Proc Jpn Acad Ser B 87: 377-385.

Ma JF, Yamaji N, Mitani N, Tamai K, Konishi S, Fujiwara T, Katsuhara M, Yano M (2007b) An efflux transporter of silicon in rice. Nature 448: 209-212.

Ma JF, Yamaji N, Tamai K., Mitani N (2007a) Genotypic difference in silicon uptake and expression of silicon transporter genes in rice. Plant Physiol 145: 919-924.

Ma QF, Rengel Z, Kuo J (2002) Aluminium toxicity in rye (*Secale cereale*): Root growth and dynamics of cytoplasmic Ca^{2+} in intact root tips. Ann Bot. 89: 241-244.

Marron AO, Alston MJ, Heavens D, Akam M, Caccamo M, Holland PW, Walker G (2013) A family of diatom-like silicon transporters in the siliceous loricate choanoflagellates. Proc R Soc B 280: 20122543.

Marschner H (1997) Mineral Nutrition of Higher Plants, 2nd edition, Academic Press, London.

Matsumoto H (2000) Cell biology of aluminum toxicity and tolerance in higher plants. Int. Rev. Cytol. 200: 1-46.

Maurel C, Verdoucq L, Luu DT, Santoni V (2008) Plant aquaporins: membrane channels with multiple integrated functions. Annu. Rev. Plant Biol. 59: 595-624.

Maxwell DP, Wang Y, McIntosh L (1999) The alternative oxidase lowers mitochondrial reactive oxygen production in plant cultures. Proc. Natl. Acad. Sci. USA 96: 8271-8276.

Mengel K, Kirkby EA (2001) Principles of Plant Nutrition. Kluwer Academic Publishers, Dordrecht.

Milla MAR, Butler ED, Huete AR, Wilson CF, Anderson O, Gustafson JP (2002) Expressed sequence tag-based gene expression analysis under aluminum stress in rye. Plant Physiol. 130: 1706-1716.

Mitani N, Chiba Y, Yamaji N, Ma JF (2009b) Identification and characterization of maize and barley Lsi2-like silicon efflux transporters reveals a distinct silicon uptake system from that in rice. Plant Cell 21: 2133-2142.

Mitani N, Ma JF (2005) Uptake system of silicon in different plant species. J Exp Bot 56: 1255-1261.

Mitani N, Yamaji N, Ago Y, Iwasaki K, Ma JF **(**2011a) Isolation and functional characterization of an influx silicon transporter in two pumpkin cultivars contrasting in silicon accumulation. Plant J 66: 231-240.

Mitani N, Yamaji N, Ma JF (2008) Characterization of substrate specificity of a rice silicon transporter, Lsi1. Pflügers Archiv 456: 679-686.

Mitani N, Yamaji N, Ma JF (2009a) Identification of maize silicon influx transporters. Plant Cell Physiol 50: 5-12.

Mitani N, Yamaji N, Ma JF (2011b) Silicon efflux transporters isolated from two pumpkin cultivars contrasting in Si uptake. Plant Signal Behav 6: 991-994.

Mitani N, Yamaji N, Ma JF (2016) High silicon accumulation in the shoot is required for down-regulating the expression of Si transporter genes in rice. Plant Cell Physiol. 57: 2510-2518.

Mitani N, Yamaji N, Zhao FJ, Ma JF (2011c) The aromatic/arginine selectivity filter of NIP aquaporins plays a critical role in substrate selectivity for silicon, boron, and arsenic. J Exp Bot 62: 4391-4398.

Montpetit J, Vivancos J, Mitani N, Yamaji N, Rémus-Borel W, Belzile F, Ma JF, Bélanger RR (2012) Cloning, functional characterization and heterologous expression of TaLsi1, a wheat silicon transporter gene. Plant Mol Bio 79: 35-46.

Mora ML, Alfaro MA, Jarvis SC, Demanet R, Cartes P (2006) Soil aluminium availability in Andisols of Southern Chile and its effect on forage production and animal metabolism. Soil Use Manage 22: 95-101.

Mora ML, Baeza G, Pizarro C, Demanet R (1999) Effect of calcitic and dolomitic lime on physicochemical properties of a Chilean Andisol. Commun Soil Sci Plan 30: 427-439.

Mora ML, Rosas A, Ribera A, Rengel Z (2009) Differential tolerance to Mn toxicity in perennial ryegrass genotypes: involvement of antioxidative enzymes and root exudation of carboxylates. Plant Soil 320: 79-89.

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22: 867-880.

Nanayakkara UN, Uddin W, Datnoff L (2008) Application of silicon sources increases silicon accumulation in perennial ryegrass turf on two soil types. Plant Soil 303: 83-94.

Neumann D, De Figueiredo C (2002) A novel mechanism of silicon uptake. Protoplasma 220: 59-67.

Nikolic M, Nikolic N, Liang Y, Kirkby EA, Römheld V (2007) Germanium-68 as an adequate tracer for silicon transport in plants. Characterization of silicon uptake in different crop species. Plant Physiol 143: 495-503.

Noctor G, Foyer CH (1998) A re-evaluation of the ATP: NADPH budget during C3 photosynthesis. A contribution from nitrate assimilation and its associated respiratory activity? J. Exp. Bot. 49: 1895-1908.

Obihara CH, Russell EW (1972) Specific adsorption of silicate and phosphate by soils. J Soil Sci 23: 105-117.

Okuno T, Furusawa, I (1977) A simple method for the isolation of intact mesophyll protoplasts from cereal plants. Plant Cell Physiol 18: 1357-1362.

Ouellette S, Goyette MH, Labbé C, Laur J, Gaudreau L, Gosselin A, Dorais M, Deshmukh R, Bélanger R (2017) Silicon transporters and effects of silicon amendments in strawberry under high tunnel and field conditions. Front Plant Sci. 8: 949.

Owino-Gerroh C, Gascho GJ (2004) Effect of silicon on low pH soil phosphorus sorption and on uptake and growth of maize. Commun Soil Sci Plan 35: 2369-2378.

Panda SK, Matsumoto H (2010) Changes in antioxidant gene expression and induction of oxidative stress in pea (*Pisum sativum L.)* under Al stress. Biometals 23: 753-762.

Pardo MT, Guadalix ME (1990) Phosphate sorption in allophanic soils and release of sulphate, silicate and hydroxyl. J Soil Sci 41:607-412.

Parfitt RL (1978) Anion adsorption by soils and soil materials. Adv Agron 30: 1-50.

Pavlovic J, Samardzic J, Maksimović V, Timotijevic G, Stevic N, Laursen K H, Hansen TH, Husted S, Schjoerring JK, Liang Y, Nikolic M (2013) Silicon alleviates iron

deficiency in cucumber by promoting mobilization of iron in the root apoplast. New Phytologist, 198: 1096-1107.

Peng CL, Ou ZY, Liu N, Lin GZ (2005) Response of flag leaves of super high-yielding rice Pei'ai 64S/E32 and liangyoupeijiu to high temperature. Rice Sci. 12: 179-186.

