Silicon effects on arsenic uptake in rice, exodermis development and expression of genes related to suberin and lignin metabolism

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Dipl.-Biol. Alexander Tobias Fleck
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Referent: Prof. Dr. Manfred K. Schenk, Leibniz Universität Hannover

Korreferent: Prof. Dr. Lukas Schreiber, Rheinische Friedrich-Wilhelms-Universität Bonn

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Zusammenfassung

Silizium (Si) ist das zweithäufigste Element in der Erdkruste und ist als Kieselsäure in der Bodenlösung nahezu überall pflanzenverfügbar. Alle Pflanzen enthalten Si und können anhand ihrer Si-Konzentration im Spross als Si-Akkumulatoren, intermediäre Typen oder Nicht-Akkumulatoren bzw. Si-Exkluder klassifiziert werden. Si wird nicht als essentiell für höhere Pflanzen angesehen, ist aber nutzbringend für Pflanzen, indem es das Wachstum fördert und unterschiedlichen abiotischen und biotischen Stress vermindert. Positive Si-Effekte wurden vor allem in Si-Akkumulatoren wie Reis, Weizen oder Rohrzucker beobachtet und sind meistens auf den Spross beschränkt.

In dieser Arbeit wurde untersucht, welche Auswirkung die Si-Ernährung auf die Wurzeln von Reispflanzen hat. Von besonderem Interesse waren hierbei die Ausbildung des Casparischen Streifens und die Transkription von Genen, die mit der Synthese von Suberin und Lignin (den Hauptbestandteilen des Casparischen Streifens) in Beziehung stehen. Zudem wurden die Dosis-Wirkung von Si und die Si-Kinetik nach kurzzeitiger Si-Applikation erforscht. Darüber hinaus wurde untersucht, welche Wirkung Si auf die Wurzeln anderer Si-Akkumulatoren sowie intermediärer Typen und eines Si-Exkluders ausübt und wo Silizium in Reis-, Mais- und Zwiebelwurzeln abgelagert wird.

Reis kann große Mengen Arsen (As) akkumulieren, was zum einen an der hohen Verfügbarkeit von Arsenit (As(III)) unter den chemischen Bedingungen beim Nassanbau von Reis liegt sowie an der effektiven Aufnahme von As(III) durch die Reiswurzeln. Es ist bekannt, dass Si die As-Konzentration im Reisspross reduziert, wobei der zugrunde liegende Mechanismus ungeklärt ist. Daher wurde untersucht, wie sich die Gabe von Si zu Nassreisböden auf die As-Konzentration in verschiedenen Pflanzenteilen von Reis und die As-Spezies im Weißreis auswirkt. Außerdem wurde die As(III)-Aufnahme von Reiswurzeln in Abhängigkeit von verschiedenen Si-Behandlungen in Nährlösung ermittelt.

Zentrale Ergebnisse dieser Arbeit sind:

(I) Si verminderte die Diffusion von Sauerstoff aus der Reiswurzel. Dies ging mit einer verstärkten Ausbildung der Casparischen Streifen in der Exodermis und Endodermis einher sowie mit einer stärkeren Lignifizierung des Sklerenchyms der Reiswurzel. Zudem erhöhte Si die Transkriptmenge von 12 Genen, die mit der Synthese von Suberin und Lignin in

Verbindung gebracht werden, sowie die eines Gens, das für ein leucine-rich repeat (LRR)-Protein kodiert, dem regulatorische Funktionen zugeschrieben werden.

(II) Die Bildung des Casparischen Streifens in der Reiswurzel wurde mit zunehmender Si-Versorgung gefördert bis hin zu einer Konzentration von 12 mg Si L^{-1}, welche in der Bodenlösung vieler Mineral-Böden vorliegt. Die Verstärkung des Casparischen Streifens in der Reiswurzel wurde erst 48 Stunden nach Si-Applikation beobachtet, was darauf hindeutet, dass Si die Genexpression nicht direkt beeinflusst.

(III) Si förderte die Entwicklung des Casparischen Streifens in der Exodermis nicht nur in den Wurzeln von Reispflanzen, sondern auch in den Wurzeln anderer Pflanzenarten einschließlich weiterer Si-Akkumulatoren sowie intermediärer Typen und eines Si-Exkluders. Dies legt den Schluss nahe, dass der zugrunde liegende Mechanismus eher chemischer als genetischer Natur ist.

(IV) Si-Ablagerungen in den Wurzeln von Reis-, Mais- und Zwiebelpflanzen wurden vor allem in der Exodermis gefunden. Dies untermauert die Hypothese, dass Si chemisch mit aromatischen Substanzen interagiert und so die Bildung der Casparischen Streifen in der Exodermis fördert.

(V) Die Gabe von Si zu Nassreisböden erhöhte die Konzentration von As in der Bodenlösung, verminderte jedoch die As-Gehalte in Reisstroh, Spelze, Braunreis und Weißreis. Außerdem reduzierte Si die Konzentration von As(III) im Weißreis, während die Konzentration anderer As-Spezies (Arsenat und Dimethylarsinsäure) unbeeinflusst blieb. Dies deutet darauf hin, dass Si nur die Aufnahme von As(III) verminderte, jedoch nicht die anderer As-Spezies.

(VI) Die As(III)-Aufnahme wurde durch ein kontinuierliches Si-Angebot während der Vorkultur vermindert, nicht aber durch ein Si-Angebot während der As(III)-Exposition. Somit kann eine kompetitive Hemmung des As(III)-Transports durch Kieselsäure nicht verifiziert werden. Die Aufnahme von Strontium, das sich hauptsächlich apoplastisch bewegt, wurde durch die Si-Behandlungen nicht beeinflusst, weshalb als Grund für eine verminderte As(III)-Aufnahme ein reduzierter apoplastischer Flux ausgeschlossen werden kann. Vielmehr scheint die Ursache für eine reduzierte As(III)-Aufnahme eine verminderte Anzahl der As(III)-Transporter Lsi1 und Lsi2 aufgrund der Si-Vorkultur zu sein.

Abstract

Silicon (Si) is the second most abundant element in the earth's crust and available for plants nearly everywhere in form of silicic acid in the soil solution. All plants contain Si in their tissue and can be classified as Si accumulators, intermediate type plants, or non-accumulators or excluders according to their shoot Si concentration. Si is not considered essential for higher plants but it is beneficial to plants as it improves the growth and alleviates several abiotic and biotic stresses such as metal toxicities or diseases. Beneficial effects were observed especially in Si accumulators such as rice, wheat or sugar cane and were usually restricted to the shoot.
In this work, the effect of Si nutrition on the root of rice plants was studied. Special attention was paid to the formation of the casparian band and the transcription of genes related to the synthesis of suberin and lignin, which are the main components of the casparian band. Also, the dose response of Si and the Si kinetic after short term Si supply were investigated. Furthermore, the effect of Si supply on the roots of other plant species including Si accumulators, intermediate types and a Si excluder was studied and the deposition of Si in roots of rice, maize and onion was investigated.
Rice can accumulate high levels of arsenic (As) due to the high availability of arsenite (As(III)) under the chemical conditions in rice paddy soils and the highly efficient uptake of As(III) by the rice root. It is known that Si reduces the As concentration in rice shoots, but the underlying mechanisms remain unclear. Thus, the effect of Si application to rice paddy soils on the As level in different plant parts of rice and the As speciation in polished rice was investigated. Moreover, the As(III) uptake of rice plants grown in nutrient solution with different Si treatment was studied.

Central results of this work are:

(I) Si supply reduced the diffusion of oxygen out of the rice roots. This was accompanied by a promoted formation of casparian bands in the exodermis and endodermis and a stronger lignification of the sclerenchyma in the rice root. Moreover, Si increased the transcript level of 12 rice genes related to the synthesis of suberin and lignin, and of a gene coding for a leucine-rich repeat (LRR) protein that is supposed to have a regulatory function.

(II) The formation of casparian bands in the exodermis of rice roots was enhanced with increasing Si supply up to 12 mg Si L^{-1}, which is a concentration present in the soil solution of most soils. The effect on the casparian band development was visible not until 48 hours after Si supply, suggesting that Si does not directly affect the gene expression.

(III) Si promoted the development of casparian bands not only in the rice root but also in the roots of other plant species including Si accumulators, intermediate type plants and Si excluders. This indicates that the underlying mechanism is rather chemical than genetic.

(IV) Si depositions in the roots of rice, maize and onion plants were found primarily in the exodermis. This further supports the hypothesis that Si chemically interacts with aromatic compounds to promote the formation of casparian bands.

(V) Si application to rice paddy soils increased the concentration of As in the soil solution but decreased the As level in the rice straw, husk, brown rice and polished rice. Additionally, Si reduced the As(III) level in the polished rice, while the concentrations of other As species (arsenate and dimethylarsinic acid) were not affected. This indicates that Si decreases the uptake only of As(III), but not of other As species.

(VI) The As(III) uptake was decreased by continuous Si supply during the preculture, but was not affected by the presence of Si during the As exposure. Thus, a competitive inhibition of the arsenite uptake by silicic acid could not be verified. The uptake of strontium, which moves mainly in the apoplast, was not affected by the Si treatments, and so the reason for the reduced As(III) uptake was not a decreased apoplastic flux. Rather, the limited As(III) uptake likely was the result of a decreased abundance of the As(III) transporters Lsi1 and Lsi2 because of the Si preculture.

Schlagworte: Silizium, Arsen, Casparischer Streifen
Keywords: Silicon, arsenic, Casparian band

Contents

Abbreviations

4CL	4-coumarate-CoA ligase
ABC transporter	ATP binding cassette transporter
Al	aluminium
As	arsenic
As(III)	arsenite
As(V)	arsenate
AT	acyltransferase
BLAST	Basic Local Alignment Search Tool
°C	degree Celsius
C	carbon
C3H	coumaroyl-CoA-3-hydroxylase
C4H	cinnamate 4-hydroxylase
cDNA	complementary DNA
COMT	caffeic acid O-methyltransferase
d.m.	dry matter
DMA	dimethylarsinic acid
DNA	deoxyribonucleic acid
drt	distance from root tip
DW	dry weight
FAD	fatty acid desaturase
FAE	fatty acid elongase
Fe	iron
Ge	germanium
ICP-MS	inductively coupled plasma mass spectrometry
IRRI	International Rice Research Institute
KCS	β-ketoacyl-CoA synthase
LA-ICP-MS	laser ablation-inductively coupled plasma-mass spectroscopy
LRR	leucine-rich repeat family protein
MMA	monomethylarsonic acid
N	nitrogen
NCBI	National Center for Biotechnology Information
P	phosphorus

P450	cytochrom P450 monooxygenase
PAL	phenylalanine ammonia-lyase
POD	peroxidase
ppm	parts per million (= mg L^{-1} or mg g^{-1})
Pt	platinum
qRT-PCR	quantitative Real Time-PCR
RLK	receptor-like kinase
RNA	ribonucleic acid
ROL	radial oxygen loss
RT	room temperature
SE	standard error
SEM-EDX	electron microscope energy-dispersive X-ray microanalysis
Si	silicon
Sr	strontium
SSH	subtractive suppression hybridization
TFA	trifluoroacetic acid
TIGR	the Institute of Genomic Research
UV	ultraviolet

General Introduction

Silicon concentrations in soil and plant

The earth crust consists to 28-31 % of silicon (Si) rendering it the second most abundant element in soils, exceeded only by oxygen in weight proportion (Exley, 1998; Epstein, 1999). The span of Si as soil constituent comprises < 1 up to 45 % of dry weight and the most abundant minerals containing Si are quartz and the aluminosilicates alkali feldspar and plagioclase (Exley, 1998; Sommer *et al*., 2006). In soil solution at pH < 9, Si is present as (ortho)silicic acid, $Si(OH)_4$, and the typical concentrations range between 0.1 and 1.4 mM with an average of 0.5 to 0.7 mM, which is in the same order of magnitude as major plant nutrients like potassium or calcium (Epstein, 1994). In soil solution of flooded soils even higher Si concentrations up to 1.8 mM were measured, which is near to the maximum solubility of Si in water of about 2.0 mM (Sjöberg, 1996; Bogdan and Schenk, 2008). Since Si is nearly ubiquitous in soil solution, it is not surprising that all plants contain Si in different amounts. Si concentrations in plants range from less than 0.5 to 100 mg g^{-1} shoot dry weight (Epstein, 1999). In general, Si levels in leaves or non-woody plant parts are highest in liverworts and horsetails, followed by mosses, angiosperms, gymnosperms, and ferns (Ma and Takahashi, 2002; Hodson *et al*., 2005). Within the angiosperms, Si concentrations in dicotyledons are generally lower than in monocotyledons and within the latter, the *Poaceae* exhibit the highest shoot Si concentration (Epstein, 1999; Hodson *et al*., 2005). Several important crops like rice, maize, wheat, barley and sorghum belong to the *Poaceae*, whereof rice exhibits the highest concentration of up to 100 mg Si g^{-1} shoot dry weight (Epstein, 1999; Ma *et al*., 2002). According to their shoot Si concentration plants can be classified as Si accumulators, intermediate types and non-accumulators or excluder species when they contain more than 10, 5 – 10 or less than 5 mg Si g^{-1} shoot dry weight, respectively (Takahashi *et al*., 1990; Epstein, 1999).

Silicon uptake by plants

Plants can take up Si in form of silicic acid over the roots. During the last years, several plant Si transporters have been identified. Firstly, in 1997 a group of Si transporters were described in diatoms, for those Si is essential and which build their frustules from SiO_2 (Hildebrand *et al*., 1997). However, these transporters are absent in higher plants. Evidence for an active Si uptake in rice, tomato and cucumber was given by Mitani and Ma (2005). They demonstrated that radial Si transport from the external solution via root cortical cells towards xylem was

mediated by a transport system with a K_m value of 0.15 mM. This value is several times higher than that of transporters for other minerals such as phosphorus (Ma *et al*., 2004).

The first Si transporter in vascular plants was identified in rice plants by Ma *et al*. in 2006. They used Germanium (Ge), which acts as a Si analogue and is toxic to plants, to isolate a rice mutant showing no symptoms of toxicity (brown spots on the leaves) after Ge treatment (Ma *et al*., 2002). The identified mutant was shown to be defective in taking up Si. Mapping of the gene controlling the Si uptake led to identification of the Si transporter Lsi1 (Ma *et al*., 2006). This transporter belongs to the Nod26-like major intrinsic proteins, a subgroup of aquaporins (water channel proteins), and is permeable to silicic acid in both directions.

A second Si transporter in rice, Lsi2, was identified one year later (Ma *et al*., 2007). Lsi2 is a putative anion transporter that actively transports silicic acid. Both transporters are located in the plasma membrane of the exodermis and the endodermis of rice, but their subcellular localization is different: Lsi1 is located on the distal side and Lsi2 on the proximal side of both, exodermis and endodermis (Fig.1) (Ma and Yamaji, 2008).

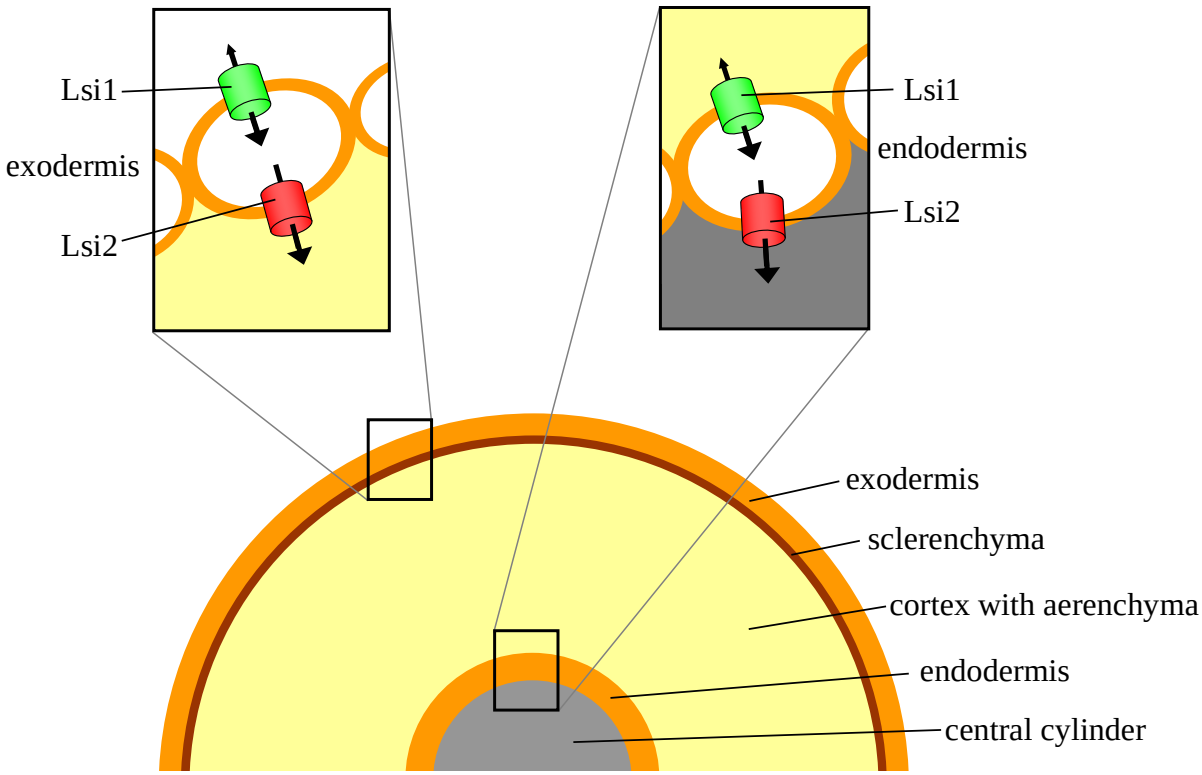

Fig. 1: Schematic overview of a cross section of an adventitious rice root with the localization of Lsi1 and Lsi2 in both exodermis and endodermis. The arrows indicate the transport direction of silicic acid.

Lsi2 shows only efflux activity to silicic acid and actively transports silicic acid from exodermal cells into the cortex, resulting in a low Si concentration in the symplast of exodermal cells. According to the Si concentration gradient Lsi1 is able to facilitate the influx of silicic acid from the external solution into the symplast. The same takes place in the endodermis, so Si is efficiently transported towards the central cylinder by the combined action of Lsi1 and Lsi2. Both genes are expressed in adventitious roots and expression is decreased by continuous Si supply (Ma and Yamaji, 2008).

A third transporter in rice, Lsi6, was identified in 2008, and this transporter is a homolog of the influx transporter Lsi1 (Yamaji *et al*., 2008). However, Lsi6 does not contribute significantly to Si uptake because it is only weakly expressed in roots. Instead, Lsi6 is expressed in the leaf sheath and leaf blade and therefore, it is supposed to unload Si from the xylem. Moreover, Lsi6 is expressed in the node I (the node beneath the rice flag leaf) and mediates the transfer of Si from large vascular bundles, which come from the root, to diffuse vascular bundles that direct to the panicles (Yamaji and Ma, 2009).

Homologous genes to the rice gene *Lsi1* were found in other Si accumulating plants: *Lsi1* and *Lsi6* in maize (Mitani *et al*., 2009a), *Lsi1* in barley (Chiba *et al*., 2009) and *Lsi1* in wheat (Montpetit *et al*., 2012). Also, for the first time in a dicotyledon plant, a gene homologous to the rice *Lsi1* was identified in pumpkin (Mitani *et al*., 2011). All these transporters function as influx transporter for silicic acid, but the pumpkin *Lsi1* shows no polar subcellular localization in the plasma membrane and the expression of the maize *Lsi1* and *Lsi6* as well as the barley *Lsi1* is not decreased by continuous Si supply (Chiba *et al*., 2009; Mitani *et al*., 2009a). Also homologous genes to *Lsi2* were found in maize and barley (Mitani *et al*., 2009b) as well as in cucumber (Mitani-Ueno *et al*., 2011). They also show active efflux transport activity for silicic acid but in contrast to rice Lsi2, maize and barley Lsi2 are localized only in the in the plasma membrane of endodermal cells without polar localization (Mitani *et al*., 2009b).

Taken together, coupling of the distal localized passive influx transporter Lsi1 and the proximal localized active efflux transporter Lsi2 in both, exodermis and endodermis, allows the highly efficient Si uptake in rice, enabling rice to accumulate highest shoot Si levels among crops (Ma *et al*., 2011).

The Si concentration in the root however, is much lower than in the shoot, because Si is rapidly translocated to the xylem and hence, root Si level is not a suitable indicator for Si accumulation (Epstein, 1999). Si in the root is supposed to be deposited in the cell walls of the root tissue (Epstein, 1994) and was found as precipitated silica in the exodermis and

endodermis of rice roots as well as in the sclerenchyma (Shi *et al*., 2005; Gong *et al*., 2006; Moore *et al*. 2011). Moreover, the deposition of silica was shown to be inducible by lignin *in vitro* (Fang and Ma, 2006). Si also can form complexes as six-coordinated Si with the phenol catechol (Barnum, 1970, 1972).

Silicon effects on plants and animals

Despite its high concentration in plants, Si is not considered essential for plants except for some algae, including diatoms, and horsetails (Epstein, 1999). According to the definition of Arnon and Stout (1939) an element is essential when "a deficiency of it makes it impossible for the plant to complete the vegetative or reproductive stage of its life cycle". For Si, this proof has not been provided, presumably because it is nearly impossible to establish a Si-free environment, where such experiments could be performed. Si is present in dust, it dissolves from glass and when setting up a nutrient solution, impurities in the nutrient salts and residual amounts of Si in demineralized water contribute also to a Si contamination, which is the reason for Si to be denoted as an "ubiquitous contaminant" (Epstein, 1994).

In contrast to plants, Si is considered essential for animals, including humans (Epstein, 1994; Exley, 1998). Si is supposed to play an important role in the formation of bones and connective tissues and Si deficiency in animals resulted in several diseases (Carlisle, 1972; Schwarz and Milne, 1972). Although no direct evidence for the essentiality of Si for humans is existent, Si was shown to be involved in the synthesis of collagen and to increase bone mineral density of humans, thus possibly reducing the risk of osteoporosis (Jugdaohsingh, 2007). Furthermore, an alleviating impact of Si on Alzheimer's disease and dementia by reducing aluminium absorption in the intestinal tract is discussed (Rondeau *et al*., 2009; Domingo *et al*., 2011). The main Si sources for humans are bananas, green bean and beer, but absorption of Si from solid food is often poor, probably because Si is present as polymerized silica (Jugdaohsingh *et al*., 2002; Sripanyakorn *et al*., 2009). In beer, Si is present as silicic acid, which is well absorbed during digestion and therefore moderate beer consumption was recommended for high Si intake (González-Muñoz *et al*., 2008; Casey and Bamforth, 2010).

Although not proven to be essential for plants, Si is considered to be a beneficial element and was called "quasi-essential" (Epstein, 1999). Si enhances growth and yield, improves mechanical strength and thus prevents lodging of rice plants (Epstein, 1999; Ma and Yamaji, 2006). Si increased resistance against fungal pathogens in rice, wheat and cucumber (Fawe *et al*., 1998; Kim *et al*., 2002; Rémus-Borel *et al*., 2005). In rice leaves, Si is deposited as silica or opal and forms together with cutin a cuticle-silica double layer, and enhanced resistance

against leaf pathogens was attributed to this Si-induced leaf fortification assigning a mainly mechanical role to Si (Kim *et al*., 2002). In further studies, elevated production of peroxidases, phytoalexins and antimicrobial compounds as well as increased activity of chitinases and peroxidases were attributed to Si, hence, allocating Si a more active role in inducing resistance (Rodrigues *et al*., 2004, 2005; Cai *et al*., 2008). Si altered the expression of nearly 4000 genes of which many were defense-related in *Arabidopsis thaliana* plants infected with fungal pathogens while Si changed expression only of 2 genes in non-infected plants (Fauteuex *et al*., 2006). In contrast, Si supply resulted in differential gene expression in rice plants both infected or not with a fungal pathogen (Brunings *et al*., 2009). However, the exact mechanisms how Si reduces leaf penetration of fungal organisms are still debated (Hayasaka *et al*., 2008; Sun *et al*., 2010).

Also biotic stress caused by herbivorous insects such as stem borers or phloem feeders can be reduced by Si, although it remains unclear whether this is due to increased physical resistance or enhanced chemical defense reactions (Reynolds *et al*., 2009). Furthermore, Si enhances resistance against abiotic stress. In several plants, Si reduced metal toxicity of aluminium, cadmium, manganese, and zinc as well as uptake of arsenic (Guo *et al*., 2005; Shi *et al*., 2005; Li *et al*., 2011; Singh *et al*., 2011; Song *et al*., 2011). Si was also effective against salinity and drought stress in rice, wheat, barley, cucumber and tomato (Liang *et al*., 2007). The extent of the beneficial effects differed between plant species and was usually more visible in plants with high Si accumulation in shoot (Ma *et al*., 2001).

Rice cultivation and adaptation of the rice root to flooded soils

Rice belongs to the family *Poaceae* within the monocotyledons. The rice genome comprises 389 Mb, which is the smallest among the cereals, and is totally sequenced resulting in rice being an ideal model plant for genomic research of the monocotyledons (Droc *et al*., 2006).

Rice is the major food crop consumed by millions of people contributing around 20 % to the human calorie intake worldwide and actually 50 % in Southeast Asian, respectively (IRRI). There are two domesticated rice species, the Asian rice (*Oryza sativa* L.) and the African rice (*Oryza glaberrima* L.) (Sweeney and McCouch, 2007). Within the Asian rice, the two subspecies Indica and Japonica are most common and are grown either as upland (dryland) or lowland (wetland / paddy) cultivation, whereof the paddy rice cultivation is the most used form (DeDatta, 1981). Rice develops different kinds of roots: the radicle or seminal root, which is the first root emerging from the seed, the mesocotyl roots and the adventitious (or nodal) roots, which account for the main part of the mature rice plant (Yoshida, 1981).