Pinhero RG, Rao MV, Paliyath G, Murr DP, Fletcher RA (1997) Changes in activities of antioxidant enzymes and their relationship to genetic and paclobutrazol-induced chilling tolerance of maize seedlings. Plant Physiol. 114: 695-704.

Pontigo S, Godoy K, Jimenez H, Gutierrez-Moraga A, Mora M, Cartes P (2017) Silicon-mediated alleviation of aluminum toxicity by modulation of Al/Si uptake and antioxidant performance in ryegrass plants. Front Plant Sci. 8: 642.

Pontigo S, Ribera A, Gianfreda L, Mora ML, Nikolic M, Cartes P (2015) Silicon in vascular plants: uptake, transport and its influence on mineral stress under acidic conditions. Planta 242: 23-37.

Poschenrieder C, Gunse B, Corrales I, Barcelo J (2008) A glance into aluminum toxicity and resistance in plants. Sci. Total Environ. 400: 356-368.

Prabagar S, Hodson MJ, Evans DE (2011) Silicon amelioration of aluminium toxicity and cell death in suspension cultures of Norway spruce (*Picea abies* L. Karst.). Environ Exp Bot 70: 266-276.

Prychid CJ, Rudall PJ, Gregory M (2004) Systematics and biology of silica bodies in monocotyledons. Bot Rev 69: 377-440.

Rains DW, Epstein E, Zasoski RJ, Aslam M (2006) Active silicon uptake by wheat. Plant Soil 280: 223-228.

Raven JA (2001) Silicon transport at the cell and tissue level. In: Datnoff LE, Snyder GH, Korndorfer GH (eds) Silicon in Agriculture. Elsevier Science BV, Amsterdam, pp 41-55.

Raven JA (2003) Cycling silicon- the role of accumulation in plants. New Phytol 158: 419-430.

Rengel Z (2000) Uptake and transport of manganese in plants. In: Sigel A, Sigel H (eds) Metal Ions in Biological Systems. Marcel Dekker, New York, pp 57-87.

Ribera A, Inostroza-Blancheteau C, Cartes P, Rengel Z, Mora ML (2013) Early induction of Fe-SOD gene expression is involved in tolerance to Mn toxicity in perennial ryegrass. Plant Physiol Bioch 73: 77-82.

Ribera-Fonseca A, Rumpel C, Mora ML., Nikolic M, Cartes P (2018) Sodium silicate and calcium silicate differentially affect silicon and aluminium uptake, antioxidant performance and phenolics metabolism of ryegrass in an acid Andisol. Crop Pasture Sci. 69: 205-215.

Rios JJ, Martínez-Ballesta, MC, Ruiz JM, Blasco B, Carvajal M (2017) Silicon-mediated improvement in plant salinity tolerance: the role of aquaporins. Front Plant Sci. 8: 948.

Robson AD, Pitman MG (1983) Interactions between nutrients in higher plants. In: Läuchli A, Bieleski RL (eds) Encyclopedia of Plant Physiology. Springer-Verlag, Berlin 15: 147-180.

Rogalla H, Römheld V (2002) Role of leaf apoplast in silicon-mediated manganese tolerance of *Cucumis sativus* L. Plant Cell Environ 25: 549-555.

Romero A, Munévar F, Cayón G (2011) Silicon and plant diseases. A Review. Agron Colomb 29: 473-480.

Ryan PR, Delhaize E (2010) The convergent evolution of aluminium resistance in plants exploits a convenient currency. Funct. Plant Biol 37: 275-284.

Ryan PR, Tyerman SD, Sasaki T, Furuichi T, Yamamoto Y, Zhang WH, Delhaizeet E (2011) The identification of aluminium-resistance genes provides opportunities for enhancing crop production on acid soils. J Exp Bot., 62: 9-20.

Ryden JC, McLauchlin JR, Syers JK (1977) Mechanisms of phosphate sorption by soils and hydrous ferric oxide gel. J Soil Sci 28: 72-92.

Sasaki T, Yamamoto Y, Ezaki B, Kasuara M, Ahn SJ, Ryan P, Delhaize E, Matsumoto H (2004) A wheat gene encoding an aluminum-activated malate transporter. Plant J. 37: 645-653.

Schachtman DP, Reid RJ, Ayling SM (1998) Phosphorus uptake by plants: from soil to cell. Plant Physiol 116: 447-453.

Schröder HC, Perović-Ottstadt S, Wiens M, Batel R, Müller IM, Müller WEG (2004) Silica transport in the demosponge *Suberites domuncula*: fluorescence emission analysis using the PDMPO probe and cloning of a potential transporter. Cell Tissue Res 316: 271-280.

Shaff JE, Schultz BA, Craft EJ, Clark RT, Kochian LV (2010) GEOCHEM-EZ: a chemical speciation program with greater power and flexibility. Plant Soil 330: 207-214.

Shahnaz G, Shekoofeh E, Kourosh D, Moohamadbagher B (2011) Interactive effects of silicon and aluminum on the malondialdehyde (MDA), proline, protein and phenolic compounds in *Borago officinalis* L. J Med Plants Res 5: 5818-5827.

Shen X, Xiao X, Dong Z, Chen Y (2014) Silicon effects on antioxidative enzymes and lipid peroxidation in leaves and roots of peanut under aluminum stress. Acta Physiol. Plant. 36: 3063-3069.

Shetty R, Frette X, Jensen B, Shetty NP, Jensen JD, Jørgensen HJL, Newman MA, Christensen LP (2011) Silicon-induced changes in antifungal phenolic acids, flavonoids, and key phenylpropanoid pathway genes during the interaction between miniature roses and the biotrophic pathogen Podosphaera pannosa. Plant Physiol. 157: 2194-2205.

Shi Q, Bao Z, Zhu Z, He Y, Qian Q, Yu J (2005) Silicon mediated alleviation of Mn toxicity in *Cucumis sativus* in relation to activities of superoxide dismutase and ascorbate peroxidase. Phytochemistry 66: 1551-1559.

Singh S, Tripathi DK, Singh S, Sharma S, Dubey NK, Chauhan DK, Vaculík M (2017) Toxicity of aluminium on various levels of plant cells and organism: a review. Environ. Exp. Bot. 137: 177-193.

Singh VP, Tripathi DK, Kumar D, Chauhan DK (2011) Influence of exogenous silicon addition on aluminium tolerance in rice seedlings. Biol Trace Elem Res 144: 1260-1274.

Sivaguru M, Ezaki B, He ZH, Tong H, Osawa H, Baluška F, Volkmann D, Matsumoto H (2003) Aluminum-induced gene expression and protein localization of a cell wall-associated receptor kinase in Arabidopsis. Plant Physiol. 132: 2256-2266.

Slinkard K, Singleton VA (1977) Total phenol analysis: automation and comparison with manual methods. Am. J. Enol. Vitic. 28: 29-55.

Snyder GH (1991) Developed of a silicon test for Histosol-grown rice. Belle Glade EREC Research Report EV-1991-2. University of Florida, Belle Glade, USA, pp 29-39.

Song A, Xue G, Cui P, Fan F, Liu H, Yin C, Sun W, Liang Y (2016) The role of silicon in enhancing resistance to bacterial blight of hydroponic-and soil-cultured rice. Sci Rep. 6: 24640.

Sui H, Han BG, Lee JK, Walian P, Jap BK (2001) Structural basis of water-specific transport through the AQP1 water channel. Nature 414: 872-878.

Takahashi E, Ma JF, Miyake Y (1990) The possibility of silicon as an essential element for higher plants. J Agr Food Chem 2: 99-122.

Takahashi MA, Asada K (1983) Superoxide anion permeability of phospholipid membranes and chloroplast thylakoids. Arch. Biochem. Biophys. 226: 558-566.

Tamai K, Ma JF (2003) Characterization of silicon uptake by rice roots, New Phytol 158: 431-436.

Taylor GJ, Foy CD (1985) Mechanisms of aluminum tolerance in Triticum aestivum L. (wheat). II. Differential pH Induced by Spring Cultivars in Nutrient Solutions. Am. J. Bot. 72: 695-701.

Tripathi DK, Bashri G, Singh S, Singh S, Ahmad P, Prassad SM, et al. (2016). "Efficacy of silicon against aluminum toxicity in plants: An overview," in Silicon in Plants: Advances and Future Prospects, eds. D. K.Tripathi, V. P. Singh, P. Ahmad,

D.K. Chauhan, S. M Prasad (Boca Raton, FL: CRC Press), 355-366. doi:10.1201/9781315369310-20

Tripathi DK, Singh VP, Prasad SM, Chauhan DK, Dubey NK, Rai AK (2015) Silicon-mediated alleviation of Cr (VI) toxicity in wheat seedlings as evidenced by chlorophyll florescence, laser induced breakdown spectroscopy and anatomical changes. Ecotoxicol. Environ. Saf. 113:133-144.

Vaculík M, Landberg T, Greger M, Luxová M, Stoláriková M, Lux A (2012) Silicon modifies root anatomy, and uptake and subcellular distribution of cadmium in young maize plants. Ann Bot. 110: 433-443.

Van der Vorm PDJ (1980) Uptake of Si by five plant species, as influenced by variation in Si-supply. Plant Soil 56: 153-156.

Vance CP, Uhde-Stone C, Allan DL (2003) Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. New Phytol 157: 423-447.

Vatansever R, Ozyigit II, Filiz E, Gozukara N (2017) Genome-wide exploration of silicon (Si) transporter genes, Lsi1 and Lsi2 in plants; insights into Si-accumulation status/capacity of plants. BioMetals, 30: 185-200.

Vega I, Nikolic M, Godoy K, Mora ML, Cartes P. Silicon improves the production of high antioxidant- or structural- phenolic compounds in barley cultivars under aluminium stress. Submitted to Crop Pasture Sci.

Von Uexküll HR, Mutert E (1995) "Global extent, development and economic impact of acid soils", in Plant-soil interactions at low pH: Principles and management. eds. R.A., Grundon, N.J., Rayment, G.E., Probert, M.E. Springer Netherlands. 5-19.

Vulavala VK, Elbaum R, Yermiyahu U, Fogelman E, Kumar A, Ginzberg I (2016) Silicon fertilization of potato: expression of putative transporters and tuber skin quality. Planta 243: 217-229.

Wallace IS, Roberts DM (2004) Homology modeling of representative subfamilies of Arabidopsis major intrinsic proteins. Classification based on the aromatic/arginine selectivity filter. Plant Physiol 135: 1059-1068.

Wang HS, Yu C, Fan PP, Bao BF, Li T, Zhu ZJ (2015) Identification of two cucumber putative silicon transporter genes in *Cucumis sativus*. Plant Growth Regul. 34: 332-338.

Wang Y, Stass A, Horst WJ (2004) Apoplastic binding of aluminum is involved in silicon-induced amelioration of aluminum toxicity in maize. Plant Physiol 136: 3762-3770.

Wickramasinghe DB, Rowell DL (2006) The release of silicon from amorphous silica and rice straw in Sri Lankan soils. Biol Fert Soils 42: 231-240.

Wu B, Beitz E (2007) Aquaporins with selectivity for unconventional permeants. Cell Mol Life Sci 64: 2413-2421.

Wu JW, Shi Y, Zhu YX, Wang YC, Gong HJ (2013) Mechanisms of enhanced heavy metal tolerance in plants by silicon: A review. Pedosphere 23: 815-825.

Yamaji N, Chiba Y, Mitani N, Ma JF (2012) Functional characterization of a silicon transporter gene implicated in silicon distribution in barley. Plant Physiol 160: 1491-1497.

Yamaji N, Ma JF (2007) Spatial distribution and temporal variation of the rice silicon transporter Lsi1. Plant Physiol 143:1306-1313.

Yamaji N, Ma JF (2009) A transporter at the node responsible for intervascular transfer of silicon in rice. Plant Cell 21: 2878-2883.

Yamaji N, Ma JF (2011) Further characterization of a rice silicon efflux transporter, Lsi2. Soil Sci. Plant Nutr. 57: 259-264.

Yamaji N, Mitani N, Ma JF (2008) A transporter regulating silicon distribution in rice shoots. Plant Cell 20: 1381-1389.

Yamaji N, Sakurai G, Mitani-Ueno N, Ma JF (2015) Orchestration of three transporters and distinct vascular structures in node for intervascular transfer of silicon in rice. Proc .Natl. Acad. Sci. U S A, 112: 11401-11406.

Yamaji N, Sasaki A, Xia J, Yokosho K, Mitani N, Ma JF (2013) Role of node-located transporters in mineral distribution in rice. In: Proceeding of XVII International Plant Nutrition Colloquium: Plant nutrition for nutrient and food security, pp 129-130.

Yamamoto Y, Kobayashi Y, Devi SR, Rikiishi S, Matsumoto H (2002) Aluminum toxicity is associated with mitochondrial dysfunction and the production of reactive oxygen species in plant cells. Plant Physiol. 128: 63-72.

Yamamoto Y, Kobayashi Y, Devi SR, Rikiishi S, Matsumoto H (2003). Oxidative stress triggered by aluminum in plant roots. Plant Soil 255: 239-243.

Yamamoto Y, Kobayashi Y, Matsumoto H (2001) Lipid proxidation is an early symptom triggered by aluminium, but not the primary cause of elongation inhibition in pea roots. Plant Physiol 125: 199-208.

Yan GC, Nikolic M., Ye MJ, Xiao ZX, Liang YC (2018) Silicon acquisition and accumulation in plant and its significance for agriculture. J. Integr. Agr. 17:2138-2150.

Zhang G, Cui Y, Ding X, Dai Q (2013) Stimulation of phenolic metabolism by silicon contributes to rice resistance to sheath blight. J. Plant Nutr. Soil Sci. 176: 118-124.

Zhu ZJ, Wei GQ, Li T, Qian QQ, Yu JQ (2004) Silicon alleviates salt stress and increases antioxidant enzymes activity in leaves of salt-stressed cucumber (*Curcumis sativa* L.). Plant Sci 167: 527-533.