Paddy rice is grown in flooded soils under usually anaerobic conditions (Ponnamperuma, 1984). Rice is adapted for growth in flooded fields by the development of an aerenchyma, which is a gas filled tissue inside of the adventitious roots that allows the diffusion of gases within the root and hence, facilitates internal aeration (Colmer, 2003a; Colmer *et al.* 2006). According to its concentration gradient, oxygen diffuses from the aerenchyma to the anaerobic rhizosphere, a process called radial oxygen loss (ROL). To prevent ROL and keep the oxygen status in the roots constantly high, the rice root develops a barrier, which is attributed to a suberized exodermis with casparian bands and to a lignified sclerenchyma (Armstrong, 1979; Armstrong *et al*., 2000; Kotula and Steudle, 2008). This barrier against ROL is present at the basal parts of the root while the younger, apical zone of the root is not protected from ROL by this barrier (Armstrong, 1971; Colmer, 2003b).

The barrier was shown to be inducible by salinity and drought stress (North and Nobel, 1995; Schreiber *et al*., 1999). Moreover, rice roots grown in anaerobic nutrient solution contained higher amounts of suberin and lignin in the outer root parts forming a stronger barrier to ROL (Kotula *et al*., 2009a, b). Root cross sections can be stained with berberine-aniline blue and examined under UV-light to visualize suberin and lignin (Brundrett *et al*., 1988) (Figure 2).

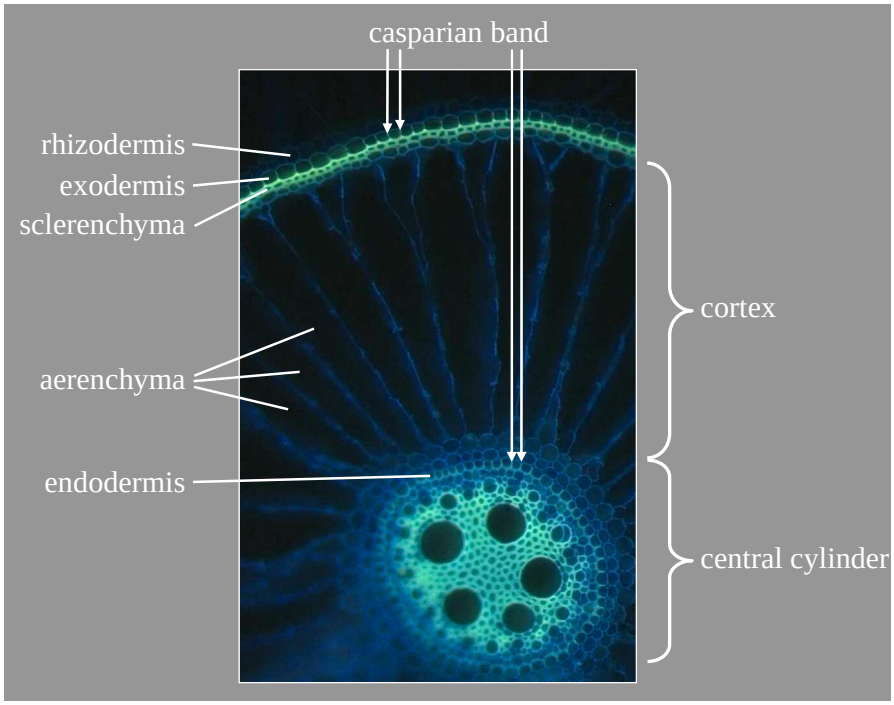

Fig. 2: Cross section of an adventitious rice root in 20 cm distance from the root tip stained with berberine-aniline blue and photographed under UV-light. The casparian bands in the exodermis and endodermis exhibit a white color and are marked with arrows. The lignified sclerenchyma has a yellow-green color.

Lignin and suberin

Lignin is a biopolymer consisting of a complex mixture of phenolic compounds that derive mostly from the three monolignols p-coumaryl, coniferyl and sinapyl alcohol. The monolignols are synthesized in the symplast via the phenylpropanoid pathway starting from phenylalanine and are released into the apoplast, where they polymerize to lignin (Boerjan *et al*., 2003; Goujon *et al*., 2003).

Suberin is a biopolymer that contains a polyaliphatic and a polyaromatic domain (Kolattukudy, 1984). Like lignin, suberin is composed of monomers, which are synthesized in the symplast, released into the apoplast and form suberin after polymerization. The components of the suberin monomers are ferulic acid, which is delivered as intermediate from the phenylpropanoid pathway, fatty acid derivates and glycerol (Franke *et al*., 2005; Franke and Schreiber, 2007). Figure 3 gives an overview of the suberin and lignin synthesis and involved enzymes and proteins. Table 1 includes the number of annotated genes in the rice genome, which are coding for these proteins, and references that assign them a role in suberin and lignin synthesis.

Tab. 1: Enzymes and proteins involved in suberin and lignin synthesis, references to protein involvement in suberin and lignin synthesis and number of annotated genes in the rice genome.

Abbreviation	**Full name**	**Reference**	**Number of annotated genes in rice**
4CL	4-coumarate-CoA ligase	Goujon *et al*., 2003	14
ABC transporter	ATP binding cassette transporter	Franke and Schreiber, 2007	36
AT	acyltransferase	Franke and Schreiber, 2007	66
C3H	coumaroyl-CoA-3-hydroxylase	Boerjan *et al*., 2003	1
C4H	cinnamate 4-hydroxylase	Boerjan *et al*., 2003	1
COMT	caffeic acid O-methyltransferase	Goujon *et al*., 2003	10
FAD	fatty acid desaturase	Franke *et al*., 2005	1
FAE	fatty acid elongase	Franke *et al*., 2005	4
KCS	β-ketoacyl-CoA synthase	Franke and Schreiber, 2007	5
PAL	phenylalanine ammonia-lyase	Goujon *et al*., 2003	9
P450	cytochrom P450 monooxygenase	Franke *et al*., 2005	6
POD	peroxidase	Franke and Schreiber, 2007	40 (expressed in roots)

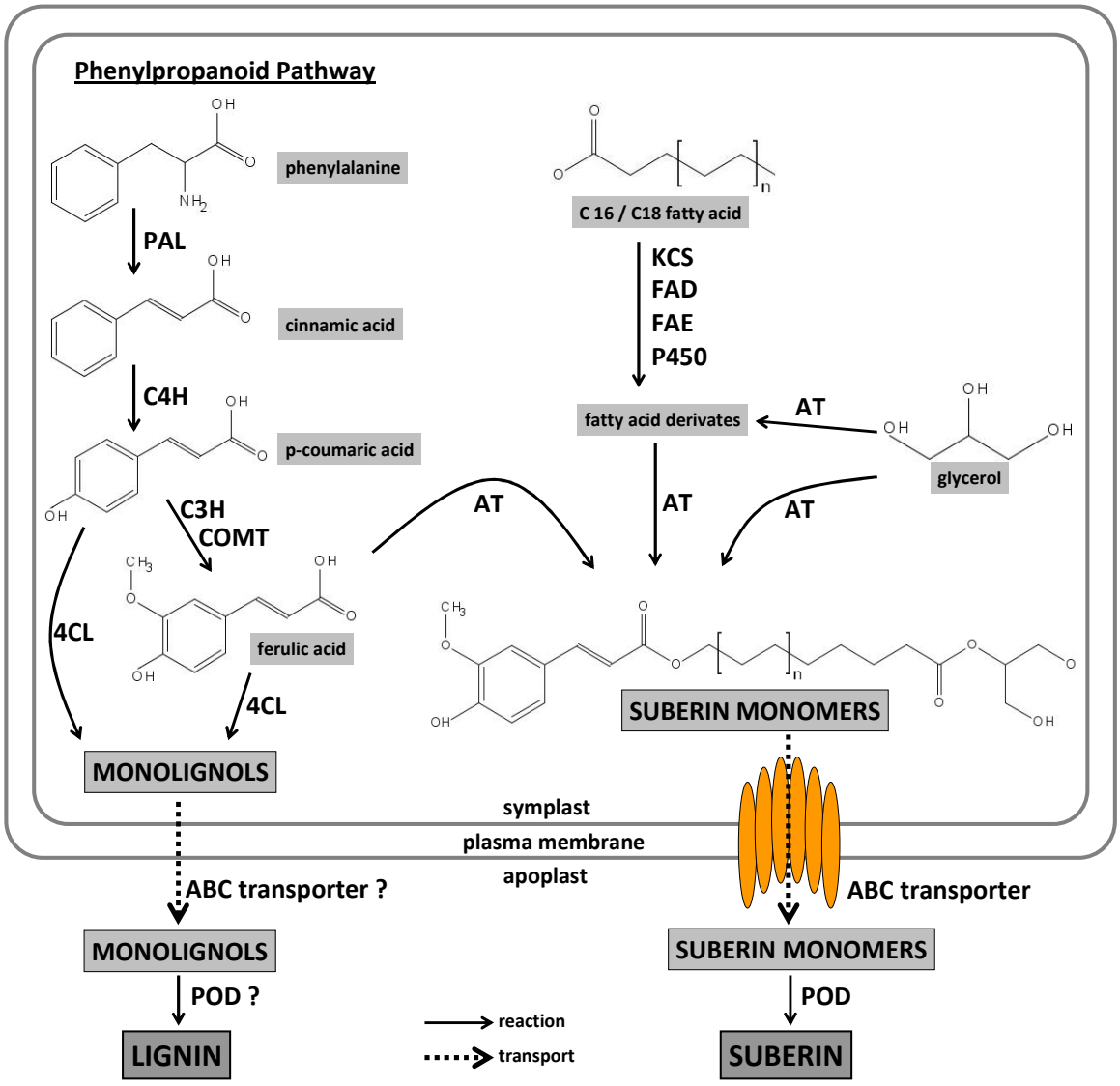

Fig. 3: Schematic overview of suberin and lignin synthesis. Full names of the abbreviations are given in Tab. 1. Figure is adapted from Fleck *et al.*, 2011 (Chapter I).

Casparian bands in the exodermis not only pose a barrier against ROL, but also reduce apoplastic bypass flow. The development of casparian bands in rice roots correlated with a reduced Na uptake and reduced salinity stress (Krishnamurthy *et al.*, 2009, 2011). Also the uptake of other minerals including As might be limited by casparian bands in the exodermis.

Arsenic dynamics in flooded soils and arsenic in rice

When fields are flooded, as it normally is the case for paddy rice fields, they are subjected to several chemical changes. The diffusion of oxygen in water is much slower than in air and once the remaining oxygen is depleted by microorganisms and plant roots, microorganisms are able to use other oxidized molecules as terminal electron acceptor for respiration (Ponnamperuma, 1984). The order of reduction is according to the redox potential and the

first molecules being reduced under anaerobic conditions are NO_3^- and MnO_2 at positive redox potential values, followed by Fe(III), while SO_4^{2-} and CO_2 are reduced at lower redox values (Marschner, 2012). The reduction of iron- and manganese-(hydr)oxides is accompanied by a release of arsenate, phosphate and silicic acid into soil solution because these were adsorbed to the (hydr)oxides before (Dedatta, 1981; Sommer *et al*., 2006).

The increased arsenic (As) concentration in soil solution after flooding exhibits a problem especially for rice cultivation, since As is a toxic and carcinogenic metalloid, which is normally present in a much higher concentration in rice than in other crops (Williams *et al*., 2007; Su *et al*., 2009). The As concentration in the grain is lower than in the leaves, however, rice diet exhibits a major source of human As intake and poses a serious health risk for the population of Southeast Asia (Meharg, 2004; Kile *et al*., 2007).

The most abundant form of As in aerobic soil is arsenate, As(V), which is present only in low concentrations in soil solution (Inskeep *et al*., 2001). However, after flooding As is released into soil solution and As(V) is reduced to arsenite, As(III), that is even more mobile in soil solution than As(V) (Garcia-Manyes *et al*., 2002; Takahashi *et al*., 2004). As can also be methylated by microorganisms to monomethylarsonic acid (MMA) or dimethylarsinic acid (DMA), and these organic As forms are considered to be less toxic to human than As(III) and As(V) (Zhao *et al*., 2009).

Not only the high availability of As in flooded paddy rice soils favors the high As level in rice, but also the efficient uptake of As by rice plants. As(III) is taken up into the rice root and transported to the xylem by the Si transporters Lsi1 and Lsi2 and hence, Si and As share the same uptake system in rice (Ma *et al*., 2008). Lsi1 also has the capability to transport MMA and DMA into rice roots (Li *et al*., 2009a).

The beneficial effect of Si on rice in alleviating metal toxicity was demonstrated for many metals. Si also reduced shoot and root As concentration of rice grown in nutrient solution with As(V) or As(III) (Guo *et al*., 2005, 2009). Also, Si concentration in soil solution was negative correlated with As concentration in polished rice and straw when rice was grown on paddy rice soils (Bogdan and Schenk, 2008). The supply of paddy rice soils with Si strongly decreased As concentration in straw and husk, but weakly decreased the As concentration in brown rice (Li *et al.*, 2009b). This decrease was due to a reduced inorganic As level in brown rice, while the DMA concentration was increased. However, this may not reflect the situation in the polished rice, since the bran, which is removed from the brown rice to get the polished rice, contains more As than the endosperm (Lombi *et al*., 2009). Moreover, the analytical method used did not allow to distinguish between the inorganic As species As(III) and As(V).

Aims of this work

Although the beneficial effects of Si on rice are known for a long time, the underlying mechanisms are not clear. To clarify the role of Si on rice root development, the following topics were experimentally examined in this work:

- Effect of Si on radial oxygen loss (ROL) and barrier formation against ROL in rice roots
- Si effect on expression of genes related to synthesis of suberin and lignin

Although beneficial effects of Si were observed best in plants with high Si accumulation in shoot, Si could have an impact also on non-accumulators or Si excluders, since roots are quasi constantly exposed to media containing silicic acid and the cell walls of the exodermal cells are in contact with the external solution. Thus, the following topic was studied:

- Si effect on roots of Si accumulators and non-accumulators

Si reduces As concentration in rice shoot, but the way how Si affects the As uptake remains to be investigated. Thus, the following topic was explored:

- Si effect on the As uptake and on the As speciation in polished rice

Chapter I

Silicon enhances suberization and lignification in roots of rice (*Oryza sativa*)

Alexander T. Fleck[1], Thandar Nye[1], Cornelia Repenning[2], Frank Stahl[2], Marc Zahn[1], Manfred K. Schenk[1]

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[1] Institute of Plant Nutrition, Faculty of Natural Sciences,
Leibniz University Hannover, Herrenhäuser Str. 2, 30419 Hannover, Germany.
[2] Institute of Technical Chemistry, Faculty of Natural Sciences,
Leibniz University Hannover, Callinstr. 3, 30167 Hannover, Germany

Abstract

The beneficial element silicon (Si) may affect radial oxygen loss (ROL) of rice root depending on suberization of exodermis and lignification of sclerenchyma. Thus, the effect of Si nutrition on oxidation power of rice root, suberization and lignification was examined. In addition, Si-induced alterations of transcript levels of 265 genes related to suberin and lignin synthesis were studied by custom-made microarray and quantitative Real Time-PCR. Without Si supply, oxidation zone of 12 cm long adventitious roots extended along the entire root length but with Si supply oxidation zone was restricted to 5 cm behind the root tip. This pattern coincided with enhanced suberization of exodermis and lignification of sclerenchyma by Si supply. Suberization of exodermis started with and without Si supply in 4-5 cm and 8-9 cm distance from the root tip, respectively. Si significantly increased transcript abundance of 12 genes, while two genes had a reduced transcript level. A gene coding for a leucine-rich repeat protein exhibited a 25-fold higher transcript level with Si nutrition. We present physiological, histochemical and molecular-biological data assigning Si an active impact on rice root anatomy and gene transcription.

Introduction

Silicon (Si) is the second most abundant element in soils and nearly ubiquitously plant available. In soil solution, Si is present as silicic acid, $Si(OH)_4$, at pH < 9, at concentrations between 0.1 and 2.0 mM, which is in the same order of magnitude as potassium, calcium, and other major plant nutrients (Epstein, 1994; Bogdan and Schenk, 2008).

Although all soil borne plants contain Si in their tissues with concentrations ranging from 0.1 % up to 10 % in dry matter, Si is not considered as an essential element according to the definition by Arnon and Stout (1939). Albeit not essential, Si is a beneficial element because it supports the healthy development of many plants species, in particular of graminaceae like rice. Si enhances growth and yield, improves the mechanical strength and thus prevents lodging, and increases the resistance to biotic and abiotic stresses like diseases and pests as well as salinity, drought stress and metal toxicities, respectively (Epstein, 1994, 1999; Ma and Yamaji, 2006).

The view how Si affects plants has changed from a passive manner to a more active way, as the alleviative impact of silicic acid on rice plants infected by the rice blast fungus *Magnaporthe grisea* has first been attributed to Si-induced cell wall fortification of rice leaves (Kim *et al*., 2002), while results from further studies suggest that Si enhances phytoalexin and peroxidase transcript levels (Rodrigues *et al*., 2004, 2005) in infected rice leaves. In cucumber and wheat plants, Si increased resistance to the fungal infection powdery mildew has been attributed to boosted production of phytoalexins in infected leaves, too (Fawe *et al*., 1998; Rémus-Borel *et al*., 2005). Moreover, Si nutrition increased lignin content and enhanced activities of peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase (PAL) in rice leaves exposed to rice blast (Cai *et al*., 2008). In maize plants, Si alleviated Al toxicity that was attributed to mediated phenol metabolism as Si treatment stimulated release of phenolic compounds in roots of maize under Al stress (Kidd *et al*., 2001). Generally, the beneficial effects of Si are most obvious in plants encountering stress situations.

Rice is often grown in flooded soils under usually anaerobic and reducing conditions (Ponnamperuma, 1984). Rice as well as other wetland species has adapted to low-oxygen environment by internal aeration of root via the aerenchyma – a tissue containing gas filled spaces, which provides a low-resistance pathway for diffusion of oxygen within the root (Colmer, 2003a, Colmer *et al.* 2006). To counteract the diffusion of oxygen from the root to the anaerobic rhizosphere, called radial oxygen loss (ROL), rice root develops a barrier

(Armstrong, 1979). This barrier against ROL is present at the basal parts of the root whereas no barrier protects the apical zone of the root from ROL (Armstrong, 1971; Colmer, 2003b). In general, densely packed cells, suberin depositions and lignification in outer cell layers are thought to serve barrier formation (Sorrell, 1994; Armstrong *et al*., 2000). In rice roots, barrier against ROL is attributed to suberized exodermis and lignified sclerenchyma cells (Kotula and Steudle, 2008). One of the factors controlling barrier formation was found to be the aeration of growth solution. Rice root grown under anaerobic conditions contained higher amounts of suberin and lignin in the outer root parts forming a stronger barrier to ROL (Kotula *et al*., 2009a,b).

Lignin and suberin metabolism in plants share the phenylpropanoid pathway resulting in monolignols, which are secreted to the apoplast and polymerized to lignin (Boerjan *et al*., 2003). Suberin monomers are composed of fatty acid derivates, glycerol and ferulic acid, with the latter being an intermediate of the phenylpropanoid pathway (Franke and Schreiber, 2007). The suberin monomers are released to the apoplast via ATP-binding cassette (ABC) transporter and polymerized by class III peroxidases (POD) to suberin. Parts of this metabolic pathway were enhanced by Si supply in plants encountering stress (Kidd *et al*., 2001; Cai *et al*., 2008). As early as 1961, Okuda and Takahashi reported that the addition of silicon to nutrient solution increased the oxidation power of rice roots leading to an oxidation of Fe^{2+} and Mn^{2+} and subsequent precipitation on the root surface and hence to a reduced Fe and Mn uptake of the rice plant.

To further investigate the Si effect on rice root we conducted experiments with rice plants grown in nutrient solution either with (+Si) or without silicic acid (-Si) and explored the oxidation power of the root, the development of exodermis, endodermis and sclerenchyma as well as the impact of Si on transcription of candidate genes.

Materials and Methods

Plant cultivation

Rice *(Oryza sativa* L. cv. Selenio) seeds were germinated in tap water for seven days and then placed between two layers of filter paper standing in tap water for additional seven days.

Each five seedlings were transferred to 5 liter pots containing non-aerated nutrient solution (mM: 1.43 NH_4NO_3, 0.32 NaH_2PO_4 x 2 H_2O, 0.51 K_2SO_4, 1 $CaCl_2$ x 2 H_2O, 1.6 $MgSO_4$ x 7 H_2O; µM: 1.82 $MnSO_4$, 0.03 $(NH_4)_6Mo_7O_{24}$, 9 H_3BO_3, 0.3 $ZnSO_4$ x 7 H_2O, 0.15 $CuSO_4$ and 35.81 Fe as sequestrene). Nutrient solution was changed every seven days for the first 14 days, and then every four days. Plants were harvested after 28 days.

For determination of oxidation power, histochemical analysis and microarray experiments, plants were supplied with Si in form of K_2SiO_3 at concentrations 0 ppm Si (control) and 50 ppm Si (1.78 mM) and potassium in the control treatment was balanced with K_2SO_4 supply. For evaluation of microarray results by quantitative Real Time-PCR (qRT-PCR), Si was applied as silica gel yielding a concentration in the range of 30 to 40 ppm Si. The pH-value of the nutrient solution was adjusted to 6.0 by addition of 10 % (v/v) H_2SO_4 and 0.75 M KOH. The plants were grown in a growth chamber (photoperiod: 14 h light, 10 h dark; temperature 25°C day / 20°C night; relative humidity 75 %; light intensity 220 µmol m^2 s^{-1}).

The oxygen concentration in the nutrient solution was measured with an optical sensor using the Fibox 3 system (Presens, Regensburg, Germany). Oxygen concentration was reduced to 3 to 4 mg L^{-1} O_2 within 24 hours after renewing nutrient solution and remained at this level. To determine root growth, adventitious roots were marked 1 cm behind the tip with a waterproof marker and root length growth was measured after 24 hours.

Determination of silicon in plant material and nutrient solution

Plant matter was dried at 60°C for four days and ground. Plant dry matter was digested overnight in a mixture of 1 M HCl and 2.3 M HF (1:2) (Novozamsky *et al.*, 1984). After addition of 3.2 % boric acid, dye reagent (0.08 M sulphuric acid and 2 % ammonium heptamolybdate), 3.3 % tartaric acid and 0.4 % ascorbic acid, silicon concentration in digested plant material and nutrient solution was photometrically determined at 811 nm.

Visualization of oxidation power in FeS medium

To visualize the oxidation power, adventitious roots were embedded in semisolid agar medium containing FeS. The medium was prepared by adding 0.8 % agar to iron-free nutrient solution and subsequent heating to solubilize the agar. The solution was amended with 1.4 g L^{-1} $FeSO_4$ x 7 H_2O and 0.32 g L^{-1} Na_2S whereupon a black FeS precipitation developed (Trolldenier, 1988). Finally, the solution was buffered by addition of 0.5 g L^{-1} $CaCO_3$ and adjusted to pH 6.0. Single adventitious root of 42 days old plants was placed between two plastic plates (5 cm x 14 cm; 0.5 cm apart), which were sealed with plasticine, while the rest of the root system remained in nutrient solution. The media was poured between the plastic plates and the top was sealed with paraffin wax. The plates were covered with black foil and photos of the plates were taken 24 h and 48 h after embedding in agar. For each treatment, four roots were investigated.

Histochemical examination of adventitious roots

For quantification of suberin layers and detection of lignification, free hand cross sections of adventitious rice roots of 42 days old plants were taken at 1-2, 4-5, 8-9 and 12-13 cm distance from root tip (drt) and stained with 0.1 % (w/v) berberine hemi-sulphate for 60 minutes and with 0.5 % (w/v) aniline blue for further 30 minutes (Brundrett *et al*., 1988*)*. Stained sections were mounted in 0.1 % (w/v) $FeCl_3$ in 50 % (v/v) glycerine and viewed under an Axioskop fluorescence microscope (Zeiss, Jena, Germany) with UV illumination using excitation filter G 365, chromatic beam splitter FT 395 and barrier filter LP 420. Pictures were taken with the AxioCam MRc (Zeiss) and picture recording software (AxioVision Ac, Version 4.4, Zeiss). Under UV light, suberin exhibited a blue-white colour and lignin was yellowish-green.

Both, -Si and +Si treatments were replicated four times with each replicate consisting of 10 plants. Per plant, one root without lateral roots was cut into four segments (1-2, 4-5, 8-9 and 12-13 cm drt), and from each segment one cross section was taken. Under microscope, 10 cells per cross section were examined and the degree of suberization in the anticlinal cell walls was determined and allocated to one of four stages: 0 % (stage I), 0-25 % (II), 25-50 % (III) and 50-100 % (IV) suberization of the length of the anticlinal cell wall.

Collection of genes if interest

To study an effect of Si nutrition on transcription of genes related to suberin and lignin synthesis, a list of candidate genes was created. A combined model of the suberin and lignin synthesis pathways was drawn containing 12 protein groups (Fig. 5). These proteins were used as search terms in the search for the putative function of genes in the Rice Genome

Annotation Database of the Institute of Genomic Research (TIGR), *http://www.tigr.org/tdb/e2k1/osa1*. Resulting hits were gathered in the list of candidate genes.
A hypothetical synthesis pathway for suberin in *Arabidopsis* (Franke and Schreiber, 2007) attributes key functions to acyltransferases (AT), ABC transporter, POD and ß-ketoacyl-CoA synthases (KCS). A query in the TIGR database for AT and ABC transporter resulted in 62 and 36 hits, respectively. For POD, there is a special database available on *http://peroxibase.isb-sib.ch*. A search herein for genes that are expressed in rice roots resulted in 40 hits. Five genes were obtained from a list of rice KCS available on *http://www.izmb.de/schreiber/elogases.shtml*. Bernards *et al.* (2004) suggested that NADPH oxidases are involved in the suberin synthesis, and a query in the TIGR database provided one rice gene with corresponding annotation.
In the biosynthesis pathway of monolignols, the precursors of lignin, major functions are attributed to PAL, 4-coumarate-CoA ligases (4CL) and caffeic acid *O*-methyltransferases (COMT) (Goujon *et al*., 2003). A putative function search for rice genes with corresponding annotation in the TIGR database resulted in 8, 14 and 10 hits, respectively. Nine additional genes were gathered from a list of candidate genes for lignin synthesis in *Arabidopsis* (Goujon *et al*., 2003). Sequences of *Arabidopsis* genes were compared to rice genome by BLAST (Basic Local Alignment Search Tool) search on *http://www.tigr.org/tdb/e2k1/osa1*. The result with the highest score was chosen and annotated function for the rice gene was adopted.
Moreover, a SSH-library with genes differentially expressed in suberized tissue compared to non suberized tissue in *Quercus suber* provided additional candidate genes (Soler *et al*., 2007). Comparing gene sequences of the library with rice genome by BLAST search provided additional 79 candidate genes. BLAST search resulting in genes with unknown function annotation were not used as candidate genes. After deleting duplicate genes from function search and BLAST search, list of candidate genes contained 265 rice genes.

Microarray analysis

Adventitious roots were harvested at 0-2 cm and 4-6 cm drt and frozen immediately in liquid nitrogen. For RNA isolation, roots were ground under liquid nitrogen and total RNA was isolated using TRIsure® Reagent (Bioline, Luckenwalde, Germany) following the instructions of the manufacturer. For cDNA synthesis and hybridization with chips, RNA had to be concentrated. Therefore, RNA of each 10 biological replicates was pooled in equal amounts and precipitated by addition of 0.1 volume 5 M NaCl and 2 volumes 96 % ethanol.

Pooled samples were stored overnight at -70°C and then centrifuged for 10 minutes at 16,500 g. The pellet was washed with 500 µl 70 % ethanol and centrifuged for 7 minutes at 16,500 g. The washed pellet was air-dried for 5 minutes and dissolved in H_2O.

For each of the 265 candidate genes, a 50mer oligonucleotide with an aminolink-C6-modification at 5'-end was designed and synthesized (Ocimum Biosolutions, Ijsselstein, Netherlands). To minimize cross-hybridization with non-target transcripts, the criteria used for oligonucleotide designing were a maximum overall similarity to all other genes in rice genome of 75 % and the exclusion of complementary sequences with more than 15 contiguous bases (Kane *et al*., 2000). Synthetic 50mer oligonucleotides were spotted in triplicates on aldehyde modified glass slides (VSS25, CEL Associates, Inc., Pearland, TX, USA) using an Affymetrix 417 arrayer. During reverse transcription 8 µg of purified total RNA was copied into fluorescein (Fl) and biotin (B) labeled cDNA. The Fl and B labeled cDNA of both samples were hybridized simultaneously in one experiment to the same array. After hybridization, the unbound and unspecifically fixed cDNA was removed from the array by three stringent washing steps with sodium chloride-sodium citrate buffer in decreasing concentrations. Specifically bound Fl and B labeled cDNAs were sequentially detected with a series of conjugate reporter molecules according to the tyramide signal amplification process, ultimately with Tyramide-Cy3 and Tyramide-Cy5. First, the DNA-chip was incubated with Anti-Fl-horseradish peroxidase (HRP) antibody-enzyme conjugate, which specifically binds to the hybridized Fl labeled cDNA probe. The enzyme portion of the conjugate, horseradish peroxidase, catalyzes the deposition of Cy3 labeled tyramide amplification reagent. The reaction results in the immediate deposition of numerous Cy3 labels adjacent to immobilized HRP. Thereby, the amount of tyramide (Cy3) is greatly amplified relative to cDNA hapten (fluorescein). After inactivation of the residual HRP, the added streptavidin-HRP binds to biotin labeled cDNA and catalyzes the deposition of Cy5 labeled tyramide amplification reagent. The array obtained through this process was subsequently scanned for the two distinct fluorescent dyes (Cy3 and Cy5), of the cDNA derived from the +Si roots and the –Si roots.

The scanning process of the hybridized chips with the Axon 4000B scanner (Axon Instruments, Foster City, CA, USA) included a six fold scan of each chip at different settings, both changing the photomultiplier tube and the laser power settings. The following primary analysis served as a quantification method and was performed with the software tool Gene Pix Pro 6.0™. Next, the secondary analysis was conducted using the data from the primary analysis. Therefore, data from different scans were first normalized by using the median of all

background intensities, which were therefore checked for outliers. Three replicates for each gene were tested for outliers. Outliers amongst the gene replicates were eliminated according to the outlier test by Nalimov and the remaining data were averaged. The ratio of the two states for each individual gene was calculated. Finally, a t-test (5 % probability of error) was applied in order to detect differentially expressed genes between the groups of samples. The results of the microarray data are given as evidence for changes in transcript abundance. The microarray data have been submitted to the NCBI Gene Expression Omnibus (Edgar *et al*., 2002) and are accessible through GEO Series accession number GSE23723 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23723).

qRT-PCR

Total RNA (1 µg) and random hexamer primers were used to synthesize first-strand cDNA using the Revert Aid^{TM} H Minus Kit (Fermentas, St. Leon-Rot, Germany) following manufacturer's instructions.

In qRT-PCR experiments, 80 ng cDNA was used as template in a 25 µl reaction-mix containing 2.5 µl 10x buffer, 3.6 mM $MgCl_2$, 0.2 mM dNTPs mix (Fermentas), 0.25 µl 1:1000 diluted SYBR-Green (Invitrogen, Carlsbad, CA, USA), 0.75 U HotStart-Taq-Polymerase (DNA cloning service, Hamburg, Germany), 0.25 µM forward and 0.25 µM reverse primers. The qRT-PCR runs were performed in the CFX96 cycler (Bio-Rad, München, Germany), using an initial 95°C-step for 10 min, followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds, and a final melting curve procedure with a stepwise increment of 1°C ranging from 60°C to 95°C.

Prior to primer design, sequences of genes of interest were blasted against rice genome on *http://rice.plantbiology.msu.edu/* and *http://blast.ncbi.nlm.nih.gov/* to obtain gene families or genes with similar sequences. For designing gene member specific primer suitable for qRT-PCR assay, gene member specific alignments were created with Vector NTI software (Invitrogen). Heterologous parts of the gene member sequence were used for primer design using Primer 3 (Rozen and Skaletsky, 2000). All primers had to fulfil internal quality standards, which included an amplicon size of 70 to 200 base pairs, a primer melting temperature of 59 to 61°C, a primer size of 20 to 24 nucleotides and a primer GC content of 45 to 65 %. Moreover, primer efficiencies were tested with a 5-fold dilution series with 5 ng/µl cDNA as highest concentration and only primer pairs with efficiency of 90 % or above were used for qRT-PCR. The sequences of forward and reverse primer pairs are shown in table 1.

The constitutively expressed eukaryotic elongation factor 1-alpha (eEF 1-α) (LOC_Os03g08010) was used as endogenous control due to its stable transcript abundance in rice (Jain *et al*., 2006; Jain, 2009). For each target in qRT-PCR, three technical and three biological replicates were used. Relative quantity was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Tab. 1: Sequences of primers used for qRT-PCR.

Gene ID	**forward primer (5' → 3')**	**reverse primer (5' → 3')**
LOC_Os01g22230	CGGCCGTTGGATGCGAGTGATTT	GATCGACACGACACGACGACACT
LOC_Os01g24010	CCTTGCTACAGCCGGCACCT	ACATGTTCGCGGGCACCCTG
LOC_Os01g42410	GGACGCATTGAAAGAGAAGC	TTGGTCAGCAATATGGGACA
LOC_Os01g67540	TGAGGCAACCGGGTGCATACCTTA	AAGCCACACGGCGCACTTTCTT
LOC_Os02g05630	GTGGTGGTCGTCGTCTTCTT	CCCATAACTGAACTTGCCGT
LOC_Os02g06250	TACCACTGTCGCCACCACTAACCA	AACGACGGCAACTCCGTGGAAA
LOC_Os02g41680	TCACAAGCTCAAGCACCATC	CTCACCAAGCTTCTTGGCAT
LOC_Os03g08010	TCAAGTTTGCTGAGCTGGTG	AAAACGACCAAGAGGAGGGT
LOC_Os05g20100	GCGACGGAGCTGTTGGGCTT	GGCACCGGCTGCCATTCCTT
LOC_Os06g16350	CAGCGCCATGGACAGCCACA	ACGGTGTCGGCCGTGGAGTA
LOC_Os06g22080	GATCGCCAGGAACGTCGCCC	TGCCCTGTTGGGATCGAAGCAC
LOC_Os06g32990	CGGCGAGCTTGTGCTGACCT	GAGTCCAAGGGTCCGGGCGT
LOC_Os06g39520	TGTGAAGAGCGCTGGGCTGCTA	TCACCCCAGCACGGATAACGCT
LOC_Os07g44560	TGCAGCACAACGACAACGGCAT	GCTGCAACAGCGACGAACGTCATA
LOC_Os08g02110	TCCTGAATTGCCCGCCTTAGCTCT	TCACAAAGACGCGGCCACGAAA
LOC_Os09g32964	CGCTCTTCTTCGAGGCGTTCAAGG	CTCCATGCTTCCCGGACTTGACTC
LOC_Os10g30610	ATCATCTAATGAGGCACGGC	TCATTGTCTGGCTGCAGAAC
LOC_Os11g14050	ATCAGGCACCATACCAAGCCAGC	TGGGAGGAATGCCGCCAGTGAAA

Results

The Si concentration of Si treated plants was much higher than of –Si plants. Si concentration in root and shoot of +Si plants was 6 to 20 and 53 to 65 mg Si / g DW, while –Si plants contained only 0.6 to 1.7 and 1.9 to 2.6 mg Si / g DW in root and shoot, respectively.

Oxidation power

Adventitious roots of plants differentially supplied with silicic acid were embedded for 24 hours in semisolid agar medium containing FeS for visualization of oxidation power. Roots of control (–Si) plants developed a bright zone (oxidized zone) in the surrounding medium over the whole length of the root from tip to basal parts (0-12 cm) (Fig. 1). In contrast, when plants were supplied with Si, bleaching of the medium was restricted to the first 5 cm, while basal parts of the root did not brighten the medium. The oxidation pattern did not differ between the four replicates, and was the same after 48 hours embedding in FeS agar.

Fig. 1: Oxidation power of adventitious rice roots as affected by silicic acid supply 24 hours after embedding in FeS-agar medium. Plants were grown for 28 days in nutrient solution without Si or with 50 ppm Si. Figure is adapted from Nye (2009).

Histochemical analysis of suberization and lignification

Suberization and lignification in adventitious roots were studied by staining root sections with berberine-aniline blue and examination under a fluorescence microscope. In 1-2 cm drt, no suberin and lignin depositions could be detected in the outer parts of –Si and +Si roots (Fig. 2). In 4-5 cm drt, suberin and lignin deposits were visible in the exodermis of +Si plants, but not in control plants. In 8-9 cm drt, suberin and lignin also occurred in -Si plants, but weaker than in the same section of +Si plants. With increasing distance from the root tip, suberization of exodermis and lignification of sclerenchyma were enhanced in both treatments. The suberization of the anticlinal cell walls of the exodermis started from the proximal side, the sclerenchyma. The length of suberized cell walls was rated for quantification of suberization of the exodermis.

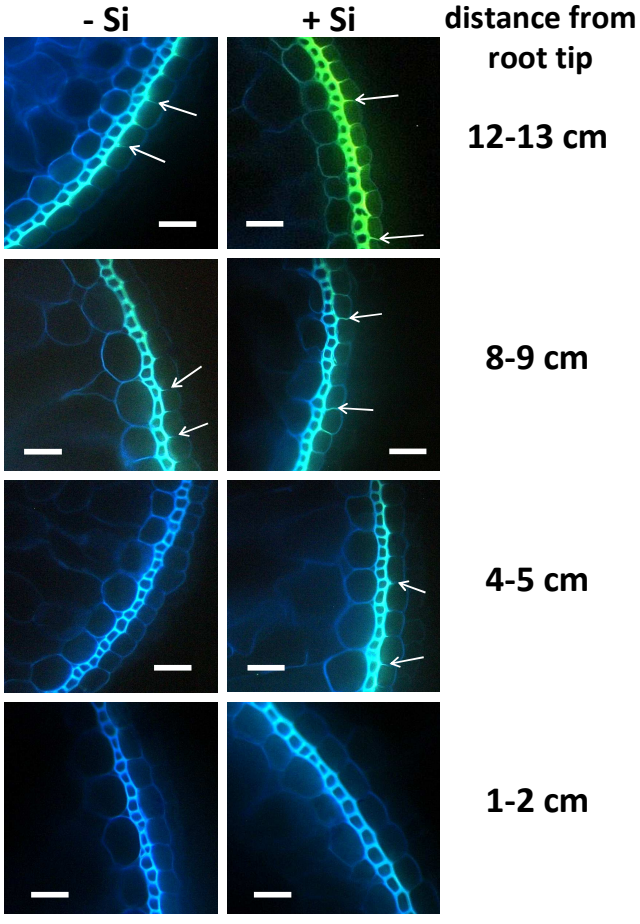

Fig. 2: Influence of silicic acid supply on suberization of exodermal cell layer and lignification of sclerenchyma of adventitious rice roots of plants grown in nutrient solution without Si or with 50 ppm Si for 28 days. Root sections were stained with berberine-aniline blue and examined under fluorescence microscope. Suberized anticlinal cell walls are blue-white-coloured and indicated by arrows, whereas lignified sclerenchyma has a yellow colour. Scale bars = 20 µm. Figure is adapted from Nye (2009).

In –Si plants, the exodermal cell walls exhibited no suberin layers (stage I) in 1-2 cm and 4-5 cm drt (Fig. 3). In 8-9 cm drt, nearly all cell walls were suberized to 25 % (stage II) of cell wall length, and in 12-13 cm drt, half of the anticlinal cell wall was suberized (stage III) for about 80 % of the cells. In contrast, in plants supplied with silicic acid, suberization started already in 4-5 cm drt and was more pronounced in the following sections. In 12-13 cm drt, nearly all anticlinal exodermal cell walls were fully suberized (stage IV). In the inner part of the root, no suberin could be detected in root section 1-2 cm drt of –Si plants while in the same root section of +Si plants, suberin in endodermis was visible over the entire length of anticlinal cell walls (Fig. 4). In the following root sections (4-5, 8-9, 12-13 cm drt) of both, –Si and +Si plants, all anticlinal endodermal cell walls were fully suberized (data not shown).

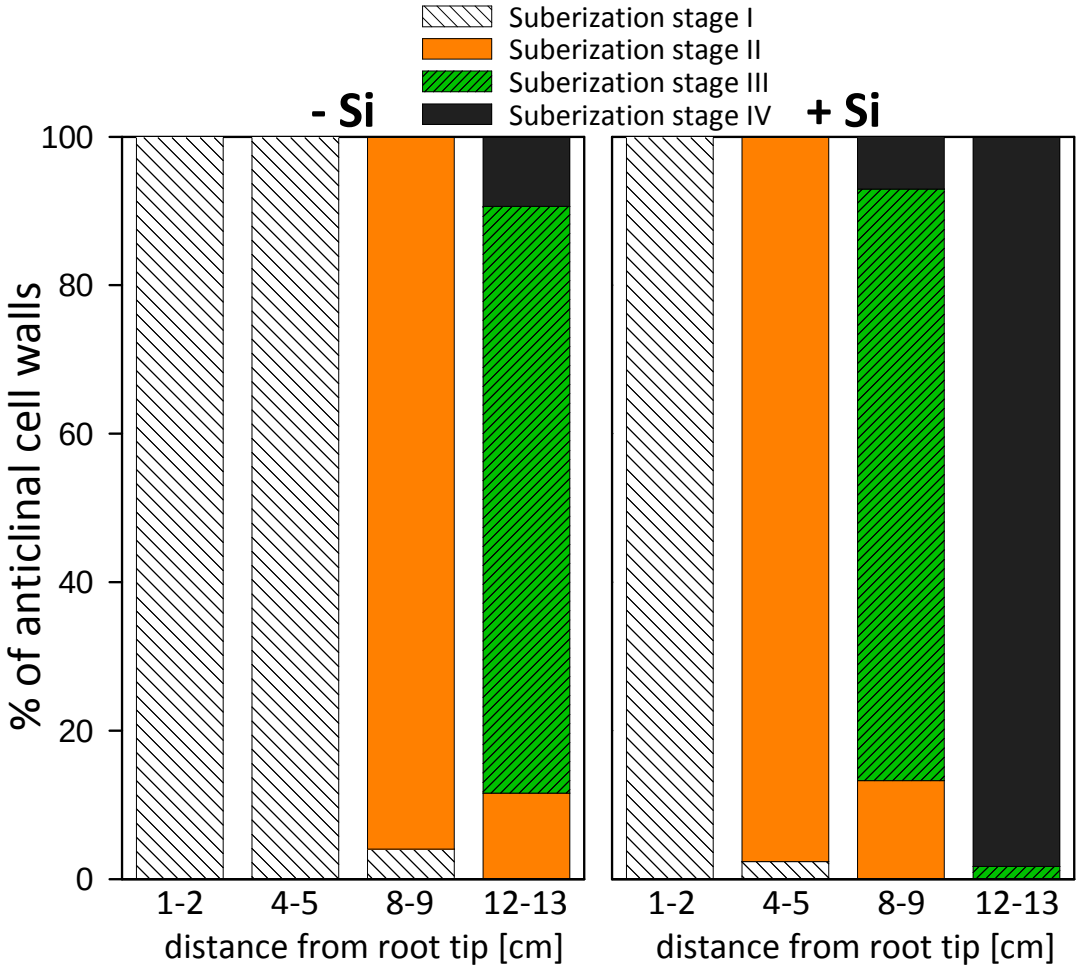

Fig. 3: Suberization of anticlinal cell walls of exodermis along adventitious roots of rice plants as effected by silicic acid supply. Plants were grown in nutrient solution without or with 50 ppm Si for 28 days. Total number of evaluated cell walls per treatment and section = 400. Suberization stages are: I = 0 %, II = 0-25 %, III = 25-50 %, IV = 50-100 % suberization of the length of the anticlinal cell wall. Data are adapted from Nye (2009).

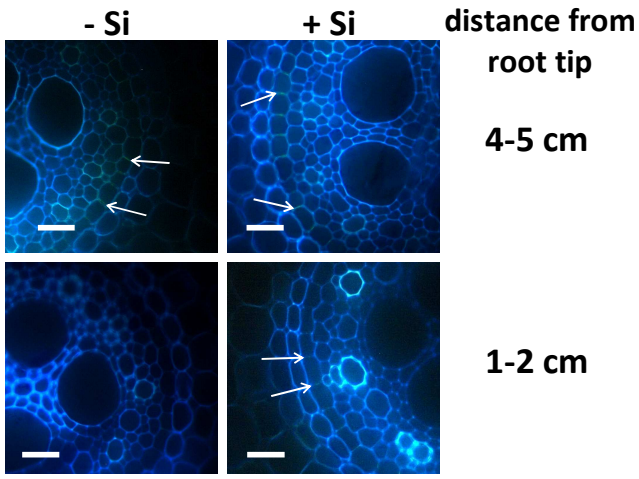

Fig. 4: Influence of silicic acid supply on suberization of endodermal cell layer of adventitious rice roots of plants grown in nutrient solution without Si or with 50 ppm Si for 28 days. Root sections were stained with berberine-aniline blue and examined under UV-light. Suberin is blue-white-coloured and indicated by arrows. Scale bars = 20 µm. Figure is adapted from Nye (2009).

Transcription analysis

Relative transcription levels of 265 genes in rice roots subjected to different Si treatments were investigated by using custom-made microarray. In the root tip (0-2 cm drt), transcript rate of eight genes was found to be regulated significantly by silicic acid supply (Table S1); transcript abundance of four genes decreased by a factor of 1.4 to 1.9 while transcripts of four genes were 1.4 to 2.5-fold up regulated. Among the latter, two genes are coding for AT, one for an ABC-transporter and one for a POD.

In root segment 4-6 cm drt, 19 gene transcripts showed significant regulation by Si treatment (Table S2); transcripts of four genes were down regulated by a factor of 1.6 to 2.8, and transcripts of 15 genes were up regulated from 1.5-fold to 2.9-fold. Among the genes with elevated transcript level, four AT, four ABC-transporter and two POD were found.

To gain independent biological replicates, further experiments were undertaken. RNA was isolated from root sections 0-2 cm and 4-6 cm drt and used for cDNA synthesis. For confirmation of microarray results, cDNA from –Si and +Si roots was used in qRT-PCR to detect Si induced changes in transcript abundance of 19 genes. 12 genes exhibited significantly increased transcript abundance by silicic acid supply, whereas transcripts of two genes were less abundant. The transcript level of five genes was not altered by Si treatment (Table 2).

The tendency of gene regulation by silicic acid supply as observed in the microarray analysis could be verified for 13 of the 19 genes in qRT-PCR analysis. The highest Si-induced change

in transcript ratio was detected for a gene coding for a leucine-rich repeat family protein (LRR), which was over 25-fold more abundant in 4-6 cm drt of +Si plants than in control plants.

Tab. 2: Relative quantity (RQ) of transcripts in root segment 0-2 cm and 4-6 cm distance from root tip (drt) as affected by silicic acid. Stars indicate significant regulation at $\alpha < 0,05$, t-test, $n = 3$.

Gene ID	**RQ +Si / -Si in 0-2 cm drt**	**RQ +Si / -Si in 4-6 cm drt**	**Gene annotation**
LOC_Os02g41680	2,10*	3,29*	phenylalanine ammonia-lyase
LOC_Os01g67540	1,95*	2,24*	4-coumarate-CoA ligase
LOC_Os07g44560	0,69	2,91*	4-coumarate-CoA ligase
LOC_Os05g20100	2,63*	2,71*	glycerol-3-phosphate acyltransferase
LOC_Os06g22080	2,05*	2,00*	diacylglycerol O-acyltransferase
LOC_Os01g24010	1,27	1,30	ABC transporter
LOC_Os01g42410	1,56	1,19	ABC transporter
LOC_Os10g30610	5,27*	3,68*	ABC transporter
LOC_Os12g32814	1,74*	1,57*	ABC transporter
LOC_Os01g22230	0,27*	0,25*	class III peroxidase
LOC_Os06g16350	4,08*	7,11*	class III peroxidase
LOC_Os06g32990	1,05	1,95*	class III peroxidase
LOC_Os08g02110	3,40*	3,03*	class III peroxidase
LOC_Os09g32964	0,34*	0,52*	class III peroxidase
LOC_Os02g05630	2,27*	1,55	protein phosphatase 2C
LOC_Os02g06250	0,38	1,16	phytosulfokine receptor
LOC_Os06g39520	1,13	1,10	myristoyl-acyl carrier protein thioesterase
LOC_Os09g23540	0,81	1,39	dehydrogenase
LOC_Os11g14050	11,00*	25,40*	leucine-rich repeat family protein

Discussion

Rice plants grown in nutrient solution with silicic acid for 28 days accumulated between 53 and 65 mg Si / g shoot DW, which was in the same range as reported for well supplied rice plants (Epstein, 1994). Control plants grown without additional silicic acid contained only 1.9-2.6 mg Si / g shoot DW, which was about 20 to 30 times less than in +Si plants.

Oxidation power, suberization and lignification

Adventitious roots of –Si and +Si plants showed clearly different pattern of oxidation power in FeS agar (Fig. 1). Roots of –Si plants bleached the medium over the whole length suggesting that the root along the entire axis released oxygen into the surrounding medium. With roots of +Si plants, discolouration of agar was restricted to about 5 cm from the root tip while basal parts of the root did not brighten the medium. This indicated a clearly reduced ROL in older parts of roots supplied with silicic acid and hence a reduced oxidation power, which is in contrast to literature (Okuda and Takahasi, 1964, cited in: Lewin and Reimann, 1969). Okuda and Takahasi observed an increased deposition of iron and manganese oxides on the root surface of Si supplied plants and concluded that Si promoted the oxidation power of rice roots. Unfortunately, the original publication was not accessible for detailed discussion.

Generally, the low oxidation power in basal parts of the root is caused by a barrier to radial oxygen loss (ROL) from aerenchyma to rhizodermis, which is due to densely packed sclerenchyma cells with lignified secondary walls and suberized exodermis with casparian bands (Kotula and Steudle, 2008). Silicic acid supply clearly enhanced the formation of casparian bands in the exodermis and endodermis (Fig. 2, 4). Nearly all anticlinal cell walls of the exodermis were suberized for 50 % of their length (stage III) in 8-9 cm drt in +Si plants, while control plants reached this stage only in 12-13 cm drt. In this stage, +Si plants had already fully developed casparian bands (stage IV) in the exodermis. Similarly, lignification of sclerenchyma was enhanced by silicic acid supply. Both, formation of casparian bands in exodermis and lignification of sclerenchyma cells reduce ROL, but the contribution of each process is unclear (Kotula *et al*., 2009a).