Zia-ur-Rehman M, Khalid H, Akmal F, Waqar M, Rizwan M, Qayyum F, Nadeeme M (2016) Silicon and Antioxidant Defense System against Abiotic Stresses in Plants: An Overview, in Silicon in Plants: Advances and Future Prospects, eds. D. K.Tripathi, V. P. Singh, P. Ahmad, D.K. Chauhan, S. M Prasad (Boca Raton, FL: CRC Press), 321-342.

Zsoldos F, Vashegyi A, Pecsvaradi A, Bona L (2003) Influence of silicon on aluminium toxicity in common and durum wheats. Agronomie 23: 349-354.

Annex 1

Supporting information about molecular cloning of Si transporters from ryegrass

Lolium perenne silicon transporter (Lsi2) mRNA, partial cds

GenBank: KY315995.1

FASTA Graphics

Go to:

```
LOCUS       KY315995                951 bp    mRNA    linear   PLN 23-MAY-2017
DEFINITION  Lolium perenne silicon transporter (Lsi2) mRNA, partial cds.
ACCESSION   KY315995
VERSION     KY315995.1
KEYWORDS    .
SOURCE      Lolium perenne
  ORGANISM  Lolium perenne
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BOP
            clade; Pooideae; Poodae; Poeae; Poeae Chloroplast Group 2 (Poeae
            type); Loliinae; Lolium.
REFERENCE   1  (bases 1 to 951)
  AUTHORS   Pontigo,S., Godoy,K., Jimenez,H., Gutierrez-Moraga,A., Mora,M.L.
            and Cartes,P.
  TITLE     Silicon-Mediated Alleviation of Aluminum Toxicity by Modulation of
            Al/Si Uptake and Antioxidant Performance in Ryegrass Plants
  JOURNAL   Front Plant Sci 8, 642 (2017)
   PUBMED   28487719
  REMARK    Publication Status: Online-Only
REFERENCE   2  (bases 1 to 951)
  AUTHORS   Pontigo,S. and Cartes,P.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-DEC-2016) Departamento de Ciencias Quimicas y
            Recursos Naturales, Universidad de La Frontera, Avenida Francisco
            Salazar 01145, Temuco, Temuco 4811230, Chile
COMMENT     ##Assembly-Data-START##
            Assembly Method       :: Geneious v. 9.0.2
            Sequencing Technology :: Illumina
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..951
                     /organism="Lolium perenne"
                     /mol_type="mRNA"
                     /db_xref="taxon:4522"
     gene            <1..>951
                     /gene="Lsi2"
     CDS             <1..>951
                     /gene="Lsi2"
                     /note="LpLsi2"
                     /codon_start=1
                     /product="silicon transporter"
                     /protein_id="ARR96075.1"
                          /translation="YLKNAGMFKHLGTLLAWKSQGGRDLLSRVCVVTALASALFTNDT
                     CCVVLTEFVLELAAERNLPAKPFLLALASSANIGSSATPIGNPQNLVIAFNSKISFPR
                     FLLGILPAMLAGMAVNMVMLLCMYWKDLEGVSPDAVDAGKQMHAVEEGRSPSIASLKS
                     PLKSPHTLNPLNGSAINDDGESMMEENLSTKHPWFMQCTEHHRKLFLKSFAYIVTVGM
                     VVAYMVGLNMSWTAITTAIALVVVDFRDAEPCLVKVSYSLLVFFSGMFITVSGFNKTG
                     LPSAIWNFMAPYSKVNSAGGISVLSIIILLLSNFASNVPTV"
ORIGIN
        1 tacctcaaga acgccggcat gttcaagcac ctgggcaccc tgctggcgtg gaagagccag
       61 ggcggccggg acctgctcag ccgcgtctgc gtcgtcacgg ccctcgcttc ggcgctcttc
      121 acaaacgaca cctgctgcgt cgtgctcacc gagttcgtcc tcgagctcgc cgctgagcgc
      181 aacctcccgg ccaagccttt cctcctggcc ctcgcctcca gcgccaacat cggctccagc
      241 gccaccccca tcggcaaccc gcagaacctc gtcatcgcgt tcaacagcaa gatctccttc
      301 ccgaggttcc tgctcggcat cctgccggcc atgctcgccg gtatggccgt caacatggtc
      361 atgctcctct gcatgtactg gaaggacctc gagggcgtgt cccccgacgc tgtcgacgcc
      421 ggcaagcaga tgcacgccgt cgaggaaggc cggtcaccct caatcgcgtc cctcaagagc
      481 ccgctcaaga gcccgcacac tctcaaccct ctcaacggca gcgccatcaa cgacgatggt
      541 gagtcgatga tggaggagaa cctctcgacg aagcacccgt ggttcatgca gtgcacggag
      601 caccaccgga agctcttcct caagagcttc gcctacatcg tcaccgtcgg catggtggtc
      661 gcctacatgg tcgggctcaa catgtcgtgg accgccatca ccactgccat cgcgctcgtc
      721 gtcgtcgact tccgggacgc cgagccgtgc ctcgtcaagg tgtcctactc gctgctcgtc
      781 ttcttctccg ggatgttcat cacggtcagc gggttcaaca agaccggcct gcccagcgcc
      841 atctggaact tcatggcgcc ctactccaag gtcaacagcg ccggcggcat ctccgtcctc
      901 tccatcatca tcctcctcct ctccaacttc gcctccaacg taccaacagt g
//
```