Suberin deposition in the exodermis was first observed in 4-5 cm drt and 8-9 cm drt for plants grown with and without Si, respectively, while endodermal suberin layers could be seen in 1-2 cm drt and 4-5 cm drt in +Si roots and –Si roots, respectively. This is comparable to literature, as for adventitious roots of rice grown hydroponically without additional Si supply,

initiation of exodermal casparian bands was found at 3 cm drt and well developed casparian bands at 5 cm drt, while endodermal casparian bands were observed already in 2 cm drt (Ranathunge *et al*., 2003). In onion (*Allium cepa)* roots, mature casparian bands in the endodermis were observed already in 0.8 cm drt (Ma and Peterson, 2003). For adventitious roots of corn (*Zea mays*) grown in aerated nutrient solution, casparian bands in the exodermis appeared first in 2-12 cm drt, with increasing drt in older roots, whereas casparian bands in the endodermis already occurred in 1 cm drt, independent of root age. When the growth of corn roots was slowed down by addition of polyethylene glycol, casparian bands were developed within 1 cm drt (Perumalla and Peterson, 1986). Other environmental factors which reduce root growth such as salinity and drought stress were also shown to enhance the suberization of roots (North and Nobel, 1995; Gong *et al*. 2006; Schreiber *et al*., 2007). The aeration of the nutrient solution is also a factor controlling ROL of rice roots, as growth in stagnant deoxygenated 0.1 % agar nutrient solution resulted in decreased ROL and elevated levels of suberin in the exodermis and lignin in the sclerenchyma compared to plants grown in aerated nutrient solution (Colmer, 2003b; Kotula *et al*., 2009a). Enhancement of barrier formation under anaerobic conditions was accompanied by root growth reduction (Kotula *et al*., 2009a). The nutrient solution contained no Si (Kotula *et al*., 2009a) or only 2.8 ppm (Colmer, 2003b), which was low compared to the Si concentration occurring in the soil solution and in our experiments and rapid depletion due to uptake by plants was probable since nutrient solution was renewed every seven days. In contrast, our experiments were conducted with high Si concentration under hypoxic conditions (3 to 4 mg L^{-1} O_2). So it was shown that anaerobic conditions and Si nutrition separately induced a barrier against ROL. How perfect anaerobic conditions and constant high Si concentrations as occurring under field conditions act together remains to be investigated.

Since in our experiment the root growth rate was unaffected by Si supply under hypoxic conditions (–Si: 1.05 cm / 24 h ± 0.05; +Si: 1.02 cm / 24 h ± 0.06) and no other environmental stresses were present, the alteration in suberin and lignin formation can be attributed to a direct effect of silicic acid.

Increased suberin content in the root may be useful not only to reduce ROL, but also to protect from biotic and abiotic stresses, since suberin was associated with partial resistance against fungal pathogens in roots (Thomas *et al*., 2007). Furthermore, stronger barriers to ROL were correlated with higher salt tolerance in rice (Krishnamurthy *et al*., 2009).

Gene regulation as affected by silicic acid

The biopolymer lignin is a complex mixture of phenolic compounds derived mainly from three hydroxycinnamyl alcohol monomers (monolignols), p-coumaryl, coniferyl and sinapyl alcohols. The synthesis of all monolignols starts with phenylalanine, which is initially processed to cinnamic acid by PAL and to p-coumaric acid by cinnamate 4-hydroxylase (C4H) during the first steps of the phenylpropanoid pathway (Fig. 5). Thereafter, p-coumaric acid can be metabolized either by 4CL and further enzymes to the monolignol p-coumaryl alcohol or by coumaroyl-CoA-3-hydroxylase (C3H) and COMT to ferulic acid (Boerjan *et al*., 2003; Goujon *et al*., 2003). Ferulic acid can further be converted to monolignols by several enzymes including 4CL. The monolignols are transported to the apoplast, probably via ABC transporter, where POD are assumed to catalyze the formation of lignin by dehydrogenative polymerization of the monolignols (Boerjan *et al*., 2003). We found that Si significantly increased the transcript abundance of PAL and 4CL, leading to enhanced synthesis of monolignols. Increased transcript abundance of ABC transporter and POD might facilitate transport to the apoplast and subsequent polymerization of monolignols, which is in line with the enhanced lignification.

Suberin is a biopolymer which consists of a polyaliphatic and a polyaromatic domain (Kolattukudy, 1984). Similar to lignin formation, suberin evolves from suberin monomers that polymerize in the apoplast under the catalytic force of POD (Fig. 5). The monomers are made up of ferulic acid, glycerol and varying oxygenated fatty acid derivates, mainly ω-hydroxyacids and α,ω-dicarboxylic acids (Franke and Schreiber, 2007). The fatty acid modifications are attributed to KCS, fatty acid desaturases (FAD), fatty acid elongases (FAE) and cytochrom P450 monooxygenases (P450) (Franke *et al*., 2005; Franke and Schreiber, 2007). The monomers are assembled by AT in the symplast, whereupon ABC transporter release the monomers into the apoplast.

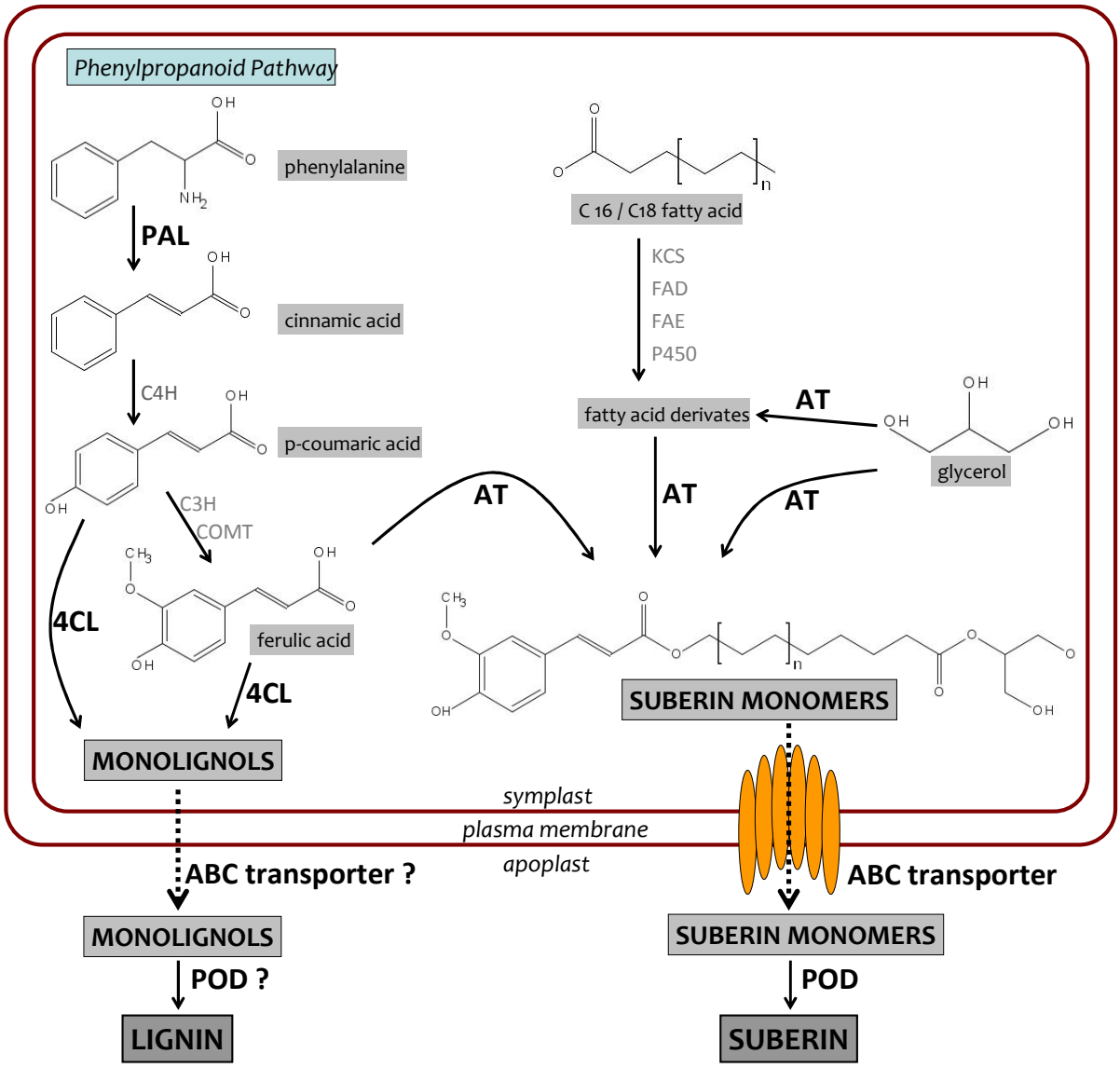

Fig. 5: Model of the suberin and lignin synthesis pathways. Bold names indicate Si-induced changes in transcript abundance. Abbreviations of the proteins are: 4CL: 4-coumarate-CoA ligase; ABC transporter: ATP binding cassette transporter; AT: acyltransferase; C3H: coumaroyl-CoA-3-hydroxylase; C4H: cinnamate 4-hydroxylase; COMT: caffeic acid O-methyltransferase; FAD: fatty acid desaturase; FAE: fatty acid elongase; KCS: β-ketoacyl-CoA synthase; PAL: phenylalanine ammonia-lyase; P450: cytochrom P450 monooxygenase; POD: peroxidase.

Si enhanced transcript abundance of AT, ABC transporter and POD, indicating an increased formation of suberin, which coincides with the histochemical observations. None of the examined genes involved in fatty acid metabolism showed significant transcription changes as effected by Si, suggesting that fatty acids derivates exhibit no bottleneck in suberin synthesis. The gene with the most highly elevated transcript level by Si supply was a leucin-rich repeat family protein (LRR). In 0-2 cm drt and in 4-6 cm drt, transcript rates were 11-fold and 25-

fold higher in +Si roots than in –Si roots, respectively. The gene was selected from a SSH-library that contained genes differentially expressed in suberized tissue compared to non suberized tissue in *Quercus suber* (Soler *et al*., 2007). LRR proteins belong to the receptor-like kinases (RLK), a major gene family with more than 1100 members in rice (Morillo and Tax, 2006). RLK are transmembrane proteins, which contain an extracellular domain that is linked via a transmembrane domain to a cytoplasmic serine/threonine protein kinase domain (Shiu *et al*., 2004). One of the motifs in the extracellular domain is the LRR-motif, and the LRR-RLKs form the biggest group of RLKs with 216 members in *Arabidopsis*, where the LRR-RLKs are best described (Diévart and Clark, 2003). LRR-RLKs are grouped into 13 subfamilies according to their LRR organization in the extracytoplasmic domain. Homologue *Arabidopsis* proteins to LOC_Os11g14050 are members of subfamily VIII-1. Since details about possible ligands for this LRR-group are not known, it would be interesting to further characterize this group and its involvement in suberin and lignin synthesis.

In the root tip, transcript level of a gene coding for a protein phosphatase 2C was significantly enhanced. Protein phosphatases are regulating proteins, and the subgroup 2C is thought to be involved in stress signal transduction (Luan, 2003). Two genes had fewer transcript levels under +Si conditions, both genes coding for POD. This must not necessarily conflict with the other results due to the wide variability of the class III peroxidase group in terms of encoded genes and substrate specifities (Marjamaa *et al*., 2009). Hence, it is not unlikely that the POD exhibiting reduced transcript levels under +Si conditions might have functions different from those in suberin and lignin synthesis.

Conclusion

The effects of silicic acid on the rice root anatomy and on the transcription of genes related to suberin and lignin synthesis are summarized in Fig. 6. Si nutrition of rice plants reduced the oxidation power of roots and enhanced development of casparian bands in exodermis and endodermis, as well as lignin depositions in the sclerenchyma. These changes are likely the reason for the reduced ROL and might be useful for the plants to grow in anaerobic soils and cope with unfavourable conditions. Increased suberization and lignification was accompanied by silicic acid triggered transcription of genes related to lignin and suberin metabolism. In addition, high impact of silicic acid supply on transcript level of a LRR-RLK gene could be observed, highlighting the possibility that this regulating protein plays a central role either in perceiving a Si signal of up to now unknown nature or in promoting suberin and lignin synthesis or in both.

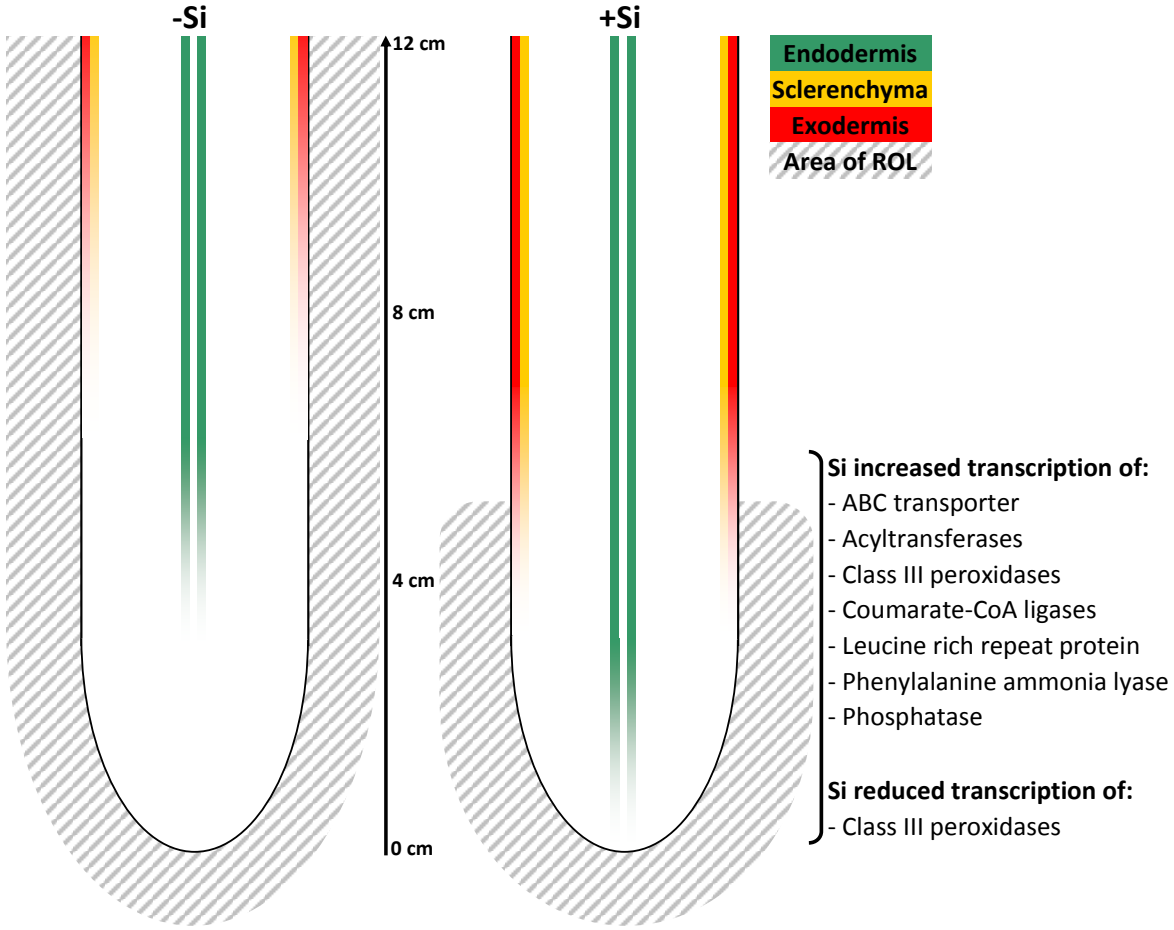

Fig. 6: Model of the rice root and transcript levels of genes as affected by silicic acid. Si supply reduced area of radial oxygen loss (ROL) to 5 cm distance from root tip and hence oxidation power. This coincided with an increased suberization of exodermis and lignification of sclerenchyma by Si treatment. Also, Si supply enhanced suberization of endodermis. These changes indicated by Si supply were related to an increased transcription of genes associated with suberin and lignin synthesis.

Supplementary material

Supplementary Tables S1 and S2 show the effect of silicic acid on relative quantity of transcripts in root segment 0-2 cm and 4-6 cm distance from root tip, respectively, as measured by microarray analysis.

Chapter II

Further characterization of silicon uptake and silicon effect on casparian bands

Alexander T. Fleck, Diane Prüß, Sascha Schulze, Manfred K. Schenk

to be sumbitted

Institute of Plant Nutrition, Faculty of Natural Sciences,
Leibniz University Hannover, Herrenhäuser Str. 2, 30419 Hannover, Germany.

Abstract

Silicon (Si) promotes the formation of casparian bands in the exodermis and endodermis in rice roots. Si is present in varying concentration in the soil solution of all soils and therefore, the effect of different concentrations of Si on the development of casparian bands in rice was investigated. Also the kinetics of the Si uptake and effect of short term Si supply on casparian band formation in rice were studied. Roots are constantly exposed to Si in the soil solution and hence, an effect of Si should be possible in the root independent from the ability of a plant to accumulate Si. Thus, we studied the effect of Si supply on casparian band formation in the roots of different plant species with varying Si shoot concentration.

The Si concentration in rice plants increased up to a Si supply of 12 mg L^{-1} and Si uptake was performed by transporters with estimated K_m and V_{max} values of 0.1 mM and 0.87 mg g^{-1} root DW h^{-1}, respectively. Casparian band formation was increased with increasing Si supply up to 12 mg Si L^{-1}. Casparian band formation in roots of Si-depleted rice plants was increased not before 48 hours after Si supply:

Si supply of rice WT, rice mutant *lsi1*, maize, nug, tradescantia and onion increased formation of casparian band in all plant species independent from the Si concentration in the shoot.

Introduction

Silicon (Si) is the second most abundant element in the earth crust with concentrations in soils ranging from below 1 to 45 % on a dry weight basis (Sommer *et al*., 2006). The soluble and plant available form of Si at pH-values below 9 is ortho-silicic acid, $Si(OH)_4$, with concentrations between 3.5 and 40 mg Si L^{-1} in soil solution (Epstein, 1999), albeit concentrations exceeding 50 mg Si L^{-1} were also found in flooded soils (Bogdan and Schenk, 2008). Si concentration varies widely not only in soil solution, but also in shoot tissue of plants, where the span is from below 1 to 100 mg g^{-1} DW (Epstein, 1999). Large amounts of Si in the shoot are found in the family *Equisetaceae* and, among the monocotyledons, in the *Cyperaceae* and *Poaceae*, the latter comprising rice (*Oryza sativa*) and maize (*Zea mays*), whereas most dicotyledons show low Si accumulation (Epstein, 1999; Hodson *et al*., 2005). The different Si concentrations have been explained by active, passive or rejective uptake modes of Si and there is evidence for all modes in different plant species, which can be divided into Si accumulators, intermediate types and non-accumulators or excluder species (Liang *et al*., 2006). The Si uptake in cucumber, tomato, maize and rice was shown to be active and to be mediated by a radial transport of Si from the external solution via root cortical cells towards xylem (Mitani and Ma, 2005; Mitani *et al*., 2009b).

Ma *et al*. (2006, 2007) identified two Si transporters in rice, Lsi1 and Lsi2, which are located in the exodermis and endodermis of rice roots and are responsible for the transport of silicic acid from the rhizosphere to the stele. In maize, two homologous genes of Lsi1 and Lsi2 have been found that also function as Si transporter (Mitani *et al*., 2009a, b).

Despite the large amount of Si in the shoots of many plants, Si is not considered essential for higher plants. However, Si exhibits a beneficial element for plants, as it reduces several abiotic and biotic stresses including drought, salinity and lodging as well as diseases caused by fungi and bacteria (Epstein, 1999). The extent of beneficial effects of Si on plants differs between species and is usually best visible in plants with high Si accumulation in shoot (Ma *et al*., 2001).

Paddy rice is grown in flooded fields under usually anaerobic and reducing conditions and rice has adapted to this environment by development of the aerenchyma that exhibits a gas filled space in the roots and facilitates the diffusion of gases within the root (Ponnamperuma, 1984; Colmer, 2003a). The diffusion of oxygen from the aerenchyma to the anaerobic rhizosphere (called radial oxygen loss; ROL) is reduced by a barrier, which is attributed to a

suberized exodermis with casparian bands and a lignified sclerenchyma (Armstrong *et al*, 2000; Kotula and Steudle, 2008).

In a study with rice grown in nutrient solution, ROL was reduced and formation of casparian bands in the exodermis was increased when Si was supplied at a concentration of 30 to 50 mg L^{-1} (Fleck *et al*., 2011). Furthermore, Si was shown to enhance transcription of genes related to the synthesis of suberin and lignin, which are the main components of casparian bands (Hose *et al*., 2001). Although Si concentrations used in this study are also found in paddy rice soils, Si levels in soil solution of many soils are lower (Epstein, 1994; Bogdan and Schenk 2008). So, this study aimed at investigating the effect of different doses of Si on rice root development. In addition, the kinetics of Si uptake and effect of short term Si supply on formation of casparian bands in the exodermis were studied.

Albeit Si concentration in shoot substantial varies between plant species, roots are continuously exposed to silicic acid in soil solution. Thus, the effect of Si on development of casparian bands should in principle occur not only in rice or other Si accumulating species, but also in low Si accumulators or even Si excluders. Thus, we studied the effect of Si supply on the roots of plant species with varying Si shoot concentrations.

Material and methods

Plant material and growth conditions

Silicon dose response experiment (first experiment)

Rice *(Oryza sativa* L. cv. Selenio) seeds were germinated in tap water for seven days and then placed between two layers of filter paper standing in tap water for additional seven days.

Seedlings were transferred to a circulation system of non-aerated nutrient solution containing in mM: 1.43 NH_4NO_3, 0.32 NaH_2PO_4 x H_2O, 0.51 K_2SO_4, 1 $CaCl_2$ x 2 H_2O, 1.6 $MgSO_4$ x 7 H_2O; in µM: 1.82 $MnSO_4$, 0.03 $(NH_4)_6Mo_7O_{24}$, 9 H_3BO_3, 0.3 $ZnSO_4$ x 7 H_2O, 0.15 $CuSO_4$ and 35.81 Fe as sequestrene. Silicon concentrations were 0, 2.5, 5, 10 and > 25 mg Si L^{-1} and Si was applied as silica gel. The pH-value was adjusted to 6.0 by addition of 10 % (v/v) H_2SO_4 and 0.75 M KOH. Nutrient solution was controlled every two days and pH and Si concentration were adapted if necessary. Plants were grown in a greenhouse with additional assimilation light (photoperiod: 16 h light, 8 h dark; temperature 26°C day / 20°C night; light intensity ~ 180 µmol m^{-2} s^{-1}) from April to May 2010.

For each Si treatment, 330 L nutrient solution were pumped from a 210 L storage tank to four 30 L cultivation containers with a flow rate of 8 ml s^{-1} and returned to the storage tank. In each cultivation container, twelve rice plants were grown and reduced to ten plants after two weeks. Plants were harvested after growing 22 days in nutrient solution.

Silicon kinetics experiment (second experiment)

Rice cultivar, germination procedure and composition of nutrient solution were the same as in the dose response experiment. Seedlings were cultivated for 25 days in nutrient solution without Si. Nutrient solution was renewed after 7, 14 and 20 days and pH was adjusted daily to 6.0 as described before. After 25 days in –Si nutrient solution, half of the plants were transferred to nutrient solution containing 40 mg Si L^{-1}, which was applied as silica gel, while the other half remained in renewed Si-free nutrient solution. The treatments consisted of four replicates with each 18 plants in 20 L pots. 6, 12, 24, 48 and 72 h after change of nutrient solution, three plants per replicate were harvested. The plants were grown in a growth chamber (photoperiod: 14 h light, 10 h dark; temperature 25°C day / 20°C night; relative humidity 75 %; light intensity 220 µmol m^{-2} s^{-1}).

Silicon effect on different plant species (third experiment)

Plant species with different shoot Si concentrations were screened for roots that have an exodermis and are suitable for free hand cross sectioning. The selected plants were rice, maize (*Zea mays*), onion (*Allium cepa*), tradescantia (*Tradescantia virginiana*) and nug (*Guizotia abyssinica*). Rice wildtype (WT) cv. Selenio was described to react upon Si supply with enhanced formation of casparian bands in the root (Fleck *et al*., 2011) and was used as positive control. The rice mutant *lsi1*, which is defective in Si uptake, was included in this experiment, too. This mutant carries a loss-of-function mutation of the gene *Lsi1* that codes for the Si transporter Lsi1 (Ma *et al.*, 2006). Seeds of *lsi1* were kindly provided by Prof. Jian Feng Ma, Okayama University, Japan.

Rice cultivars Selenio and *lsi1* were germinated as described in the dose response experiment and then cultivated in non-aerated nutrient solution. Maize was germinated between two layers of filter paper for 7 days and than cultivated in aerated nutrient solution. Onion bulbs and seeds of nug were cultivated in peat substrate for 7 and 14 days, respectively, roots were washed with tap water and plants were then transferred into aerated nutrient solution. Tradescantia was taken from a garden in Hannover, roots were washed with tap water and plants were transferred into aerated nutrient solution.

Composition of nutrient solution and pH correction were the same as described above. Si was supplied as silica gel to the high Si treatment (+Si; 26 - 41 mg L^{-1}), while no Si was applied to the control treatment (–Si) (Supplementary Fig. 5). The treatments consisted of four replicates with each 10-12 plants in 5 L pots. The plants were grown in a growth chamber (photoperiod: 14 h light, 10 h dark; temperature 25°C day / 20°C night; relative humidity 75 %; light intensity 220 µmol m^{-2} s^{-1}). Since root growth differed between the plant species (Supplementary Fig. 8), plants were cultivated in nutrient solution for 12 (tradescantia), 16 (maize, nug, onion), 21 (rice WT) and 30 days (rice *Lsi1*), respectively.

Determination of root length growth and silicon concentration

To measure root length growth, three roots per replicate were marked with a permanent marker 2 cm behind the root tip. After 48 h, distance from mark to root tip was measured and root growth was calculated.

Plant matter was dried at 60°C for four days, ground and digested overnight in a mixture of 1 M HCl and 2.3 M HF (1:2) (Novozamsky *et al*., 1984). After addition of 3.2 % (w/v) boric acid, dye reagent (0.08 M sulphuric acid and 2 % (w/v) ammonium heptamolybdate), 3.3 %

(w/v) tartaric acid and 0.4 % (w/v) ascorbic acid, Si concentration in digested plant material and nutrient solution was photometrically determined at 811 nm.