Lolium perenne silicon transporter (Lsi1) mRNA, complete cds

GenBank: KY315994.1

FASTA Graphics

Go to:

```
LOCUS       KY315994                888 bp    mRNA    linear   PLN 23-MAY-2017
DEFINITION  Lolium perenne silicon transporter (Lsi1) mRNA, complete cds.
ACCESSION   KY315994
VERSION     KY315994.1
KEYWORDS    .
SOURCE      Lolium perenne
  ORGANISM  Lolium perenne
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BOP
            clade; Pooideae; Poodae; Poeae; Poeae Chloroplast Group 2 (Poeae
            type); Loliinae; Lolium.
REFERENCE   1  (bases 1 to 888)
  AUTHORS   Pontigo,S., Godoy,K., Jimenez,H., Gutierrez-Moraga,A., Mora,M.L.
            and Cartes,P.
  TITLE     Silicon-Mediated Alleviation of Aluminum Toxicity by Modulation of
            Al/Si Uptake and Antioxidant Performance in Ryegrass Plants
  JOURNAL   Front Plant Sci 8, 642 (2017)
   PUBMED   28487719
  REMARK    Publication Status: Online-Only
REFERENCE   2  (bases 1 to 888)
  AUTHORS   Pontigo,S. and Cartes,P.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-DEC-2016) Departamento de Ciencias Quimicas y
            Recursos Naturales, Universidad de La Frontera, Avenida Francisco
            Salazar 01145, Temuco, Temuco 4811230, Chile
COMMENT     ##Assembly-Data-START##
            Assembly Method       :: Geneious v. 9.0.2
            Sequencing Technology :: Illumina
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..888
                     /organism="Lolium perenne"
                     /mol_type="mRNA"
                     /db_xref="taxon:4522"
     gene            1..888
                     /gene="Lsi1"
     CDS             1..888
                     /gene="Lsi1"
                     /note="LpLsi1"
                     /codon_start=1
                     /product="silicon transporter"
                     /protein_id="ARR96074.1"
                     /translation="MSTNSRSNSRANFSNEIHDIGAARSTTPSMYYNERSIADYFPPH
                     LLKKMVSEVVSTFLLVFMTCGAAAISASDPTRISQLGQSVAGGLIVTVMIYSVGHISG
                     AHMNPAVTLSFAVFRHFPWIQVPFYWASQFTGAICASFVLKAVLHPITEIGTTVPHGP
                     HWHSLVIEVVVTFNMMFVTLAVATDSRAVGELAGLAVGSSVCITSIFAGAVSGGSMNP
                     ARTLGPALASNHFTGLWIYFLGPVLGTLSGAWTYTFIRFEDPPKDAPQKLSSFKLRRL
                     QSQSVAATDLEDDLEHIPI"
ORIGIN
        1 atgtcgacca actcgagatc gaactccagg gcgaacttct cgaacgagat ccacgacatc
       61 ggcgcggcgc gctccaccac gcccagcatg tactacaacg agaggtctat cgcggactac
      121 ttcccgcccc acctcctgaa gaagatggtg tcggaagtgg tgtcgacgtt cctgctggtg
      181 ttcatgacct gcggggcggc ggcgatcagc gcaagtgacc ccacgcgcat atcacagctg
      241 ggacagtcgg tagccggcgg tctcatcgtg accgtgatga tctactccgt cggacacatc
      301 tccggtgcgc acatgaaccc tgccgtcacg ctctccttcg ccgtcttccg gcatttccca
      361 tggattcagg tcccgttcta ctgggcgtcg cagttcaccg gcgcgatctg cgcgtccttc
      421 gtgctcaagg cggtgctcca ccccatcacc gagatcggca ccaccgtgcc gcacggcccg
      481 cactggcact ccctcgtcat cgaggttgtc gtcaccttca acatgatgtt cgtcacctc
      541 gctgtcgcaa cggacagtag agcggtgggt gagttggccg ggttagctgt cggatcctct
      601 gtttgcatta cgtccatctt cgcaggggcg gtgtcaggcg gatcgatgaa cccggcgagg
      661 acgctgggcc cggcgctggc cagcaaccac ttcaccggcc tctggatcta cttcctcggt
      721 cccgtcctcg gcacgctctc cggagcctgg acctacacct tcatccgctt cgaggacccg
      781 cccaaggatg cgccgcagaa gctctcctcc ttcaagctcc gccggttgca gagccagtcc
      841 gtcgctgcca ccgacctcga agacgacctc gaacatatcc ccatctga
//
```

Table S1.1. NCBI BLASTp search results using the deduced amino acid sequence of LpLsi1 as query.

QUERY		SUBJECT		SCORE			IDENTITIES		
Name	**Length**	**Gene**	**Accession**	**Bit**	**Raw**	**E-Value**	**Match**	**Total**	**Pct.(%)**
LpLsi1	295	Silicon transporter [*Hordeum vulgare*]	BAH24163.1	530	1366	0.0	262	297	88
		Aquaporin NIP2-1 [Triticum urartu]	ADM47602.1	526	1356	0.0	260	296	88
		Aquaporin NIP2-1 [Zea mays]	NP_001105637.1	502	1293	4E-178	249	298	84
		Aquaporin NIP2-1 [Oryza sativa Japonica Group]	XP_015626173.1	501	1291	8E-178	239	296	81

Table S1.2. NCBI BLASTx search results using the partial cDNA sequence of LpLsi2 as query.

QUERY		SUBJECT		SCORE			IDENTITIES		
Name	**Length**	**Gene**	**Accession**	**Bit**	**Raw**	**E-Value**	**Match**	**Total**	**Pct.(%)**
LpLsi2	951	Silicon transporter [Hordeum vulgare]	BAH84976.1	550	1418	0.0	287	318	90
		Silicon efflux transporter Lsi2 [Brachypodium distachyon]	XP_003559015.1	547	1490	0.0	280	317	88
		Low silicon transporter 2 [Oryza sativa Indica Group]	CCH63884.1	493	1270	2E-171	260	321	81
		Silicon transporter [Zea mays]	NP_001183945.1	486	1251	1E-168	261	317	82

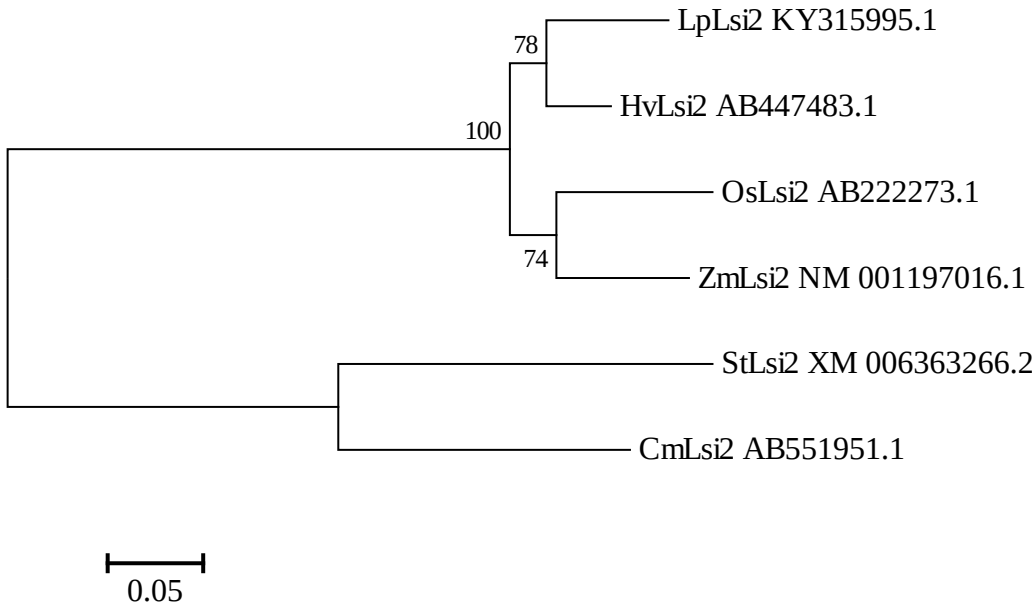

Figure S1.1. Phylogenetic tree of ryegrass Lsi2 and Lsi2-like genes reported in vascular plants. The 0.05 scale shows substitution distance. Nucleotide sequences from ryegrass, barley, rice, maize, potato and pumpkin are indicated with the prefixes Lp, Hv, Os, Zm, St and Cm, respectively. Phylogenetic analysis was performed using MEGA version 6 (Tamura et al., 2013).

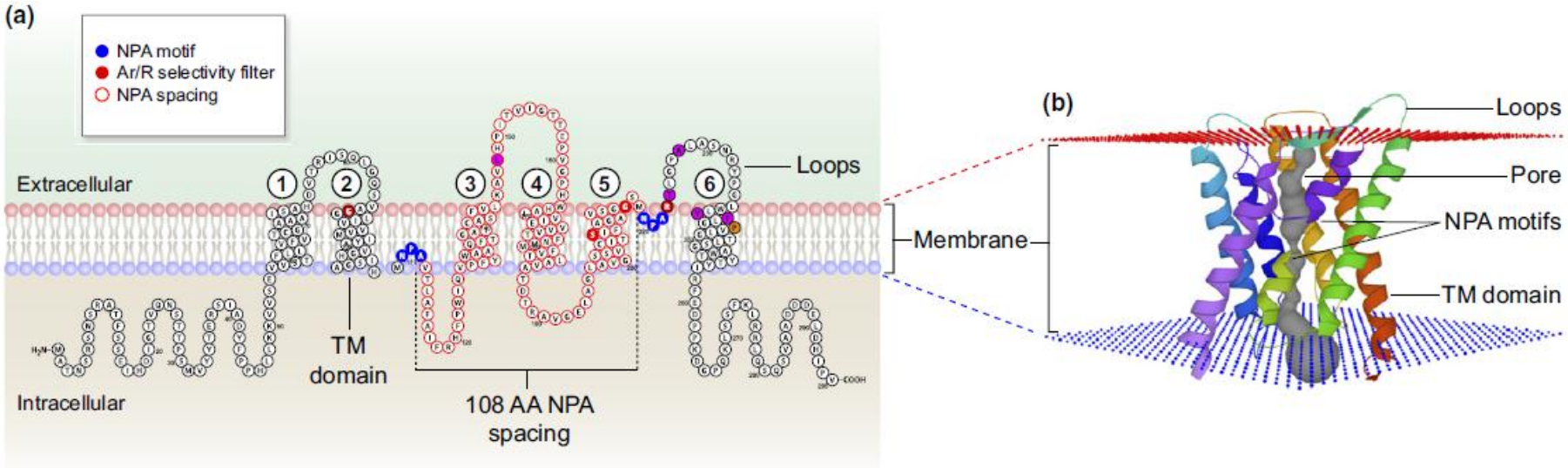

Figure S1.2. Two-dimensional (a) and three-dimensional (b) structure of wheat Si transporter (TaLsi1) showing the known features that influence solute specificity. AA, amino acid; TM domain, transmembrane domain (1-6). Taken from: Coskun et al. (2019), New Phytologist 221: 67-85.

Annex 2

Supporting information about Si uptake kinetics experiments

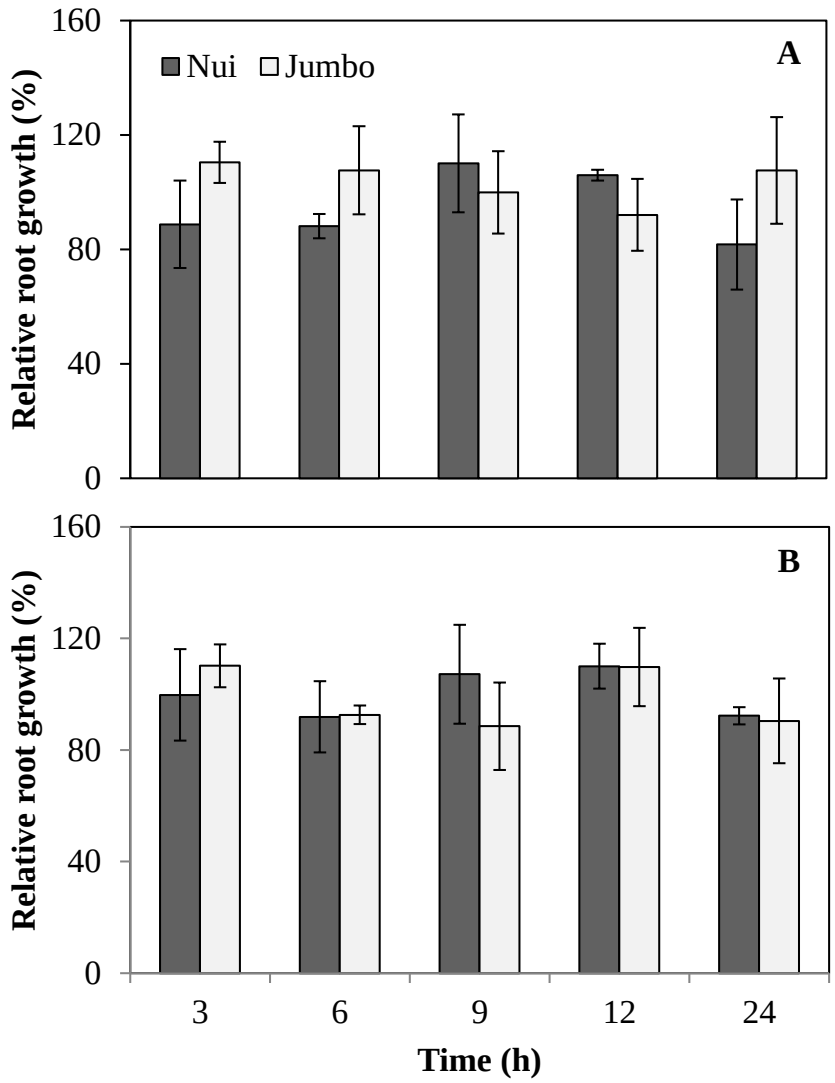

Figure S2.1. Relative root growth (%) of ryegrass cv. Jumbo and cv. Nui grown in nutrient solutions supplemented with or without 0.5 mM (A) or 2 mM (B) Si at different times (h). Data are means of three replicates ± standard error.