Determination of silicon uptake rate

Root growth was assumed to be linear during plant cultivation and the Si uptake rate in the dose response and the kinetic experiment was calculated according to Claassen, 1990:

$$Up = \frac{Si_2 - Si_1}{t_2 - t_1} \times \frac{2}{DW_2 + DW_1}$$

where Up = uptake rate [mg g^{-1} root DW h^{-1}]; $Si_{1,2}$ = Si content in shoot [mg $plant^{-1}$] at start (1) and end (2) of cultivation in nutrient solution; $DW_{1,2}$ = root dry weight at start (1) and end (2) of cultivation in nutrient solution [g $plant^{-1}$]; $t_{1,2}$ = time at start (1) and end (2) of cultivation in nutrient solution [h].

Histochemical examination of adventitious roots

For detection of casparian bands, free hand cross sections of roots without lateral roots were taken at two different distances from the root tip (drt). In the dose response and the kinetic experiment, sections of adventitious rice roots were taken at 0-2 and 4-6 cm drt.

Prior to harvest in the third experiment, root sections along the whole root were taken and checked for casparian bands. The 2 cm-zone where formation of casparian bands started was defined as root zone A and the 2 cm-zone 2 cm behind as root zone B and both zones were harvested for determination of casparian bands. Position of root zones A and B for each species are described in results.

Root cross sections were stained with 0.1 % (w/v) berberine hemi-sulphate for 60 minutes and with 0.5 % (w/v) aniline blue for further 30 minutes (Brundrett *et al*., 1988). Stained sections were mounted in 0.1 % (w/v) $FeCl_3$ in 50 % (v/v) glycerine and viewed under an Axioskop fluorescence microscope (Zeiss, Jena, Germany) with UV illumination using excitation filter G 365, chromatic beam splitter FT 395 and barrier filter LP 420. Pictures were taken with the AxioCam MRc (Zeiss) and picture recording software (AxioVision Ac, Version 4.4, Zeiss). Under UV light, suberin exhibited a blue-white colour. The development of casparian bands in the anticlinal exodermal cell walls was determined and allocated to one of four stages: 0 % (stage I), 0-25 % (II), 25-50 % (III) and 50-100 % (IV) development of casparian bands in the anticlinal cell wall of the exodermis. The degree of development of casparian bands was calculated using the arithmetic mean. For this purpose, the number of cells of each stage were multiplied by their respective stage (stage I = 1, II = 2, III = 3, IV = 4), the products were

summed and the sum was divided by the total number of cells. The degree of development of casparian bands was minimum 1 and maximum 4, relating to no or full development of casparian bands, respectively.

From each replicate in the dose response experiment, ten roots without lateral roots were taken for cross sectioning and each ten cells from ten cross sections were examined. In the second and third experiment, ten roots without lateral roots per replicate were taken for cross sectioning and each 20 cells from five cross sections were used for microscopic examination. In all experiments, degree of development of casparian bands was calculated on basis of 400 cell walls per treatment.

Statistical analysis

In all experiments, treatments were replicated four times and mean of the treatments were compared with t-test or tukey-test after ANOVA with $p < 0.05$ using R software (R Development Core Team, 2011).

Results

Silicon dose response

Si concentration in nutrient solution was constant during the cultivation period for all Si levels except the highest Si treatment, where it increased continuously (Supplementary Fig. 1). The Si concentrations were on average 0.7, 2.5, 4.9, 12.1 and 25.9 mg L^{-1} for the Si 0, 2.5, 5, 10 and >25 treatments, respectively. From day 8 to 21, Si concentration in the highest treatment was between 25 and 35 mg L^{-1}. Shoot and root yield of rice plants after 22 days differential Si treatment was not significantly affected by Si supply (Supplementary Fig. 2).

The Si concentration in root was lowest for the plants without Si supply and increased up to the 5 mg Si L^{-1} treatment, but this change was not significant (Fig. 1). Shoot Si concentration was more than one order of magnitude higher than in root and increased from 14 mg g^{-1} DW in the Si 0 treatment up to 100 mg g^{-1} DW in the second highest Si treatment, while higher Si supply was not effective. When drawing a nonlinear regression between Si supply and Si uptake, the estimated K_m and V_{max} values were 2.83 mg L^{-1} (equivalent to 0.1 mM) and 0.87 mg g^{-1} root DW h^{-1}, respectively (Fig. 2).

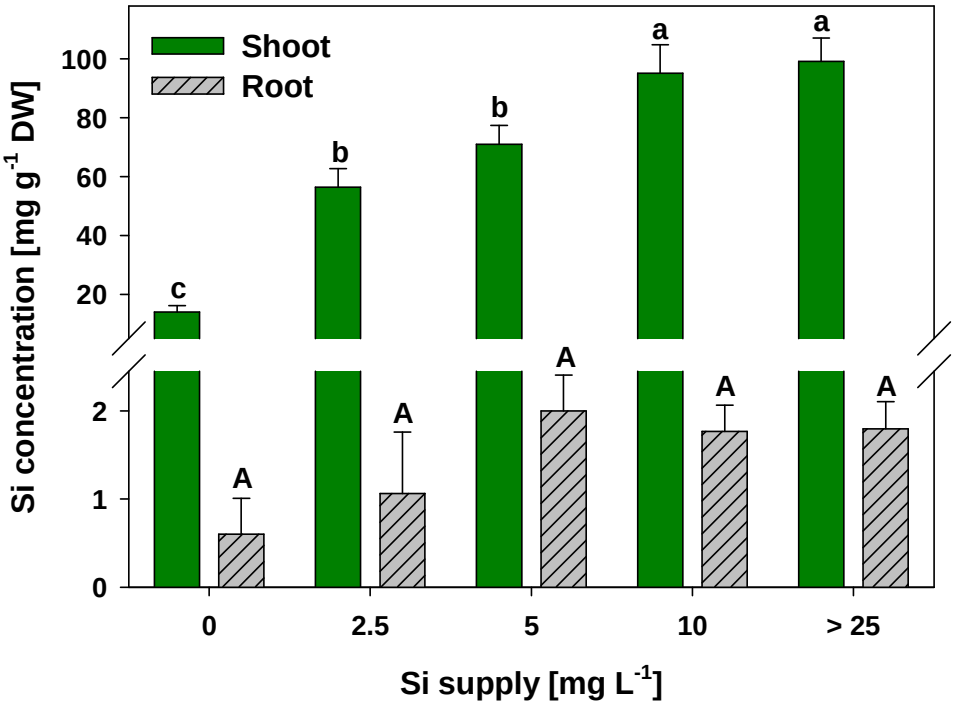

Fig. 1: Si concentration in shoot and root of rice plants as affected by Si supply in nutrient solution. Data are mean ± SE, n = 4. Different capital and small letters indicate significant difference between Si treatments in shoot or root, respectively; tukey-test with $p < 0.05$. Data are adapted from Prüß (2010).

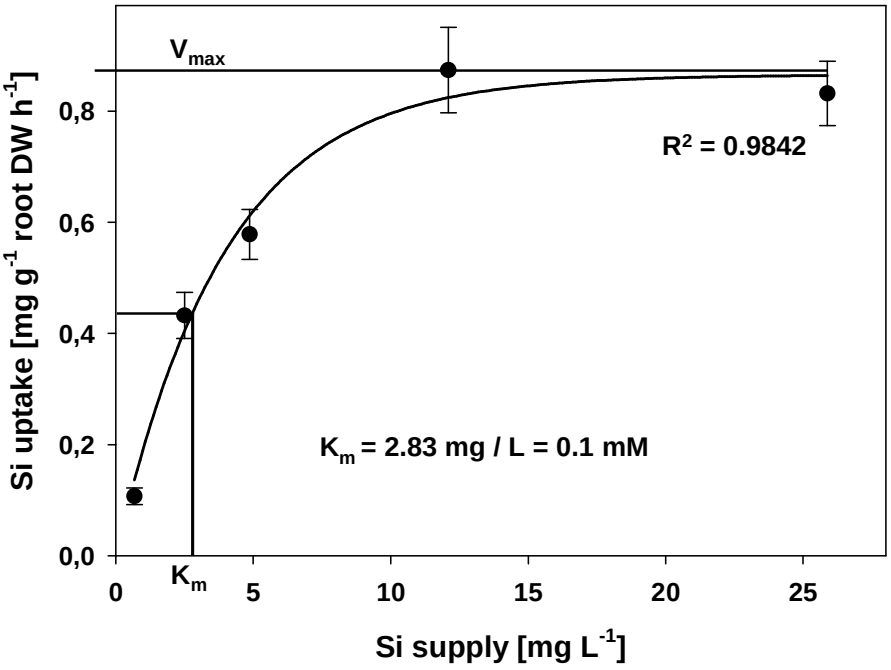

Fig. 2: Silicon uptake of rice roots grown in nutrient solution with different Si concentrations. Data are adapted from Prüß (2010).

Development of casparian bands in the exodermis in 4-6 cm distance from root tip (drt) was lowest in the Si 0 treatment and increased up to the Si10 treatment, while the highest Si supply was not further effective (Fig. 3). Casparian bands in the exodermis in 0-2 cm drt were not observed in the 0 and 2.5 mg Si L^{-1} treatments and were only marginal in the higher Si concentrations (data not shown).

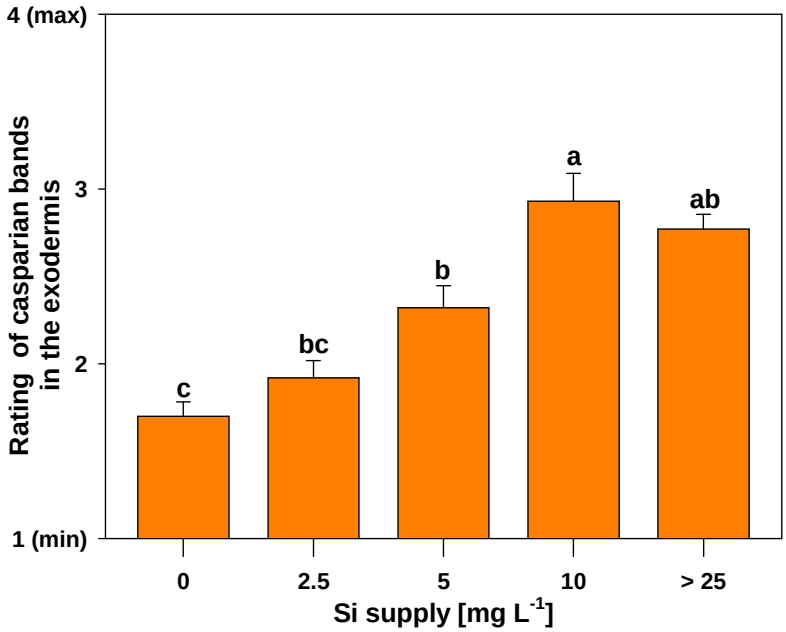

Fig. 3: Development of casparian bands in exodermis of adventitious rice root in 4-6 cm distance from root tip as affected by Si supply in nutrient solution.

Anticlinal cell walls were determined under microscope and classified in 0, 0-25, 25-50 or > 50 % developed casparian band. Shown at the x-axis is the arithmetic mean of 400 examined cell walls (1 = no casparian band; 4 = fully developed casparian band). Data are mean ± SD, n = 4. Different letters indicate significant difference between Si treatments; tukey-test with $p < 0.05$. Data are adapted from Prüß (2010).

Silicon kinetics

Si concentration in nutrient solution of the –Si treatment was permanently below 2 mg L^{-1} (Supplementary Fig. 3). At day 25, half of the plants were transferred into +Si nutrient solution, which initially contained 40 mg Si L^{-1} but contained only 7 mg Si L^{-1} after 72 h. Shoot and root dry weight was not affected by Si supply (Supplementary Fig. 4) and also root growth did not differ between Si treatments (–Si: 2.03 ± 0.13 cm 24 h^{-1}; +Si: 1.98 ± 0.03 cm 24 h^{-1}).

Si concentration in root was between 1 and 2 mg g^{-1} DW in both Si treatments and unaffected by Si supply (Fig. 4). Shoot Si concentration of control plants was constant at around 4 mg g^{-1} DW while plants supplied with Si rapidly accumulated Si in their shoots. Shoot Si concentration was doubled compared to control already after 6 h Si treatment.

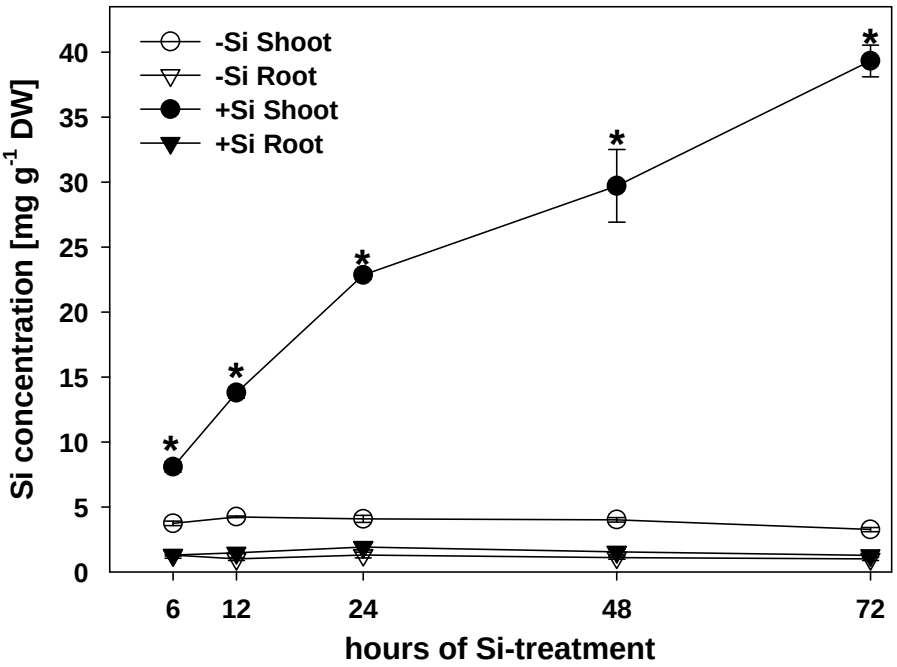

Fig. 4: Si concentration in shoot and root of rice plants as affected by short term Si supply in nutrient solution. Data are mean ± SE, n = 4. Asterisks indicate significant difference between Si treatments in shoot or root; t-test with $p < 0.05$.

Status of casparian bands in the exodermis was determined in 0-2 and 4-6 cm drt and generally, casparian bands were stronger developed in older root zones than near the root tip (Fig. 5). Within 24 h after starting the Si treatment, development of casparian bands was not altered in both root zones. However, 48 h after roots were supplied with Si, formation of casparian bands was clearly enhanced in both root zones of +Si plants and increased further during the course of the experiment.

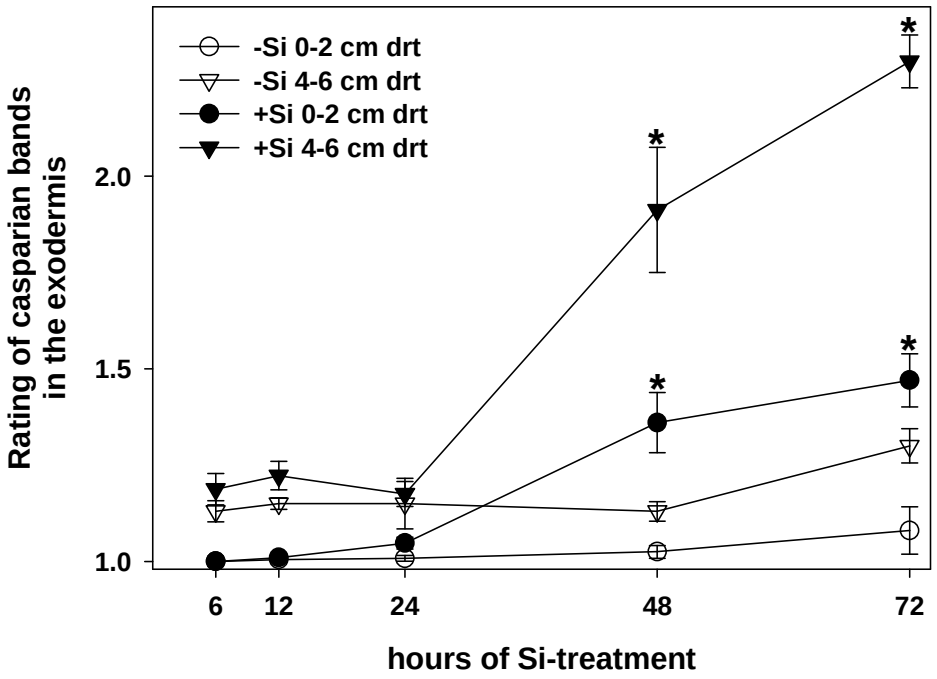

Fig. 5: Development of casparian bands in exodermis of adventitious rice root in 0-2 and 4-6 cm distance from root tip as affected by short term Si supply in nutrient solution.

Anticlinal cell walls were determined under microscope and classified in 0, 0-25, 25-50 or > 50 % developed casparian band. Shown at the x-axis is the arithmetic mean of 400 examined cell walls (1 = no casparian band; 4 = fully developed casparian band). Data are mean ± SD, n = 4. Asterisks indicate significant difference between Si treatments in a root section; t-test with $p < 0.05$.

Silicon effect on silicon accumulators and non-accumulators

Six plant species and cultivars differing in their shoot Si concentration were cultivated in nutrient solution either with or without Si. In the control treatment without additional Si supply, Si concentration in nutrient solution was below 1 mg L^{-1}, while it was on average 32 mg L^{-1} in +Si treatment (Supplementary Fig. 5).

Shoot DW of rice WT, rice mutant *lsi1*, maize, nug and tradescantia was unaffected by Si treatment, while onion shoot DW was increased by Si supply (Supplementary Fig. 6). Si supply also increased root DW of onion but decreased that of tradescantia (Supplementary Fig. 7). Root DW of the other plant species was not affected by Si supply.

The root growth rates differed widely between the plant species and was lowest for onion (6 mm 24 h^{-1}) and highest for nug (32 mm 24 h^{-1}) but root growth of all plants was not affected by Si supply (Supplementary Fig. 8).

Shoot Si concentration of all species grown without Si supply was similar and ranged from 0.3 to 0.5 mg g^{-1} DW (Fig. 6A). With Si supply, shoot Si concentrations in all plants were

clearly enhanced except for onion, where Si concentration in shoot was on the same level like in control plants. Root Si concentration of the plants in the –Si treatment differed between the species and ranged from 0.3 in onion to 2.7 mg Si g^{-1} DW in tradescantia (Fig. 6B). With Si supply, concentration of Si in root increased in all plants.

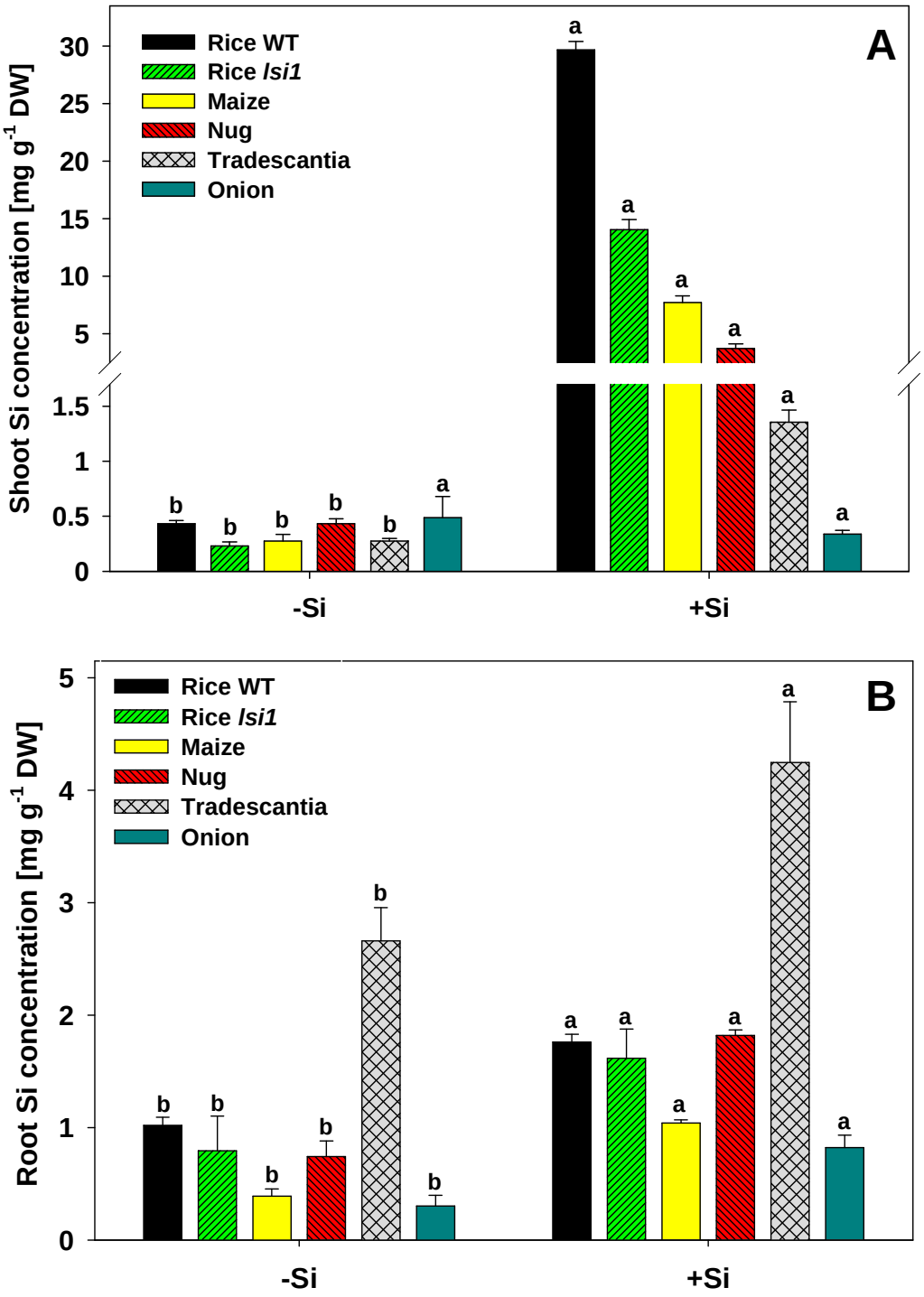

Fig. 6: Si concentration in shoot (A) and root (B) of six plant species and cultivars as affected by Si supply in nutrient solution. Data are mean ± SE, n = 4. Different letters indicate significant difference between Si treatments of a species; t-test with $p < 0.05$. Data are adapted from Schulze (2011).

Formation of casparian bands started in the plant species in different distances from the root tip. This zone A was in 0-2 cm drt for both rice cultivars and onion, in 2-4 cm drt for maize and tradescantia and in 8-10 cm drt for nug. In all plant species, casparian bands were stronger developed in older parts of the root than in younger ones (Fig. 7). Si treatment significantly increased casparian band formation in root zone A in all plant species except tradescantia and onion, while in root zone B, Si enhanced formation of casparian bands in all plant species.

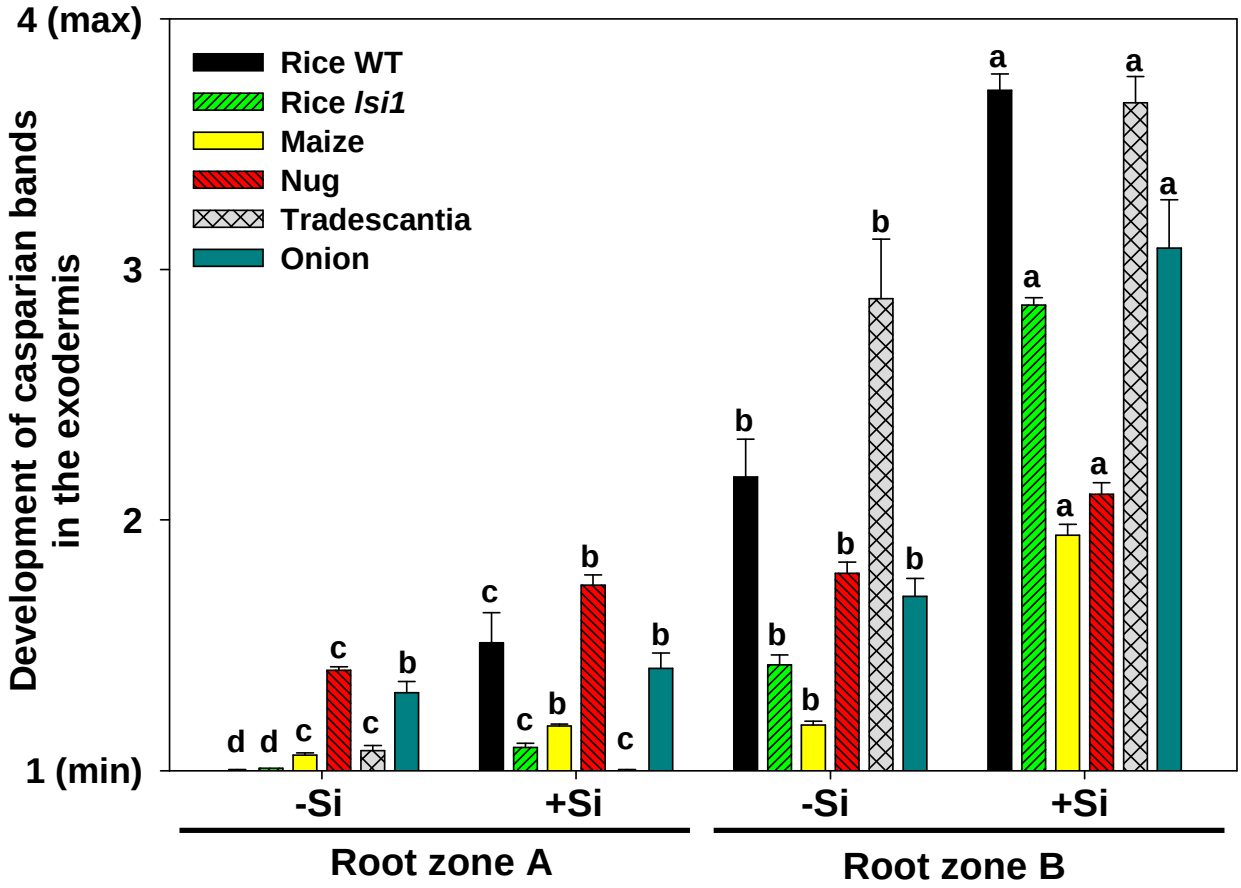

Fig. 7: Development of casparian bands in exodermis of six plant species and cultivars as affected by Si supply in nutrient solution.