Annex 3

Supporting information about Si-Al assays

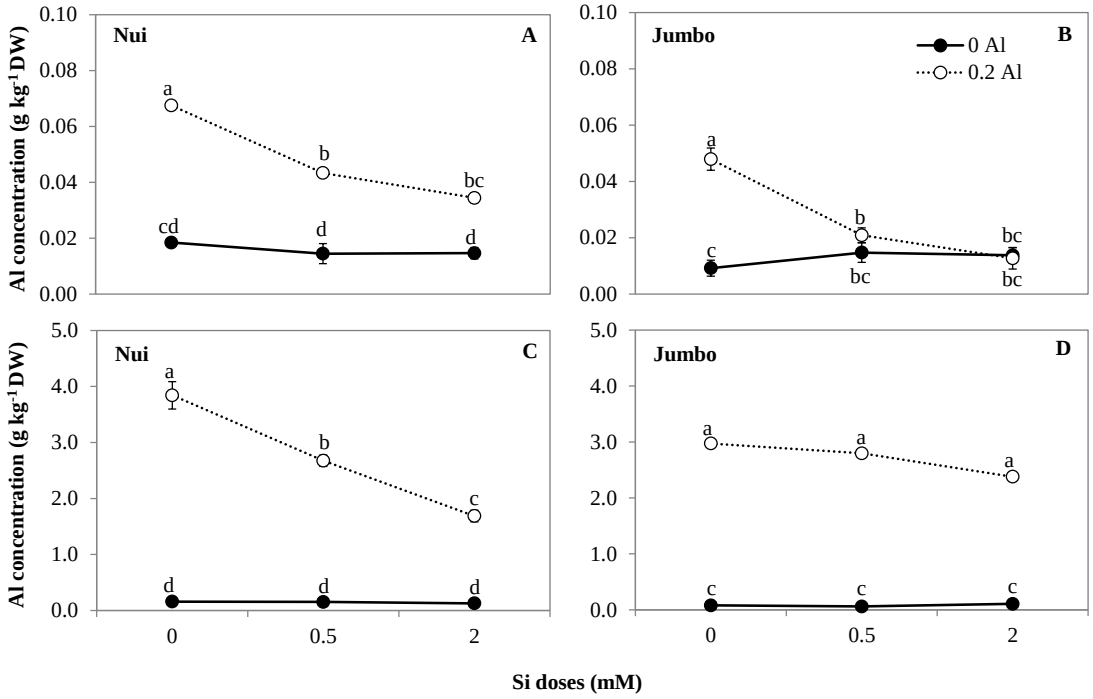

Figure S3.1. Aluminium concentration in shoots (A, B) and roots (C,D) of ryegrass cultivars Nui and Jumbo hydroponically cultivated under Al and Si treatments. Data are means of three replicates ± standard error. Different letters indicate statistically significant differences ($p \leq 0.05$) among treatments.

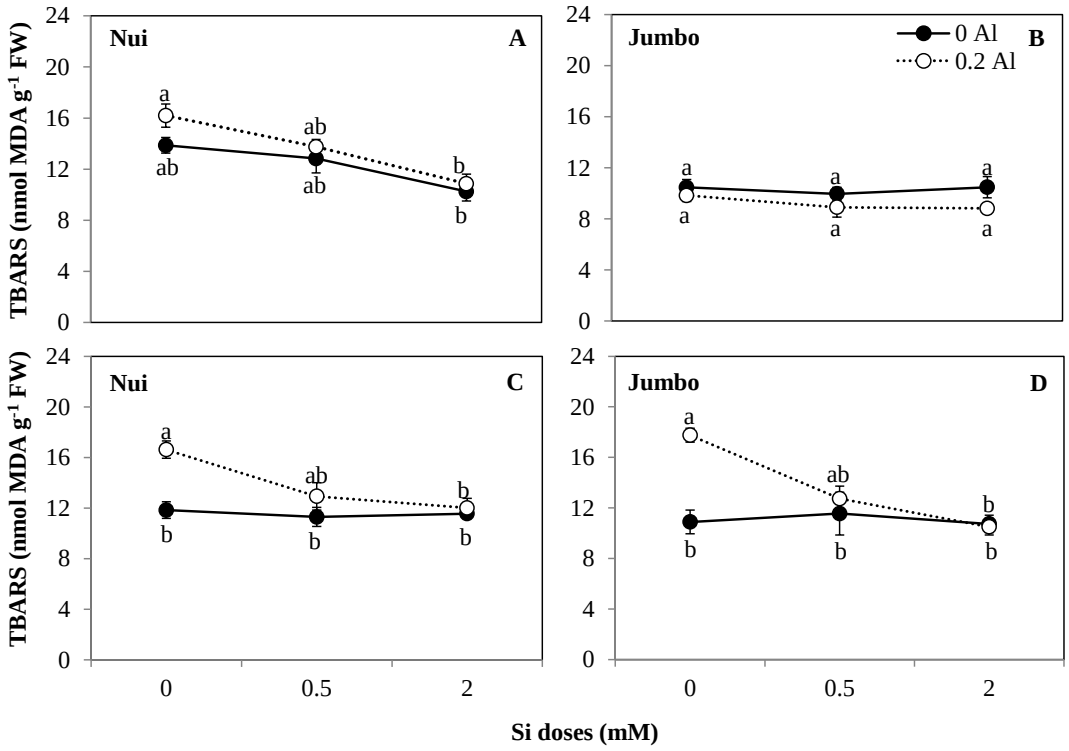

Figure S3.2. Lipid peroxidation in shoots (A, B) and roots (C,D) of ryegrass cultivars Nui and Jumbo hydroponically cultivated under Al and Si treatments. Data are means of three replicates ± standard error. Different letters indicate statistically significant differences ($p \leq 0.05$) among treatments.

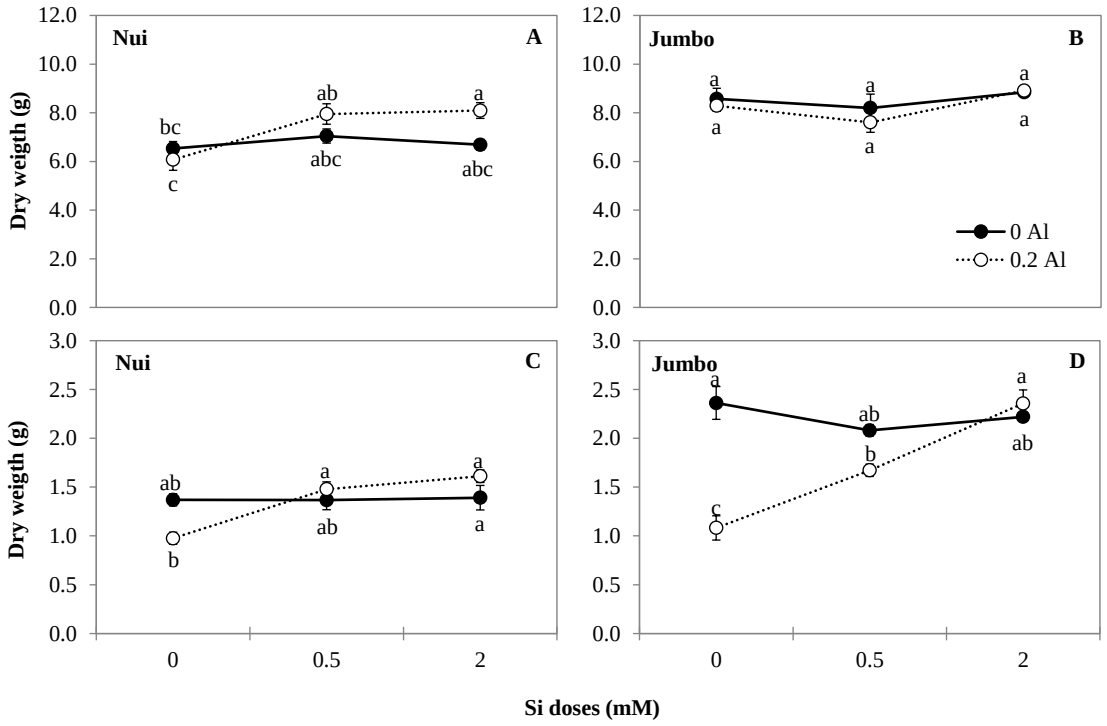

Figure S3.3. Dry matter production in shoots (A, B) and roots (C,D) of ryegrass cultivars Nui and Jumbo hydroponically cultivated under Al and Si treatments. Data are means of three replicates ± standard error. Different letters indicate statistically significant differences ($p \leq 0.05$) among treatments.

Table 1. Pearson's correlation among plant growth, Si and Al concentration and lipid peroxidation in shoots and roots of two ryegrass cultivars hydroponically cultivated under Al and Si treatments.

	Al	Si	Dry weight	TBARS
Shoots		*Nui*		
Al	1.00			
Si	-0.927**	1.00		
Dry weight	-0.849**	0.721*	1.00	
TBARS	0.946**	-0.947**	-0.757*	1.00
Roots				
Al	1.00			
Si	-0.935**	1.00		
Dry weight	-0.876**	0.823**	1.00	
TBARS	0.740*	-0.734*	-0.800**	1.00
Shoots		*Jumbo*		
Al	1.00			
Si	-0.872**	1.00		
Dry weight	-0.156	0.396	1.00	
TBARS	0.377	-0.483	0.285	1.00
Roots				
Al	1.00			
Si	-0.888**	1.00		
Dry weight	-0.901**	0.897**	1.00	
TBARS	0.818*	-0.961*	-0.851**	1.00

Asterisks indicate significance as follows: ** $p \leq 0.01$,* $p \leq 0.05$

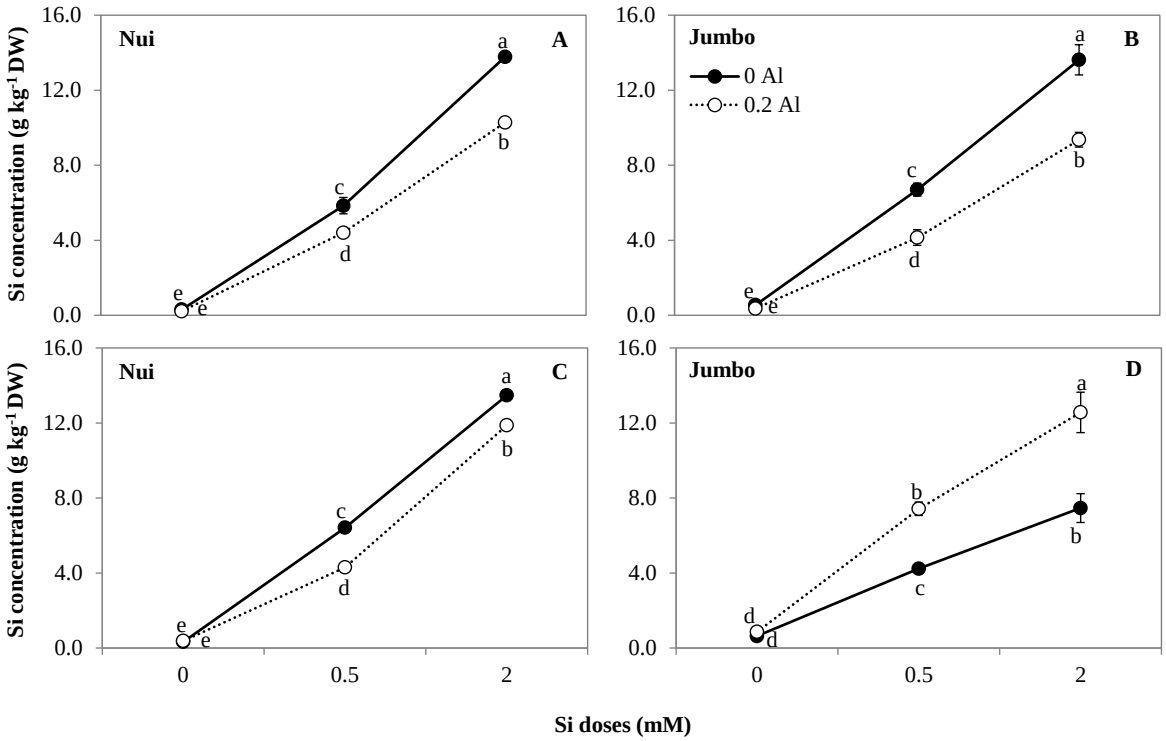

Figure S3.1. Silicon concentration in shoots (A, B) and roots (C,D) of ryegrass cultivars Nui and Jumbo hydroponically cultivated under Al and Si treatments. Data are means of three replicates ± standard error. Different letters indicate statistically significant differences ($p \leq 0.05$) among treatments.

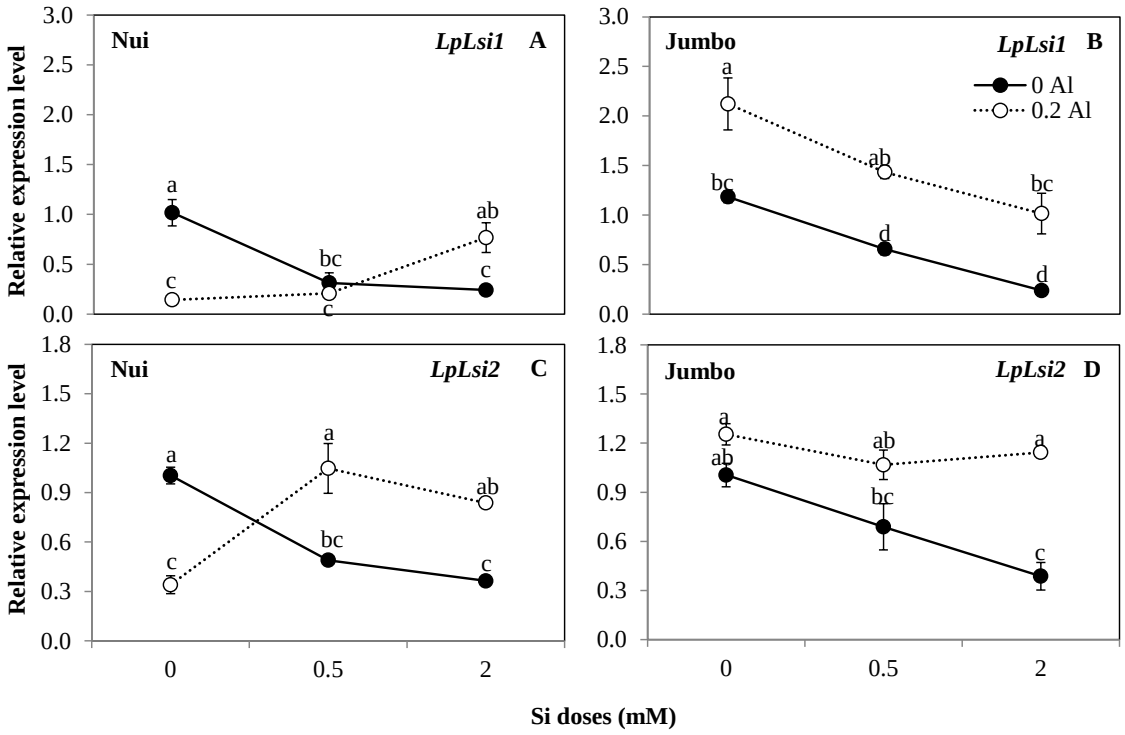

Figure S3.5. Expression analysis of *LpLsi1* (A,B) and *LpLsi2* (C,D) genes in ryegrass cultivars Nui and Jumbo hydroponically cultivated under Al and Si treatments. The expression levels were normalized in relation to *Actin* or *eEF1A(m)* gene expression. Data are means of three replicates ± standard error. Different letters indicate statistically significant differences ($p \leq 0.05$) among treatments.