Root zone where formation of casparian bands started was defined as root zone A. This was in 0-2 cm drt for rice wildtype, rice *lsi1* and onion, in 2-4 cm drt for maize and tradescantia and in 8-10 cm drt for nug. Root zone B started 2 cm behind end of root zone A. Anticlinal cell walls were determined under microscope and classified in 0, 0-25, 25-50 or > 50 % developed casparian band. Shown at the x-axis is the arithmetic mean of 400 examined cell walls (1 = no casparian band; 4 = fully developed casparian band). Data are mean ± SD, n = 4. Different letters indicate significant difference between Si treatments and root sections of a species; tukey-test with $p < 0.05$. Data are adapted from Schulze (2011).

Discussion

Silicon in nutrient solution and effect of silicon on plant growth

In all three experiments, the Si concentration in the nutrient solution was around 1 mg L^{-1} when no Si was supplied (Supplementary Fig. 1, 3, 5). This is still several times higher than the concentration of micronutrients like manganese, boron or zinc in the nutrient solution. The high Si concentration in the –Si treatments might be due to residual amounts of Si even in the demineralized water that was used for setting up nutrient solution. It is a long known problem to get nutrient solutions completely purged from Si especially when evaluating the essentiality of Si for plants (Lewin and Reimann, 1969; Epstein 1994). However the application of silica gel clearly enhanced the Si concentration in the nutrient solution in all cases compared to the control.

In the dose response experiment, the five Si treatments differed from each other in their Si concentration in nutrient solution during the whole cultivation period. Moreover, except for the highest Si treatment, where the Si concentration was continuously increasing, the Si concentration within a treatment remained on a constant level, which is a prerequisite for investigating the dose response of a chemical. This was possible by using a large volume of nutrient solution since rice plants efficiently take up Si and rapidly deprive the solution of Si (Ma and Yamaji, 2008) (Supplementary Fig. 3). In all experiments, shoot and root dry weight of rice plants was not significantly affected by Si supply (Supplementary Fig. 2, 4, 6, 7). Si is known to enhance the growth of rice grown in paddy rice fields as well as of other Si accumulating plant species such as wheat and sugar cane (Lewin and Reimann, 1969; Cheng, 1982; Ma *et al*., 1989; Epstein, 1999). However, cultivation of plants in nutrient solution in a green house or a growth chamber exhibits a low-stress environment and under these near-optimum conditions, positive effects of Si may not occur.

In the third experiment, shoot and root dry weight of all plant species was not altered by Si supply except for onion and tradescantia (Supplementary Fig. 6, 7). Root and shoot yield of onion was increased by Si supply, what is in contrast to literature (Lewin and Reimann, 1969). An explanation for the observed yield enhancement can not be given. On the other hand, tradescantia root dry weight was lower when grown in +Si nutrient solution compared to control. It is assumed, that the initial root mass was higher for the plants cultivated in –Si than in +Si nutrient solution, since tradescantia plants taken from a garden were not fully homogeneous. However, root growth rate of all plants was unaffected by Si supply (Supplementary Fig. 8).

Silicon concentration in rice plants and silicon uptake

The Si concentration in the rice roots was similar in all experiments when no Si was applied to the nutrient solution (Fig. 1, 4, 6B). The root Si concentration increased with continuous Si supply, while it was not altered within 72 h after Si supply in the kinetic experiment. The Si concentration in the shoot was an order of magnitude higher in rice plants grown in the Si 0 treatment in the first experiment than in plants grown without Si supply in the second and third experiment (Fig. 1, 4, 6A). This difference can be explained by the rapid depletion of residual Si in the –Si treatments in the second and third experiment, where plants were grown in 5 L pots, while plant in the dose response experiment were cultivated in large volumes of nutrient solution, where Si was not completely depleted and plants could constantly take up Si. With Si supply, the Si concentration in the shoot of rice plants were highest in the Si 10 treatment in the first experiment with values of 100 mg g^{-1} DW (Fig. 1), which were reported as maximum concentrations for rice plants (Epstein, 1994). With higher Si supply, there were no higher Si shoot levels, because Si uptake was already saturated at about 12 mg Si L^{-1} and K_m and V_{max} values were estimated to be 0.1 mM and 0.87 mg g^{-1} root DW h^{-1}, respectively (Fig. 2).

The Si uptake of rice has previously been shown to follow Michaelis-Menten kinetics with an estimated K_m value of 0.32 mM and a V_{max} of 6.2 mg g^{-1} root DW h^{-1} (Tamai and Ma, 2003). In a study using ^{68}Germanium as a tracer for Si, K_m value of Si transport in rice was 0.35 mM and V_{max} was 8.7 mg g^{-1} root DW h^{-1} (Nikolic *et al*., 2007). Mitani and Ma (2005) determined Si transport in rice, cucumber and tomato and found a K_m value of 0.15 mM in all the three species, whereas V_{max} was different.

While the K_m value determined in the dose response experiment is in the same order of magnitude as reported in literature, the V_{max} value in our study is lower compared to literature reports. Reason might be different experimental conditions, since in these studies plants were young (5-11 days) and Si uptake experiments were conducted for a short time (6-8 h), while in our study plants were grown with different Si levels for 22 days, implying the exposure to day and night cycles with the accompanying changes in temperature, light intensity and transpiration. Moreover, plants in the dose response experiment were continuously exposed to Si, while in the mentioned studies plants were Si-depleted because they were grown in nutrient solution without Si prior to Si uptake experiments. Therefore the transcription level of the Si transporter *Lsi1* and *Lsi2* likely was lower in our experiment compared to literature studies, since Ma *et al*. (2006, 2007) demonstrated that the expression of the Si transporters was decreased after continuous Si supply for 3 days.

Rice shoot Si levels in the kinetic experiment rapidly increased with Si supply and were twice as high in +Si plants compared to control already 6 h after differential Si treatment (Fig. 4). The estimated uptake rate of the rice plants within 24 h of Si supply is 4.5 ± 0.21 mg g^{-1} root DW h^{-1}. This is similar to the V_{max} values reported in literature presumably because both, plants in the kinetic experiments and literature were Si-depleted before Si uptake experiment (Tamai and Ma, 2003; Nikolic *et al*., 2007).

Silicon concentration and uptake in different plant species

In the third experiment, all plants exhibited similar shoot Si concentration when grown in –Si nutrient solution (Fig. 6A). However when Si was supplied, all plant species except onion had clearly enhanced shoot Si levels with the highest Si concentration in rice WT, followed by the rice mutant *lsi1*, maize, nug and tradescantia. Si concentration in onion shoot was not elevated when grown with Si, what is consistent with literature reports that describe onion as Si non-accumulator (Lewin and Reimann, 1969). Furthermore, the family *Amaryllidaceae*, to which onion belongs, has a very low ranking in the extensive list about the mean relative shoot Si concentration of 735 plant species presented by Hodson *et al*. (2005). Also the shoot Si concentration of the plant species grown with Si supply in our study is in accordance with the ranking of the species (or the respective families or orders) in this list.

The high shoot Si level in rice can be explained by the Si transporters Lsi1 and Lsi2 (Ma *et al*. 2008). The second highest Si concentration in the shoot is found in the rice mutant *lsi1*, where the gene *Lsi1* is non-functional. However, the transporter Lsi2, which actively transports Si from exodermal cells towards the cortex and from endodermal cells towards the xylem, is still functioning and enables the rice mutant to accumulate Si in the shoot. Also in maize, two Si transporters have been identified that facilitate Si accumulation of maize (Mitani *et al*., 2009a, b). For nug and tradescantia, no Si transporters are known but shoot Si concentration could be explained solely by passive Si transport via transpiration stream. The amount of Si that makes the difference between –Si and +Si shoot for a nug and tradescantia plant is contained in 66 and 24 ml nutrient solution (with average 30 mg Si L^{-1}), respectively. According to transpiration coefficient values between 200 and 300 ml H_2O g^{-1} shoot DW reported for several plant species (van der Vorm, 1980), the estimated volume transpired by nug and tradescantia during the cultivation was at least 110 ml per plant and thus, sufficient to passively transport Si into the shoot. However, it is not known whether the mobility of silicic acid in the root is similar to that of water.

Taken together, the plant species can be classified as Si accumulators (both rice cultivars, maize), intermediate type (nug, tradescantia) and excluder plants (onion) according to their shoot Si accumulation ability as suggested by Takahashi *et al*. (1990). The disability of onion to accumulate Si in the shoot did not hinder the root to accumulate a certain amount of Si, since the root Si concentration of all plants increased with Si supply (Fig. 6B).

Effect of silicon concentration and dynamics on development of casparian bands in rice roots

Continuous Si supply enhanced development of casparian bands in the exodermis of rice plants (Fig. 3, 7), what confirms the observations made previously (Fleck *et al*., 2011). The strongest promotion of casparian bands in the dose response experiment was in the Si 10 treatment, where the average Si concentration in the nutrient solution was 12 mg L^{-1} (Supplementary Fig. 1). Hence, Si is supposed to be effective in most soils, since the Si concentration in soil solution normally ranges between 3 and 20 mg L^{-1} (Epstein, 1994, 1999). Moreover, paddy rice is grown in flooded fields under anaerobic and reducing conditions, which favor the release of Si into the soil solution, and so higher concentrations even exceeding 50 mg Si L^{-1} are also reported (Ponnamperuma, 1984; Bogdan and Schenk, 2008). However, other environmental factors like anaerobic conditions or salinity were also shown to enhance formation of casparian bands, so situation under field conditions is more complex (Colmer, 2003b; Schreiber *et al*., 2007; Kotula *et al*., 2009a). In the kinetic experiment promoted development of casparian bands in the rice roots was observed not before 48 hours after Si supply (Fig. 5), suggesting that Si does not directly induce casparian band formation.

Effect of silicon on development of casparian bands in different plant species

In all experiments and all plant species, formation of casparian bands in the exodermis was stronger in older parts of the root than in younger parts (Fig. 3, 5, 7), what is in line with literature reports (Schreiber *et al*., 1999; Ranathunge *et al*., 2003, 2011; Fleck *et al*., 2011).

Without Si supply, casparian bands appeared in rice WT, rice mutant *lsi1* and onion close to the root tip, in few cm from the root tip in maize and tradescantia, while formation of casparian bands started in more basal parts of the roots in nug (root section A) (Fig. 7). An early development of casparian bands coincided with a low root growth rate (Supplementary Fig. 8), which is in accordance with reports that a reduced root growth resulted in earlier development of casparian bands in maize and rice (Perumalla and Peterson, 1986; Gong *et al*., 2006; Schreiber *et al*., 2007).

Si supply increased formation of casparian bands in the exodermis of all plant species (Fig. 7). For rice, this is in line with the dose response and the kinetic experiment and a previous study (Fleck *et al*., 2011). However, for the other plants comprising Si accumulators, intermediate type plants, and a Si excluder, this has not been shown before.

Recently, Vakulic *et al*. (2012) reported that treatment of maize seedlings with 5 mM Si resulted in the development of exodermal suberin lamellae in older root parts compared to –Si plants. However, Vakulic *et al*. used primary roots and we investigated adventitious roots.

In rice, suberization of the root exodermis combined with lignification of the sclerenchyma reduces the radial oxygen loss from the aerenchyma to the surrounding medium by forming a physical barrier against gas movement (Armstrong, 1979; Kotula and Steudle, 2008). In general, the casparian band in the exodermis limits apoplastic flow and allows for the controlled flux of solutes into the cortex (Hose *et al*., 1999). Furthermore, colonization of the cortex by fungal or bacterial organisms may be prevented by this barrier (Enstone *et al*., 2003). Thus, promoted development of casparian bands could pose an advantage for plants and this might contribute to the reported beneficial effects of Si on plant growth (Epstein, 1999; Ma *et al*., 2001).

Possible mechanisms of silicon-enhanced development of casparian band

Si might induce precipitation or cross-linking of aromatic compounds such as suberin, which contains a polyaliphatic and a polyaromatic domain (Kolattukudy, 1984), or lignin that is a complex mixture of phenolic compounds that derive mostly from the three monolignols p-coumaryl, coniferyl and sinapyl alcohol (Boerjan *et al*., 2003; Goujon *et al*., 2003). It was shown that silica gel or colloidal silica can form complexes with the phenol catechol as six-coordinated Si (Barnum 1970, 1972). There is also evidence that lignin can induce silica deposition (Fang and Ma, 2006). Inanaga *et al*. (1995) proposed that Si is associated with phenolic acids and is involved in cross-linking between lignin and carbohydrates in the cell walls of rice. Furthermore, the hemi-cellulose callose was shown to induce precipitation of silica in horsetail (*Equisetum arvense*), where Si is found along with callose in the cell walls (Currie and Perry, 2009; Law and Exley, 2011). Currie and Perry (2007) suggested that precipitated silica particles have a negative surface charge and therefore may interact with the plant cell wall.

In rice, Si was found as deposited silica in the tangential and in the radial cell walls of the endodermis (Parry and Soni, 1972; Shi *et al*., 2005). Gong *et al*. (2006) showed that silica is deposited not only in the endodermis but also in the exodermis. More recently, Moore *et al*.

(2011) confirmed that silica is deposited in the cell walls mainly of the endodermis but found also Si depositions in the cell walls of the sclerenchyma and the exodermis.

A direct effect of Si on gene expression leading to a stimulated metabolism towards the synthesis of suberin and lignin seems questionable. Continuous Si supply enhanced transcription of genes related to suberin and lignin synthesis (Fleck *et al*., 2011). However, this could be a secondary effect due to fewer amounts of suberin monomers and monolignols after their transport into the apoplast and subsequent polymerization. Furthermore, Si supply was effective on casparian band formation not before 48 hours (Fig. 5), whereas regulation of gene expression upon external changes can be very fast as observed in salt stressed rice plants, where transcription of several genes was altered within 15 minutes after onset of stress (Kawasaki *et al*., 2001).

Moreover, it can be concluded from the Si excluder onion, that a Si signal from shoot-to-root is not involved as it is known for the regulation of sulfate and phosphate uptake (Rouached *et al*., 2011), since Si promoted formation of casparian bands independent from the shoot Si level (Fig. 6A, 7). Instead, Si has to be effective in the root and a chemical mechanism of Si seems rather plausible than a direct impact of Si on gene expression because Si was effective in a range of various plant species with different genetic backgrounds.

Condensed, we hypothesize that silicon in the cell walls of the exodermis and endodermis induces precipitation of aromatic compounds or is involved in cross-linking between the cell wall and aromatic compounds leading to an enhanced formation of casparian bands. Furthermore, this mechanism should take place not only in the roots of rice and other Si accumulators, but also in the roots of plants that lack the ability to actively take up Si and accumulate it in the shoot.

Chapter III

Silicon decreases arsenic level in rice grain by limiting arsenite transport

Alexander T. Fleck[1], Jürgen Mattusch[2], Manfred K. Schenk[1]

to be submitted

Author contributions: Jürgen Mattusch: Determination of arsenic species

[1] Institute of Plant Nutrition, Faculty of Natural Sciences,
Leibniz University Hannover, Herrenhäuser Str. 2, 30419 Hannover, Germany.
[2] UFZ, Centre for Environmental Research Leipzig-Halle, Department of Analytical Chemistry, Permoserstr. 15, 04318 Leipzig, Germany

Abstract

Silicon (Si) was shown to reduce arsenic (As) levels in rice shoot and grain, however, underlying mechanisms remain unclear. In this study, we examined the effect of Si application to three rice paddy soils on the dynamics in the soil solution, As accumulation in rice straw, flag leaf, husk, brown rice and polished rice, also on As speciation in polished rice. Silicon application to soil increased the concentrations of Si, iron, As, and phosphorus in the soil solution, while the redox potential was unaffected. Arsenic concentration of straw, flag leaf and husk were reduced by half by Si application, while As concentration in brown and polished rice was decreased by 22%. The main As species in polished rice was arsenite, As(III), with a fraction of 70%, followed by dimethylarsinic acid (DMA) and arsenate, As(V), with 24 and 6%, respectively. Silicon application to the soil did not affect DMA or As(V) concentration in polished rice, while As(III) concentration was reduced by 33%. These results confirm that Si reduces As(III) uptake and translocation into the shoot. Furthermore, data indicate that decrease of As concentration in polished rice is due to decreased As(III) transport into grain. Possible underlying mechanisms are discussed.

Introduction

Rice can contain high levels of the non-threshold carcinogen arsenic (As), exceeding the amounts in other crops (Williams *et al.*, 2007; Su *et al.*, 2009). Although As concentration in the grain is lower than in the leaves, rice diet exhibits a relevant source of human As intake (Kile *et al.*, 2007). The high As levels in rice are caused by the cultivation of paddy rice in flooded fields under reducing conditions, which lead to the mobilization of As (Ponnamperuma, 1984). Arsenic is present in aerobic soils mostly as arsenate, As(V), which is bound to iron (Fe), aluminum (Al) and manganese (hydr)oxides and hence, As concentration in the soil solution is low (Inskeep *et al.*, 2001). In flooded fields after depletion of oxygen, iron (hydr)oxides are reduced to Fe^{2+} and As is released into the soil solution. Furthermore, As(V) is reduced to arsenite, As(III), which exhibits the dominant As species in flooded fields and is more mobile in the soil solution than As(V) (Garcia-Manyes *et al.*, 2002; Takahashi *et al.*, 2004). Arsenic can also be methylated by microorganisms to monomethylarsonic acid (MMA) or dimethylarsinic acid (DMA), but the inorganic As forms are the prevailing As species in the soil solution (Lomax *et al.*, 2012). In general, the organic As forms are considered less toxic to humans than As(III) and As(V) (Zhao *et al.*, 2009).

Besides the high availability of As in flooded soil, an efficient uptake of As by rice plants contributes to the large As amounts in rice. As(III) is taken up into the rice root and transported to the xylem by the transporters Lsi1 and Lsi2, that were initially identified as transporters of silicic acid (Ma *et al.*, 2008). Lsi1 is an influx transporter and is located at the distal side of both exodermis and endodermis, while the efflux transporter Lsi2 is located at the proximal side of exodermis and endodermis. Lsi1 also has the capability of transporting MMA and DMA into rice roots (Li *et al.*, 2009a).

Silicon (Si) is the second most abundant element in soils and is present in the soil solution as silicic acid at concentrations ranging between 0.1 and 2.0 mM (Epstein, 1994; Bogdan and Schenk, 2008). Silicon is beneficial to plants as it improves growth and yield and enhances the resistance to biotic and abiotic stresses, such as pests or diseases and salinity or drought stress (Epstein, 1994, 1999; Ma and Yamaji, 2006). Silicon also reduces the toxicity in rice against several metals including Al, As, cadmium (Cd), and zinc (Guo *et al.*, 2005; Shi *et al.*, 2005; Singh *et al.*, 2011; Song *et al.*, 2011). Silicon was also shown to reduce the As concentration in rice root and shoot when grown in a nutrient solution containing either As(V) or As(III) (Guo *et al.*, 2005, 2009). When rice was grown in six different rice paddy soils, the Si concentration in the soil solution was negatively correlated with the As concentration in

straw and polished rice (Bogdan and Schenk, 2008). Silicon application to soil strongly decreased the As concentration in straw and husk, while the decrease of brown rice As concentration was only small (Li *et al.*, 2009b). The latter was due to a reduced inorganic As level in brown rice, while the DMA concentration was increased. However, this may not reflect the situation in the polished rice, since the bran, which is removed from the brown rice to get the polished rice, contains more As than the endosperm (*Lombi* et al., 2009). Moreover, the analytical method used did not allow to distinguish between the inorganic As species As(III) and As(V). The decreased As concentration is supposed to be due to a competitive inhibition of the uptake of As(III), which is present as arsenious acid, by silicic acid (Guo *et al*., 2009; Li *et al*., 2009b; Zhao *et al*., 2009). Moreover, the expression of *Lsi1* and *Lsi2* was reduced by the Si supply and this could also have contributed to a decreased As uptake by Si (Ma *et al*., 2008).

This study aimed at investigating the effect of Si supply to three different rice paddy soils on the As concentration in rice straw, flag leaf, husk, brown rice, and polished rice. In addition, the concentration of As(III), As(V), DMA, and MMA in polished rice was determined.

Material and Methods

Soil

Pot experiments in the greenhouse were conducted with three Italian rice paddy soils that originated from the Po area in Northern Italy. The soils D and L have a loamy and soil G a sandy-loamy soil texture (according to US soil taxonomy). The soils differ in their aqua regia soluble As concentrations ranging from 5.0 (soil G) through 6.5 (soil D) to 15.1 mg As/kg (soil L). Detailed soil characteristics are given in Bogdan and Schenk (2008). The soils were supplied with 10 g silicagel/kg for +Si treatment, while the control soils (–Si) were not treated with Si. The soils were mixed thoroughly and 16 L of each soil was placed in a 20 L pot (diameter 36 cm, height 30 cm) to reflect rooting depth under field conditions. The soils were flooded seven days before rice cultivation.

Cultivation and harvest

Seeds of rice (*Oryza sativa* L., cv. Selenio) were germinated in tap water for seven days and 55 seedlings were transplanted in a pot and later reduced to 35. The soils were fertilized three times with urea (1 g N/pot) (Sigma-Aldrich, St. Louis, MO, USA). The experiment was conducted in the greenhouse from April to August with average temperatures around 28°C. The water level was 1 cm above the soil for the first 20 days and increased to 3 cm for the remainder of the experiment.

The plants were harvested at maturity after 147 days. The stems were cut 3 cm above the ground and the plants were separated into straw, flag leaf and grain. The straw, husk and flag leaf were dried at 60°C for five days and milled. The rice grains were dried at 40°C overnight and processed to polished rice and husk by the Rice Research Institute (Castello d'Agogna, Italy).

Soil solution and redox potential

The soil solution was collected weekly using suction cups (ecoTech, Bonn, GER) with a porous polyamide membrane (5 cm length, 0.45 µm pore size). Three suction cups per pot were embedded in 20 cm depth and the soil solution was collected by using a syringe. Each 7 ml from the syringes were transferred to a vessel that contained 250 µl 65% HNO_3. The acidified soil solution was filtered (2.5 µm, Carl Roth, Karlsruhe, Germany) and analyzed for Si, As, iron (Fe), and phosphorus (P). The pH was measured in the remaining non-acidified soil solution.

The redox potential of the soils was measured weekly using a Pt electrode embedded at a 20 cm depth. A calomel electrode (B 2810 Schott Instruments, Mainz, GER) was used as reference. The redox potential values were adjusted to the standard hydrogen electrode by adding 244 mV.

Chemical analysis

Silicic acid in the soil solution was determined photometrically at 811 nm after the addition of 3.2% boric acid, dye reagent (0.08 M sulfuric acid and 2% ammonium heptamolybdate), 3.3% tartaric acid, and 0.4% ascorbic acid (Novozamsky *et al*., 1984). Silicon in the straw was extracted by digesting dried plant matter overnight in a mixture of 1 M HCl and 2.3 M HF (1:2) (Novozamsky *et al*., 1984) and determined similar to the Si in the soil solution. Arsenic, Fe and P in the soil solution were measured with ICP-MS 7500c (Agilent Technologies, Waldbronn, Germany). Dried and milled straw, flag leaf, husk, brown rice, and polished rice were digested in a microwave (ETHOSplus, MLS GmbH, Germany) at 190°C with 4 mL HNO_3 (65%) and 1.5 mL H_2O_2 (30%) for 20 and 15 min, respectively, and analyzed for total As with ICP-MS 7500c. A certified rice reference material (NCS ZC73008, China National Analysis Center, Beijing, China) was used to check the accuracy of the As measurement, and the As measured was 97.2 ± 3.5% of the reference value (n = 4).

Arsenic for speciation in polished rice was extracted by digesting dried and milled plant matter in 0.28 M HNO_3 for 90 min at 90° C (Huang *et al*., 2010). Arsenic species were analyzed using HPLC-ICP-MS. The chromatographic separation was performed with a mixed-mode column IonPac AS7/AG7 using a nitric acid gradient (Mattusch and Wennrich, 1998). The chromatographic equipment used consisted of a HPLC Series 1100 quaternary pump, degasser and thermostated autosampler (Agilent Technologies) and was online coupled to an ICP-MS PQExcell (Thermo Scientific, Waltham, MA, USA). The element As was measured on *m/z* 75 with a dwell time of 1 s in transition mode. The main parameters were tuned daily. Chloride as potential interference $AsCl^+$ was chromatographically separated from the target analytes. An arsenic species specific calibration was performed with a LOD of 1 µg As/L for quantification. The linear dynamic range is more than three orders of magnitude.

The sum of As species in polished rice was on average 101.4 ± 2.9% of the total As in polished rice.

Statistical analysis

All soils and treatments were replicated four times and all pots were arranged in a randomized block design. Generalized additive mixed models for soil solution parameters were created and compared with a likelihood ratio test (Wood, 2006), and mean values of plant parameters were compared with Tukey's test after ANOVA with $p < 0.05$ using R software (R Development Core Team, 2011).

Results

Dynamics in soil solution

The redox potential was firstly measured seven days after the soils were flooded. The redox potential was already negative in soils G and D and further decreased within 14 days to below -200 mV, whilst the redox potential in soil L was positive at the first measurement and decreased comparatively slowly (Fig. 1A). However, the redox conditions were strongly reducing in all soils during the second half of cultivation resulting in a slight pH increase in the soil solution in all soils from 6.8 to 7.4 in soil L and from 7.0 to 7.4 in soils G and D (data not shown).

The Fe concentration in the soil solution of soils G and D was already high at day 0 and, while the redox potential strongly decreased within the next 14 days, the Fe concentration in the soil solution strongly increased and then declined continuously until the end of cultivation (Fig. 1B). The Fe concentration in the soil solution of soil L was very low at the first measurement and increased up to a maximum between days 40 and 70 and decreased subsequently, but stayed on higher levels compared to soils G and D.

The course of the As concentration in the soil solution was similar to that of the Fe concentration for the first 60 days (Fig. 1C). The As concentration in the soil solution of soils G and D was around 75 µg/L at the first measurement and more than doubled within 14 days, but decreased successively to initial values during the next six weeks, and then remained at the same level. Soil L had clearly different As dynamics compared to soils G and D, as the As concentration was only 4 µg/L soil solution at the start and increased continuously up to 250 µg/L with the +Si treatment after 100 days. Thus, the final As concentration in the soil solution was three times higher in soil L than in soils G and D. The development of the P concentration in the soil solution showed similarities with those of As (data not shown). The initial P concentration in soil L was very low and increased only slowly over time, while the P concentration in the soil solution of soils G and D rapidly increased to a maximum after 7 and 28 days, respectively, and decreased afterwards. However, the pattern was different in soil G +Si after day 80. Silicon application significantly enhanced the As and P concentrations in the soil solution in all three soils.

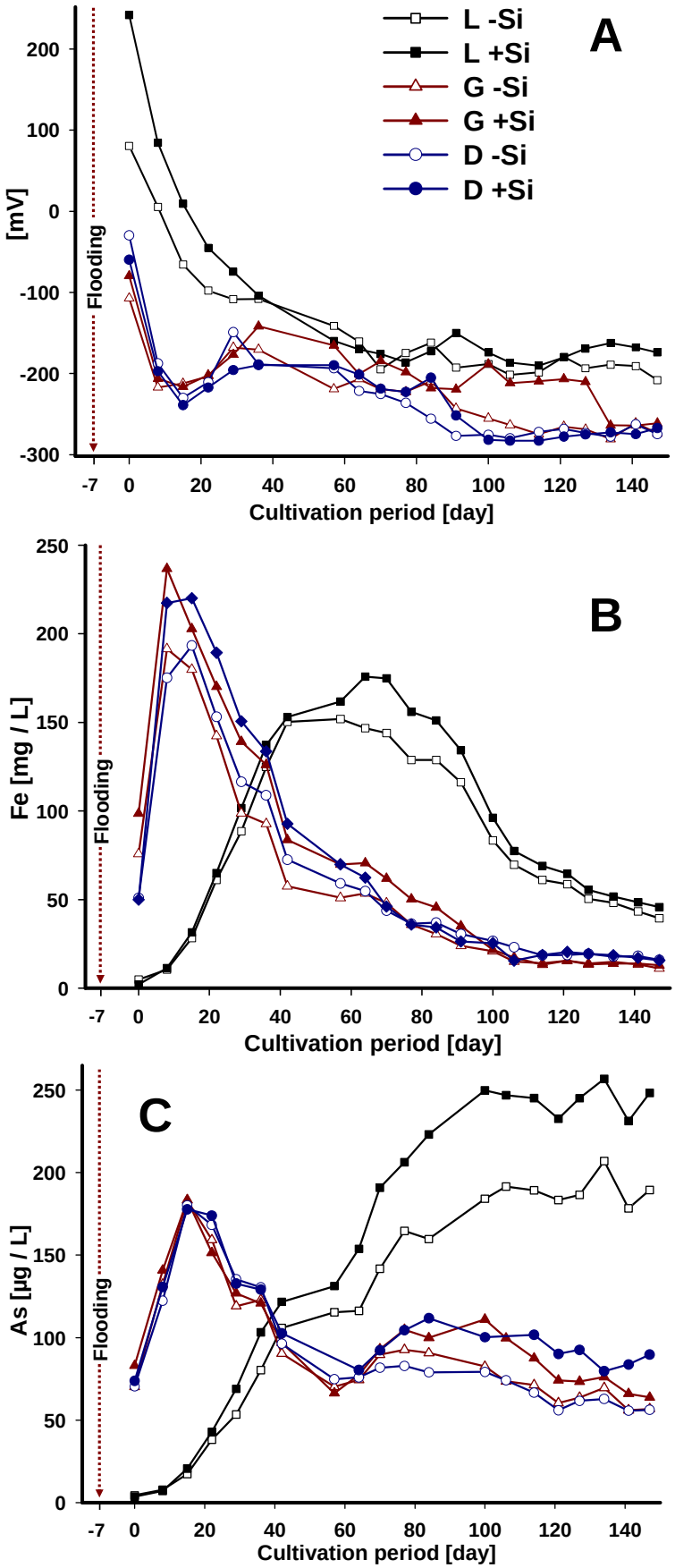

Fig. 1: Redox potential (A), Fe (B) and As (C) concentration in soil solution of three paddy rice soils as affected by Si supply during the cultivation period. Stars indicate significant difference between Si treatments of a soil; likelihood ratio test with $p < 0.05$.

The initial Si concentration in the soil solution seven days after flooding was twice as high in soils G and D compared to soil L without Si application (Fig. 2A). Over the next 21 days, the Si concentration slightly increased in all –Si soils but decreased afterwards and was on average 6 mg/L in soil L and below 4 mg/L in soils G and D from day 42 until the end of cultivation.

The Si concentration was above 20 mg/L at the first measurement in soils with Si supply and rapidly increased during the first 14 days to above 35 mg/L and did not differ between the soils. The average Si concentration in the +Si soils was between 30 and 33 mg/L and clearly higher than in –Si soils.

Yield and concentration of Si and As in plant

Silicon supply increased straw and grain yield on average by 21% and 17%, respectively, in all three soils (data not shown). The Si concentration in straw of the –Si soils was on average 15 mg/g d.m., while silica gel application significantly enhanced the Si concentration in all soils to on average 40 mg/g d.m. (Fig. 2B).

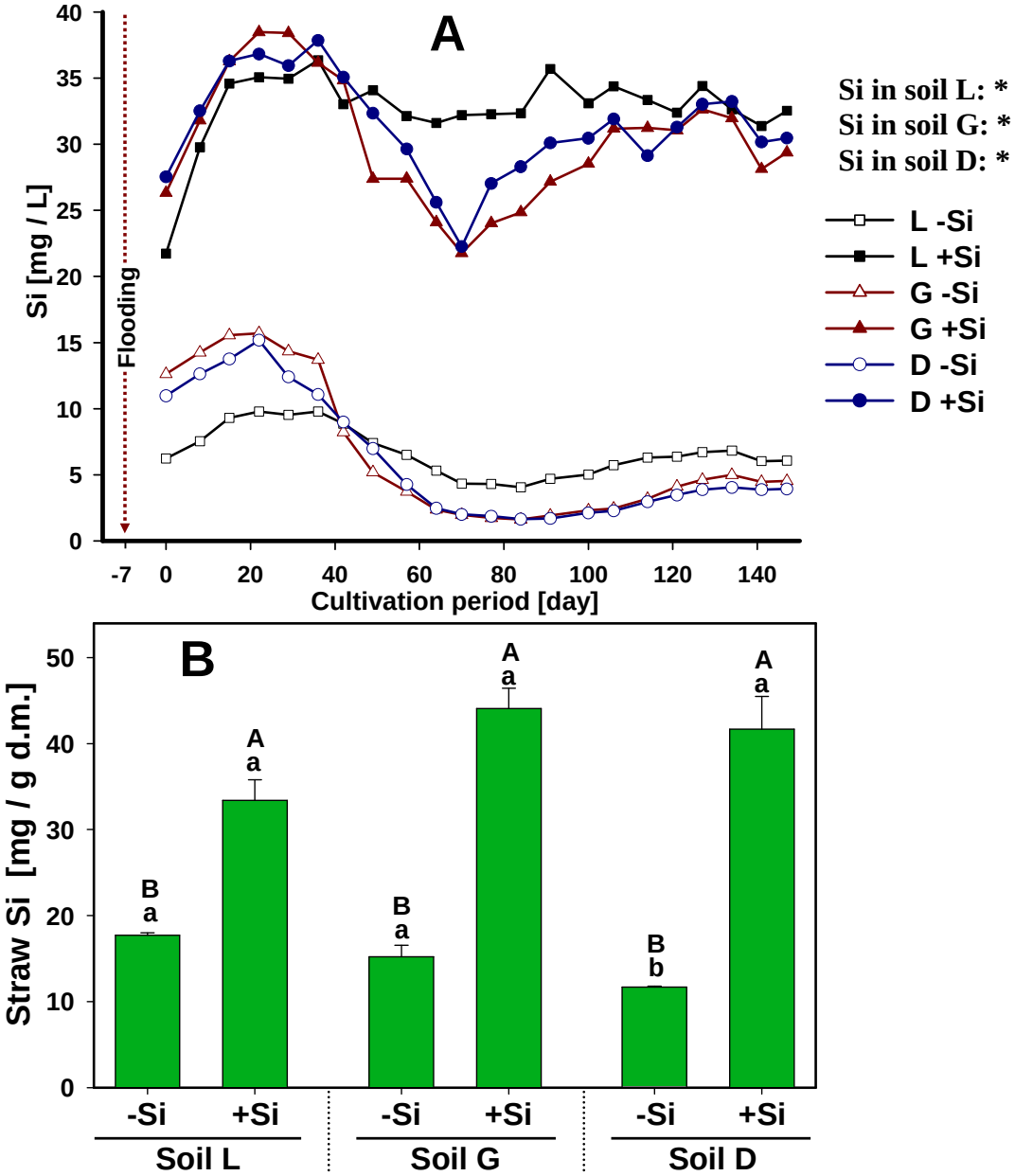

Fig. 2: Silicon concentration in soil solution (A) and in rice straw (B) grown in three paddy rice soils as affected by Si supply during the cultivation period. Stars and different capital letters indicate significant difference between Si treatments of a soil, different small letters indicate differences between the soils of a Si treatment; likelihood ratio test (A) and tukey-test (B) with $p < 0.05$. Bars are SE.

The As concentration in straw was similar in all –Si soils after 34 days and increased during the cultivation period. The As concentration in straw at day 147 was more than two times higher in soil L than in soils G and D (Fig. 3A). Husk As concentration was an order of magnitude smaller than in straw (data not shown), while flag leaf As concentration was higher compared to straw (Fig. 3B), but the pattern of As concentration between the soils was similar to straw As. Silicon application to soil decreased the As concentration of straw and flag leaf

as well as husk by half in all soils, although the As level in the soil solution was increased by Si application (Fig. 1C).

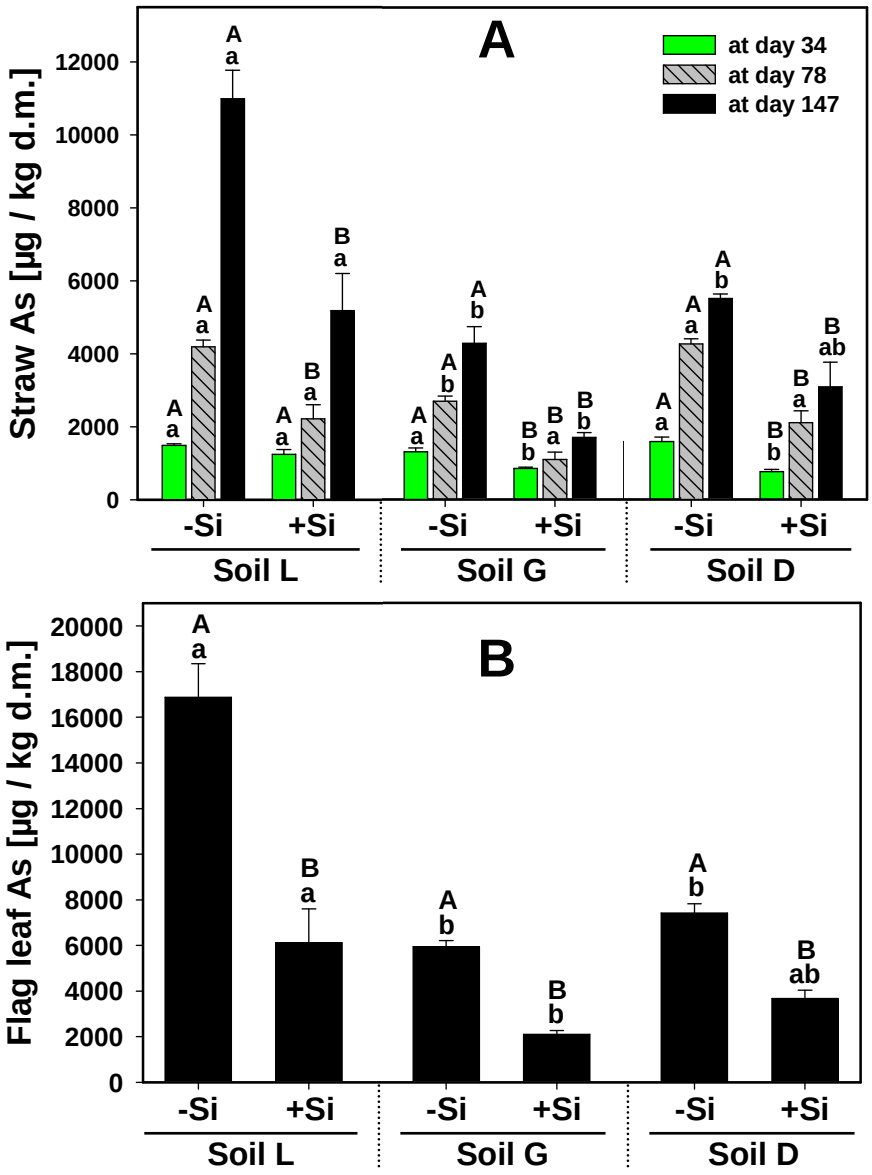

Fig. 3: As concentration in rice straw (A) after 34, 78 and 147 days of cultivation and flag leaf (B) after 147 days grown in three paddy rice soils as affected by Si supply. Different capital and small letters indicate significant difference between Si treatments of a soil at a harvest and between soils of a Si treatment at a harvest, respectively; tukey-test with $p < 0.05$. Bars are SE.

The As concentration in brown rice was an order of magnitude smaller than in straw, and the polished rice As concentration was around one third lower than in brown rice (Fig. 4A). Silicon decreased the As level in brown rice and polished rice by 23 and 22%, respectively. As(III) was the main As species in polished rice with a fraction of 61-83% of total As, while

DMA contributed 12-34% and As(V) only 4-7% (Fig. 4B). The total As concentration in polished rice did not differ between the soils, while the DMA concentration in polished rice was lower in soil L than in soils G and D (Fig. 4B). Silicon application did not affect the DMA and As(V) concentration in polished rice, but the concentration of As(III) was clearly reduced by Si supply in all soils.

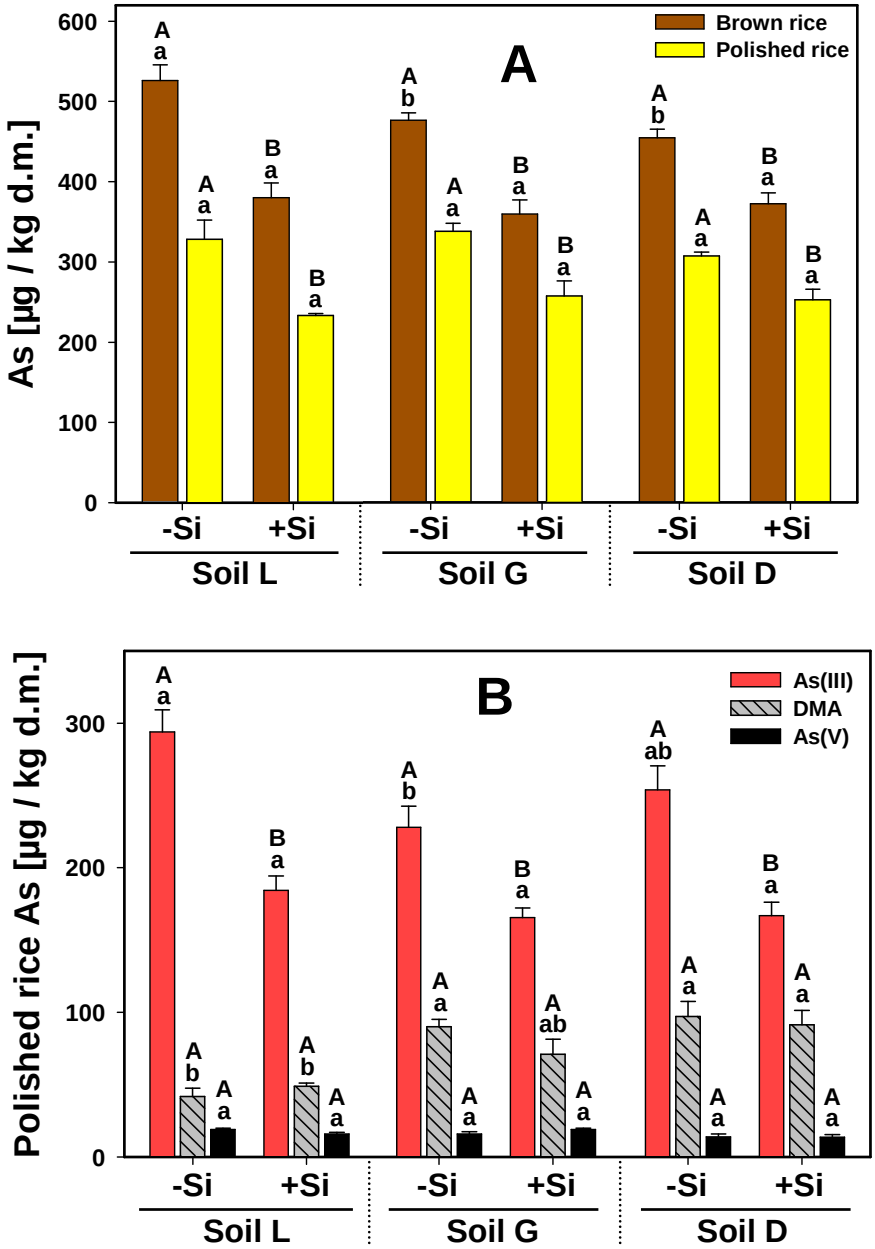

Fig. 4: Total arsenic concentration in brown rice and polished rice (A) and As species concentration in polished rice (B) grown in three paddy rice soils as affected by Si supply. Different capital and small letters indicate significant difference between Si treatments of a soil and between soils of a Si treatment, respectively; tukey-test with $p < 0.05$. Bars are SE.

Discussion

Dynamics in soil solution after flooding

After flooding, the soil was subjected to drastic changes as the redox potential decreased, while concentrations of Fe, As, Si, and P in the soil solution increased, with the changes being more rapid in soils G and D compared to soil L (Fig. 1A, B, C, 2A). The changes in the chemical composition of the soil solution after the flooding of an aerated soil are caused by changes in the redox potential (DeDatta, 1981). Oxygen diffuses in water at much slower rates than in air and, once depleted by microorganisms and plant roots, microorganisms firstly reduce NO_3^- and MnO_2 at positive redox potential values, followed by reduction of Fe(III), while SO_4^{2-} and CO_2 are reduced at negative redox values (Marschner, 2012).

The soils in this study were used previously for plant cultivation and root residues offered an easily available carbon source for microorganisms, resulting in a rapid depletion of oxygen and a subsequent rapid decrease of the redox potential. The decrease of the redox potential was slower in soil L than in soils G and D, presumably due to higher amounts of MnO_2 and thus a higher redox buffer capacity in soil L compared to soils G and D (Fig. 1A).

The rapid decrease of the redox potential was also reflected in the fast increase of the Fe concentration in the soil solution of soils G and D, whereas an increased Fe concentration in soil L was delayed since the redox potential dropped more slowly (Fig. 1A, B). The Fe concentration declined after 14 days in soils D and G and after 70 days in soil L, which might be the result of FeS precipitation because the redox potential was low enough for sulfate reduction (Inskeep *et al*., 2001; Marschner, 2012).

The As concentration in the soil solution increased in the same pattern as the Fe concentration (Fig. 1B, C). This is in line with previous reports, where As and Fe release into the soil solution were closely related since As(V) is bound to Fe (hydr)oxides and released into the soil solution when the Fe (hydr)oxides are reduced (Onken and Hossner, 1996; Bogdan and Schenk, 2008; Yamaguchi *et al*., 2011). The As concentration in the soil solution of soils G and D dropped after having reached a maximum similar to the Fe concentration but less pronounced, whereas the As concentration in the soil solution of soil L remained more or less constant (Fig. 1C). Presumably, As precipitated in the form of As(III) sulfide (Bostick *et al*., 2004), since the prevailing As form in the soil solution was probably As(III), as reported in several studies with flooded soils (Takahashi *et al*., 2004; Xu *et al*., 2008; Bogdan and Schenk, 2008; Li *et al*., 2009b). Sulfide might have had a higher affinity for Fe(II) than for As(III) and so precipitation decreased rather the concentration of Fe than As. The highest As

concentrations in the soil solution were around 250 µg/L in soil L+ and hence slightly exceeded the toxicity threshold of As(III) for rice of 180 µg/L that was reported by Hoffmann and Schenk (2011). However, the As concentrations in the other soils were continuously below this level and no plants in any soils showed toxicity symptoms (Fig. 1C).

Similar to As(V), silicic acid and phosphate are adsorbed to Fe (hydr)oxides and released into the soil solution in the course of reduction, which may explain the increasing Si concentration in the soil solution in the first 14 days, as well as the development of the P concentration in the soil solution (Fig. 1A, C, 2A) (Sommer *et al*., 2006).

The decrease of the Si concentration in the soil solution after 40 days was probably the result of high Si uptake by plants since the vegetative growth was highest in this period, while, with onset of the generative growth with flowering at around day 70, the Si concentration in +Si soils recovered. The Si concentration in the soil solution of –Si soils was low, presumably because Si uptake exceeded the rate of dissolution of silicic acid from easily available Si sources. The Si concentration in Si-treated soils was mostly above 30 mg/L during the cultivation period that is in the upper range reported for other soils and not far from the solubility maximum of Si at about 56 mg/L (Epstein, 1999).

The reason for the low P concentration in soil L compared to soils G and D is that soil L had not received any P fertilization for many years in contrast to soils G and D (Bogdan and Schenk, 2008). Decline of the P concentration after 7 and 28 days in the soil solution of soils G and D, respectively, may be due to the formation of iron phosphate (vivianite), and relatively constant P concentration after 40 days possibly reflects an equilibrium between dissolution and precipitation processes. The pH slightly increased in soils after flooding, which is known from other studies and is related to proton depletion during reduction (Liu, 1985; Xu *et al*., 2008).

Effect of Si application on soil solution, plant growth and Si concentration in plants

Soils with Si application exhibited higher concentrations of Si, Fe, As, and P in the soil solution than –Si soils (Fig. 1B, C, 2A). Li *et al*. (2009b) also reported that Si fertilization increased the As concentration in the soil solution. The higher As and P concentrations in +Si soils are probably the result of substitution of As(V) and phosphate by silicic acid because they compete for the same adsorption sites (Waltham and Eick, 2002).

The straw and grain yield increased with Si supply in all three soils (data not shown). It is well known that Si may enhance the growth of Si accumulating plant species, such as rice, wheat and sugar cane (Lewin and Reimann, 1969; Ma *et al*., 1989; Epstein 1999).

The Si concentration in straw was enhanced from around 15 mg/g d.m. in the –Si plants to on average 40 mg/g d.m. in the plants grown on soils with Si application (Fig. 2B). The latter is in the range reported for well-supplied rice plants and a high Si accumulation (Bogdan and Schenk, 2008; Tamai and Ma, 2008).

The Si uptake of rice is driven by a transport system with a K_m value of 0.15-0.32 mM that is equivalent to 4.2-9 mg Si/L (Tamai and Ma, 2003; Mitani and Ma, 2005; Nikolic *et al*., 2007). Since the Si concentration in the soil solution of +Si soils was on average above 30 mg/L and hence, several times greater than the K_m value of the transporter, it can be assumed that the Si transport process in the rice root was near saturation in the +Si soils (Fig. 2A). However, higher Si concentrations in rice shoots were reported in other studies (Li *et al*., 2009b; Norton *et al*., 2010). This may be due to rice cultivar or environmental conditions leading to higher transpiration rates and hence, better translocation of Si, since Si transport is driven by the transpiration stream.

As in plant parts

The As concentrations in plant parts at the end of cultivation were highest in the flag leaf, followed by straw, husk, brown rice, and polished rice (Fig. 3A, B, 4A). This distribution was also observed in other studies and the highest As concentrations are to be expected in the roots (Abedin *et al*., 2002a; Liu *et al*., 2006; Rahman *et al*., 2007; Xu *et al*., 2008). During the cultivation period, the straw As concentration increased in all soils (Fig. 3A). This is consistent with a study of Zheng *et al*. (2011) who reported that the As concentration in straw increased for rice plants grown under flooded conditions after flowering.

The As concentration in straw after 34 days was similar in all soils without Si supply, although the As concentration in the soil solution was higher in soils G and D than in soil L during the first weeks (Fig. 1C, 3A). This indicates that the As uptake of the young plants was already saturated at the relatively low concentration of As in the soil solution of soil L. This did not seem to be the case in older plants, since the As concentration in straw after 147 days was highest in soil L without Si supply, which coincided with the high final As concentration in the soil solution of soil L (Fig. 1C).

The flag leaf As concentrations were higher than in straw, which is in contrast to observations that flag leaves contain less As than older leaves (Zheng *et al*., 2011). However, the course of the soil parameters in that study are not known, and a decreasing As level in the soil solution during the cultivation could be the reason for a comparatively low As concentration in the youngest leaves.

The As concentration in polished and brown rice was an order of magnitude smaller than in straw, which is in accordance with previous studies (Fig. 4A) (Bogdan and Schenk, 2008; Xu *et al*., 2008). The As concentration in polished rice was around one third lower than in brown rice, which can be attributed to the removal of the bran, which contains more As than the endosperm (Fig. 4A) (Lombi *et al*., 2009).

Effect of silicic acid on As uptake and distribution in plant

The Si supply decreased straw As levels in all soils at the three different harvest dates, except in soil L after 34 days (Fig. 3A). The Si supply reduced the As concentration in both straw and husk in all soils by half at final harvest. On the other hand, Si application reduced the As concentration in brown rice and polished rice by only 23 and 22%, respectively (Fig. 4A). The Si effects observed on the As concentration in straw and grain are in line with other studies (Guo *et al*., 2005; Li *et al*., 2009b). Bogdan and Schenk (2008) showed that the Si concentration in the soil solution was negatively correlated with straw and grain As for rice grown in different rice paddy soils. Li *et al*. (2009b) observed that the As levels in straw and husk in pot experiments were reduced by 78 and 50%, respectively, when rice plants were grown with additional Si, while grain As concentrations were decreased by only 16%.

The As species in the soil solution were not determined, but it is likely that As(III) was the predominant form, as reported in several studies with flooded soils (Takahashi *et al*., 2004; Xu *et al*., 2008; Bogdan and Schenk, 2008; Li *et al*., 2009b). Moreover, any As(V) in the soil solution would have been taken up into the rice root via phosphate-transporters and rapidly reduced to As(III) inside the root (Xu *et al*., 2007). Accordingly, Zhao *et al*. (2009) reported the main As species in the xylem being As(III), regardless whether As(III) or As(V) was supplied to rice plants. Thus, in our study, the main As species transported to the shoot was most likely As(III).

As(III) is taken up by the rice root via the transporters Lsi1 and Lsi2 (Ma *et al*., 2008). These transporters are located in the exodermis and endodermis of rice roots and were initially identified as transporters of silicic acid (Ma *et al*., 2006, 2007). Lsi1 is an aquaporin and acts as a passive influx-transporter, while Lsi2 is an anion-channel that serves as an energy-dependent efflux-transporter. Lsi1 is located on the distal side and Lsi2 on the proximal side of both exodermis and endodermis, and coupling of these transporters due to their subcellular localization allows for the highly efficient uptake of As and Si by rice roots (Ma and Yamaji, 2008). After release from endodermis into stele, As(III) is translocated into the shoot via the xylem following the transpiration stream.

Ma *et al*. (2006, 2007) showed that the expression of *Lsi1* and *Lsi2* is decreased by Si supply and this may cause a lower uptake of As(III). Some authors suggest that the reason for a decreased uptake of As(III) under Si supply is due to a competitive inhibition of As(III) by silicic acid (Guo *et al*., 2009; Li *et al*., 2009b; Zhao *et al*., 2009). This could not be verified for Lsi1 since no competition for the uptake of As(III) and silicic acid could be observed in experiments with yeast cells and *Xenopus* oocytes expressing *Lsi1* (Bienert *et al*., 2008; unpublished data of Ma and Mitani in: Li *et al*., 2009a).

At Lsi2, however, which is an anion-channel, a competitive inhibition of As(III) transport by Si could take place, and Lsi2 is supposed to be crucial for As transport to the shoot in mediating As(III) efflux towards the xylem (Li *et al*., 2009a; Zhao *et al*., 2009).

As species in polished rice

The As species in polished rice were analyzed with HPLC-ICP-MS (Mattusch and Wennrich, 1998) after HNO_3 extraction. This method has the advantage of preserving not only the organic As forms DMA and MMA, but also the inorganic forms As(III) and As(V) (Huang *et al*., 2010, 2012), the latter exhibiting a problem when using other methods, such as extraction with trifluoroacetic acid (TFA) (Heitkemper *et al*., 2001; Liu *et al*., 2006; Li *et al*., 2009b). Therefore, no information about inorganic As species in the grain is available in literature. The sum of As species in polished rice was on average 101.4 ± 2.9% of the total As in polished rice determined by ICP-MS.

The dominant As species in polished rice was As(III) with a fraction of 70%, followed by DMA with 24% and As(V) with 6% (Fig. 4B), while other As species were not detected. Silicon supply did not alter DMA or As(V) concentration, while As(III) concentration was reduced by one third, so decrease of polished rice As concentration can be attributed solely to a reduced As(III) concentration.

As(III) transport into rice grain occurs nearly exclusively via the phloem, since the grain As level was reduced by 90 to 97% when phloem at the base of the panicle was destroyed (Carey *et al*., 2010; Zhao *et al*., 2012). As(III) may be loaded into the phloem in the leaves, especially the flag leaf, as well as in the stem nodes by xylem-to-phloem transfer. The transportation of As(III) from the flag leaf to the grain is disputed in literature. Carey *et al*. (2011) fed excised flag leaves with As(III), and found As in the flag leaf, while no As was detected in the grain. In contrast, Zhao *et al*. (2012) exposed excised flag leaves to radioactive ^{73}As-labelled As(III) and found 2-3% of the total absorbed As was transported to the grain. The different results between the studies were explained by the higher sensitivity of the ^{73}As

method. Furthermore, the high As concentration used by Carey *et al.* (2011) might have caused phytotoxicity (Hoffmann and Schenk, 2011).

Stem nodes are a place of high xylem-to-phloem transfer, as shown for potassium and amino acids (Marschner, 2012). Recently, Fujimaki *et al.* (2010) reported that Cd in rice coming from the root via the xylem is also transferred into the nodes from xylem to phloem prior to transport to the grain.

It is likely that Si application reduced the As(III) transfer from xylem to phloem, since As(III) is the main As form in the xylem (Zhao *et al.*, 2009) and As concentration in shoot dry matter was decreased by about 50% by Si application (Fig. 3A). Additionally, the Si transporter Lsi6 might be involved in the transport processes in the nodes. Lsi6 is supposed to unload Si from the xylem in the node beneath the flag leaf of rice plants (Yamaji and Ma, 2009). Moreover, expression of *Lsi6* in yeast and in oocytes demonstrated that Lsi6 also facilitates the transport of arsenic acid (Bienert *et al.*, 2008; Ma *et al.*, 2008).

In this study, Si supply reduced the As(III) concentration in grain while DMA was not affected (Fig. 4B). DMA is synthesized in the soil solution from soil microorganisms, while plants are not able to methylate inorganic As (Lomax *et al.*, 2012). Hence, the DMA concentration in the grain is related to the DMA level in the soil solution.

DMA is taken up by Lsi1, but, in contrast to As(III), Lsi2 does not show transport activity for DMA (Li *et al.*, 2009a). A reduced As(III) uptake by Si application is either due to the decreased expression of transporters or to competitive inhibition at Lsi2. The uptake of DMA is rather limited compared to As(III), since the DMA concentration in the soil solution is much lower (Bogdan and Schenk, 2008; Xu *et al.*, 2008) and the affinity of transporters for DMA is less (Abedin *et al.*, 2002b).

Higher DMA concentrations in the grain with more than 85% of the total As were found in other studies with paddy rice (Smith *et al.*, 2008; Li *et al.*, 2009b). The higher DMA fractions in grain in these studies compared to our study may be due to a different composition of the microbial flora with more As methylating species or due to different soil conditions that favored the growth and activity of microorganisms. In our study, DMA concentrations in polished rice were lower in plants grown in soil L than in soils G and D, possibly because of less microbial activity (Fig. 4B). Li *et al.* (2009b) found that the DMA concentration in grain was even increased by 33% when Si was applied to soils. A reason might be an enhanced methylation of inorganic As in the soil solution after Si supply.

In conclusion, we showed that Si application to rice paddy soils decreased the As concentration in rice straw and polished rice, although the As concentration in the soil

solution was enhanced by Si. The decreased As level in polished rice relied solely on a decreased As(III) concentration while As(V) and DMA were unaffected by Si. The reduced As(III) translocation to the shoot was the result of either reduced transporter density due to decreased expression of *Lsi1* and *Lsi2*, or competitive inhibition of As(III) uptake by Si at Lsi2, or a combination of both effects. This reduced translocation also explains the decreased As(III) concentration in polished rice when plants were grown on soils with Si application, while the DMA concentration was not reduced because competitive inhibition did not occur at the DMA transporting aquaporin Lsi1, and a reduced transporter expression would not have been effective for DMA due to its low external concentration.

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Chapter IV

Mechanism of silicon-mediated decrease of arsenite uptake in rice and distribution of silicon in roots of rice, maize, and onion

Introduction

Silicon (Si) is a beneficial element to many plant species by increasing growth and yield and alleviating several biotic and abiotic stresses including metal toxicities (Epstein, 1999). The Si concentration in the soil solution of six flooded soils was negatively correlated with the arsenic (As) level in straw and polished rice (Bogdan and Schenk, 2008). Si application to flooded soils reduced the As concentration in the rice shoot and polished rice, the latter caused by a decreased concentration of arsenite (Chapter III). The prevailing As species in the soil solution of flooded soils is arsenite, As(III) (Takahashi *et al*., 2004; Xu *et al*., 2008), which is taken up into the rice root by the transporters Lsi1 and Lsi2 that were initially identified as transporters of silicic acid (Ma *et al*., 2008).

It was hypothesized that Si reduces the As concentration in rice by competitively inhibiting the uptake of As(III), which exists as arsenious acid (Guo *et al*., 2009; Li *et al*., 2009b; Zhao *et al*., 2009). While for Lsi1, a competitive inhibition of As(III) uptake by Si could not be verified (Bienert *et al*., 2008), this effect could take place at Lsi2.

Moreover, Si could limit the As(III) uptake by reducing the transporter abundance, since continuous Si supply reduces the transcript level of *Lsi1* and *Lsi2* (Ma *et al*., 2006, 2007). Si also increases the formation of casparian bands in the rice root (Fleck *et al*., 2011) which could lead to a limited apoplastic flow (Krishnamurthy *et al*., 2009) and hence, decrease the uptake of As(III).

To clarify the mode of action of Si, rice plants cultivated in nutrient solution with different Si treatments were exposed to As(III) and strontium (Sr) and uptake rates were determined. Strontium was used, since it is an indicator of the apoplastic movement (Storey and Leigh, 2004).

Si promotes the development of casparian band not only in adventitious roots of rice (Fleck *et al*., 2011) but also in the roots of other plant species including maize and the Si excluder onion (Chapter II). Si increases the transcription of genes related to the synthesis of suberin and lignin, which are components of the casparian band (Fleck *et al*., 2011). However, whether Si directly affects gene transcription seems questionable, since the reaction of casparian band formation upon Si supply was delayed and Si was effective in several plant species with different genetic backgrounds (Chapter II). Instead, the effect of Si on gene transcription could be secondary. Si could increase casparian band formation rather in a chemical way by inducing the precipitation of aromatic compounds or cross-linking these with the cell wall, because Si was shown to react with aromatic compounds such as catechol

or lignin (Barnum, 1970; Fang and Ma, 2006). Moreover, Si was found as precipitated silica in the exodermis and endodermis of rice roots, where the casparian bands are located (Gong *et al*., 2006; Moore *et al*., 2011).

To further study the Si deposition, root cross sections of rice, maize and onion plants cultivated in nutrient solution with Si were investigated using laser ablation-inductively coupled plasma-mass spectroscopy (LA-ICP-MS). Moreover, rice root cross sections were investigated with a scanning electron microscope energy-dispersive X-ray microanalysis (SEM-EDX).

Material and methods

Plant material and growth conditions

Rice *(Oryza sativa* L. cv. Selenio) seeds were germinated in tap water for seven days and then placed between two layers of filter paper standing in tap water for additional seven days. Seedlings were transferred in 5 L pots with non-aerated nutrient solution containing in mM: 1.43 NH_4NO_3, 0.32 NaH_2PO_4 x H_2O, 0.51 K_2SO_4, 1 $CaCl_2$ x 2 H_2O, 1.6 $MgSO_4$ x 7 H_2O; in µM: 1.82 $MnSO_4$, 0.03 $(NH_4)_6Mo_7O_{24}$, 9 H_3BO_3, 0.3 $ZnSO_4$ x 7 H_2O, 0.15 $CuSO_4$ and 35.81 Fe as sequestrene. Silicon concentrations were 0 (-Si) and 30 mg L^{-1} (+Si) and Si was applied as silica gel. The pH-value was adjusted to 6.0 by addition of 10 % (v/v) H_2SO_4 and 0.75 M KOH. The plants were cultivated in a growth chamber (photoperiod: 14 h light, 10 h dark; temperature 25°C day / 20°C night; relative humidity 75 %; light intensity 220 µmol m^{-2} s^{-1}). After 7 days in nutrient solution seminal roots were removed and only adventitious roots were left.

For determination of Si depositions in the root, rice plants were germinated and cultivated in +Si nutrient solution as described above. Maize seeds were germinated between two layers of filter paper standing in tap water for 5 days and then transferred to aerated +Si nutrient solution. Onion bulbs were cultivated in peat substrate for 5 days, roots were washed with tap water and plants were then transferred into aerated +Si nutrient solution. The composition of the nutrient solution was the same as described above and the Si concentration was 30 mg L^{-1}. Roots of rice, maize and onion were harvested after 21 days in nutrient solution and root zones 4-6 cm (rice, onion) and 6-8 cm (maize) distance from root tip (drt) were stored in 70 % ethanol at 4°C.

Arsenic and strontium uptake experiments

After 18 days in nutrient solution whole plants were used for As and strontium (Sr) uptake experiments. Each two plants per replicate were transferred into 3 L pots with new nutrient solution with 0 (-Si) or 30 mg Si L^{-1} (+Si) generating the following treatments: -Si preculture, transfer to -Si: -Si/-Si; -Si preculture, transfer to +Si: -Si/+Si; +Si preculture, transfer to –Si: +Si/-Si. $NaAsO_2$ and $SrCl_2$ were added to the nutrient solution of each treatment yielding final concentrations of 2 µM As and 1 mM Sr. Shoots and roots were harvested after exposure of As and Sr for 48 hours.

Uptake rates of As and Sr were determined also after short term Si supply of excised roots in rhizotrons. Rhizotrons were built according to Klug and Horst (2010) with modified measures

(Fig. 1). 1.5 mm thick acrylic glass plates were glued with liquid glue for plastics (Revell GmbH & Co. KG, Bünde, Germany).

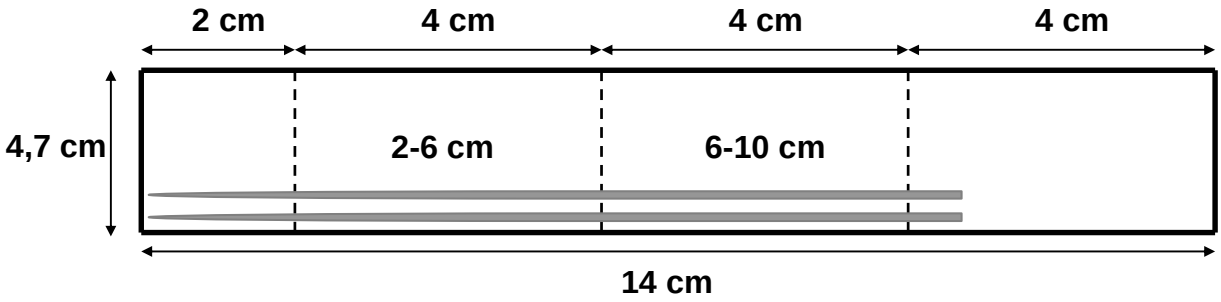

Fig. 1: Design of a rhizotron. As and Sr were added to the zones 2-6 and 6-10 cm. Roots are indicated in grey.

After 31 to 34 days in -Si or +Si nutrient solution adventitious rice roots were excised from plants cultivated either in -Si or +Si nutrient solution and 8 adventitious roots with a length of 11 cm were set into a rhizotron containing minimal nutrient solution (1 mM $CaCl_2$, 2 µM H_3BO_3) with 0 or 30 mg Si L^{-1} generating the same Si treatments as above. Lateral roots initiated at about 6 cm behind the root tip.

To avoid movement of the nutrient solution between the compartments, the borders were sealed with low-melting (melting point 42-44°C) paraffin (Merck KGaA, Darmstadt, Germany). $NaAsO_2$ and $SrCl_2$ were added to the zones 2-6 cm or 6-10 cm yielding final concentrations of 2 µM As and 1 mM Sr and roots were allowed to take up As and Sr for 4 hours. Then samples of the nutrient solution were taken to assure that the compartments were sealed properly. The amount of As and Sr in the compartments next to the application zone was on average 3.1 and 3.0 % of the total applied As and Sr, respectively. Finally, whole roots were carefully taken out of the rhizotron and blotted dry.

Chemical analysis

Plant matter was dried at 60°C for 4 days, ground and digested overnight in 65 % (v/v) HNO_3. Samples were heated for 30 min to 65°C, diluted 1:10 with ddH_2O and filtered. Concentrations of As and Sr in digested plant matter and nutrient solution were detected with ICP-MS 7500c (Agilent Technologies, Waldbronn, Germany). For determination of the As and Sr uptake rate, the root growth was assumed to be linear during As and Sr application and uptake rates were calculated according to Claassen, 1990:

$$Up = \frac{As_2 - As_1}{t_2 - t_1} \times \frac{2}{DW_2 + DW_1}$$

where Up = uptake rate [mg g^{-1} root DW h^{-1}]; $As_{1,2}$ = As content in shoot [mg $plant^{-1}$] at start (1) and end (2) of cultivation in nutrient solution; $DW_{1,2}$ = root dry weight at start (1) and end (2) of cultivation in nutrient solution [g $plant^{-1}$]; $t_{1,2}$ = time at start (1) and end (2) of cultivation in nutrient solution [h]. Sr uptake was calculated similar to the As uptake. To calculate the uptake rate per root surface in the rhizotron experiment, root length and root surface of the root sections 2-6 and 6-10 cm drt were measured using a flat bed scanner and WinRhizo software (Regent Instruments Inc., Quebec, Canada). The surface:length ratio was 0.223 (± 0.018) in 2-6 cm drt and 0.122 (± 0.005) in 6-10 cm drt.

For determination of the Si concentration in the rice root, 200 mg of dried and ground root matter was digested in 3 ml 65 % HNO_3, 2 ml H_2O and 2 ml 30 % H_2O_2 in a microwave for 12 minutes at 190°C and then diluted with 20 ml 10 % NaOH and neutralized with HNO_3 (Haysom *et al*., 2006). Si concentration was determined photometrically at 811 nm after addition of 3.2 % boric acid, dye reagent (0.08 M sulphuric acid and 2 % ammonium heptamolybdate), 3.3 % tartaric acid and 0.4 % ascorbic acid (Novozamsky *et al*., 1984).

qRT-PCR

Adventitious roots were harvested at 2-6 cm and 6-10 cm drt and frozen immediately in liquid nitrogen. Roots were ground under liquid nitrogen and total RNA was isolated using TRIsure® Reagent (Bioline, Luckenwalde, Germany) following manufacturer's instructions. Total RNA (600 ng) and random hexamer primers were used to synthesize first-strand cDNA using the Revert Aid™ H Minus Kit (Fermentas, St. Leon-Rot, Germany) following the instructions of the manufacturer.

In qRT-PCR experiments, 100 ng cDNA was used as template in a 25 µl reaction-mix containing 2.5 µl 10x buffer, 3.6 mM $MgCl_2$, 0.2 mM dNTPs mix (Fermentas), 0.25 µl 1:1000 diluted SYBR-Green (Invitrogen, Carlsbad, CA, USA), 0.75 U HotStart-Taq-Polymerase (DNA cloning service, Hamburg, Germany), 0.25 µM forward and 0.25 µM reverse primers. The qRT-PCR runs were performed in the CFX96 cycler (Bio-Rad, München, Germany), using an initial 95°C-step for 10 min, followed by 35 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds, and a final melting curve procedure with a stepwise increment of 1°C ranging from 60°C to 95°C.

Forward and reverse primers of Lsi1 and Lsi2 were used as described in studies of Ma *et al*., (2007, 2011). The eukaryotic elongation factor 1-alpha (eEF 1-α) was used as endogenous control due to its stable transcript abundance in rice (Jain *et al*., 2006; Jain, 2009). For each target in qRT-PCR, three technical and three biological replicates were used. Relative quantity was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Histochemical examination of roots

For detection of casparian bands, free hand cross sections of adventitious rice, maize and onion roots without lateral roots were taken at 4-6 cm (rice, onion) and 6-8 cm (maize) drt. Root cross sections were stained with 0.1 % (w/v) berberine hemi-sulphate for 60 minutes and with 0.5 % (w/v) aniline blue for further 30 minutes (Brundrett *et al*., 1988). Stained sections were mounted in 0.1 % (w/v) $FeCl_3$ in 50 % (v/v) glycerine and viewed under an Axioskop fluorescence microscope (Zeiss, Jena, Germany) with UV illumination using excitation filter G 365, chromatic beam splitter FT 395 and barrier filter LP 420. The development of casparian bands in the anticlinal exodermal cell walls of a plant species was compared under UV-light between -Si and +Si plants.

Embedding and sectioning of roots

Root zone 4-6 cm of rice and onion and 6-8 cm drt of maize were embedded using the Steedman`s wax protocol (Gomez *et al*., 2009) in a modified form. Roots were fixed in freshly prepared Farmer's fixative (3 parts ethanol + 1 part acetic acid) at 4°C overnight. Roots were then dehydrated at RT under rotation for each 2 hours in 75 %, 85 %, 95 % and 100 % ethanol, respectively. Molten Steedmans's wax (9 parts poly (ethylene glycol) distearate (SigmaAldrich, St. Louis, USA) + 1 part 1-hexadecanol (SigmaAldrich)) was mixed 1:1 with ethanol and roots were incubated in the mixture at 38°C overnight. Roots were then incubated three times at 38°C for each 2 hours in pure Steedman's wax. Afterwards, roots were divided in 3 mm pieces and embedded in Steedman's wax in TurbOflowII molds and cassettes (McCormick Scientific, St. Louis, USA). The wax was allowed to solidify overnight at RT. The wax blocks were cut with a Hyrax M55 rotary microtome (Zeiss, Jeny, Germany) into 20, 50 and 100 µm slices. Wax slices were dissolved by addition of ethanol and root sections were washed several times by exchanging ethanol.

Laser ablation-inductively coupled plasma-mass spectroscopy (LA-ICP-MS)

Tin (Sn) foils (Elementar Analysensysteme GmbH, Hanau, Germany) were placed on microscopy glass slides and root sections floating in ethanol were transferred to Sn foils. Evaporation of the ethanol led to a fixation of the root sections to the foil, which allowed the use of a laser for ablation. For rice and maize, 100 µm thick root sections were used and for onion, 50 µm thick slices were used. Root tissue was ablated with the solid state NYAG-laser UP193 SS (New Wave Research Co. Ltd., Cambridge, England). The laser beam was adjusted to a diameter of 75 µm and energy of 2.5 J cm^{-1} for rice and maize and to a diameter of 50 µm and energy of 4.0 J cm^{-1} for onion. The ablation chamber was coupled to the ICP-MS torch with a tygone® tube and was filled with carrier gas at a flow rate of 0.25 L min^{-1}. After the chamber was passed the flow rate was increased with makeup gas to 1.2 L min^{-1}. ^{13}C and ^{28}Si signals were detected using the quadropole ICP-MS 7500 CX (Agilent Technologies, Santa Clara, USA). Table 1 summarizes further ICP-MS settings.

Tab. 1: LA-ICP-MS parameters

ICP-MS conditions	
RF power	1300 W
Carrier gas (Ar)	0.25 L min^{-1}
Makeup gas (Ar)	0.95 L min^{-1}
Dwell time / isotope	10 ms
LA conditions	
Pulse length	1 s (rice, maize); 2 s (onion)
Pulse frequency	10 Hz

For calibration, four root samples of rice plants grown with or without Si supply were used as standards. Ground plant matter from the same root samples was compressed to pellets and each 10 spots from the pellets were analyzed for ^{13}C and ^{28}Si signals using LA-ICP-MS. The ^{28}Si:^{13}C signal ratios of the four samples were correlated with their Si concentrations using R software (R Development Core Team, 2011) and the equation of the linear regression (correlation coefficient $R^2 = 0.96$) was used to calculate the Si concentrations from the ^{28}Si:^{13}C signal ratios for all root cross sections.

Scanning electron microscope energy-dispersive X-ray microanalysis (SEM-EDX)

20 µm thick root sections of rice, maize and onion were transferred to polished aluminium sample holders (Hoffmann Group, München, Germany) and sputtered with gold for 60 s at 30 mA using a Sputter Coater (Cressington 108 auto). Samples were analyzed using a scanning electron microscope (JSM-6610 LV, JEOL, Eching, Germany). Energy-dispersive X-ray microanalysis (EDX) was done with XFlash Detector 410-M (Bruker Nano GmbH, Berlin, Germany) using Quantax Esprit 1.9 software (Bruker) with 20 kV acceleration voltage.

Statistical analysis

All Si treatments in the As and Sr uptake experiments were replicated four times and all pots were arranged in a randomized block design. Uptake rates were compared with Tukey's test after ANOVA with $p < 0.05$ and qRT-PCR data were compared with a mixed model analysis (Steibel *et al*., 2009) using R software.

Results

Arsenic and strontium uptake rates of whole plants

The As uptake rate of whole rice plants that were grown without Si supply during the preculture was not affected by Si supply for 48 hours (Fig. 2A). However, when plants were pretreated with Si for 18 days, the As uptake rate was clearly decreased. On the other hand, the Sr uptake rate was not affected by the short term Si supply or during the preculture (Fig. 2B).

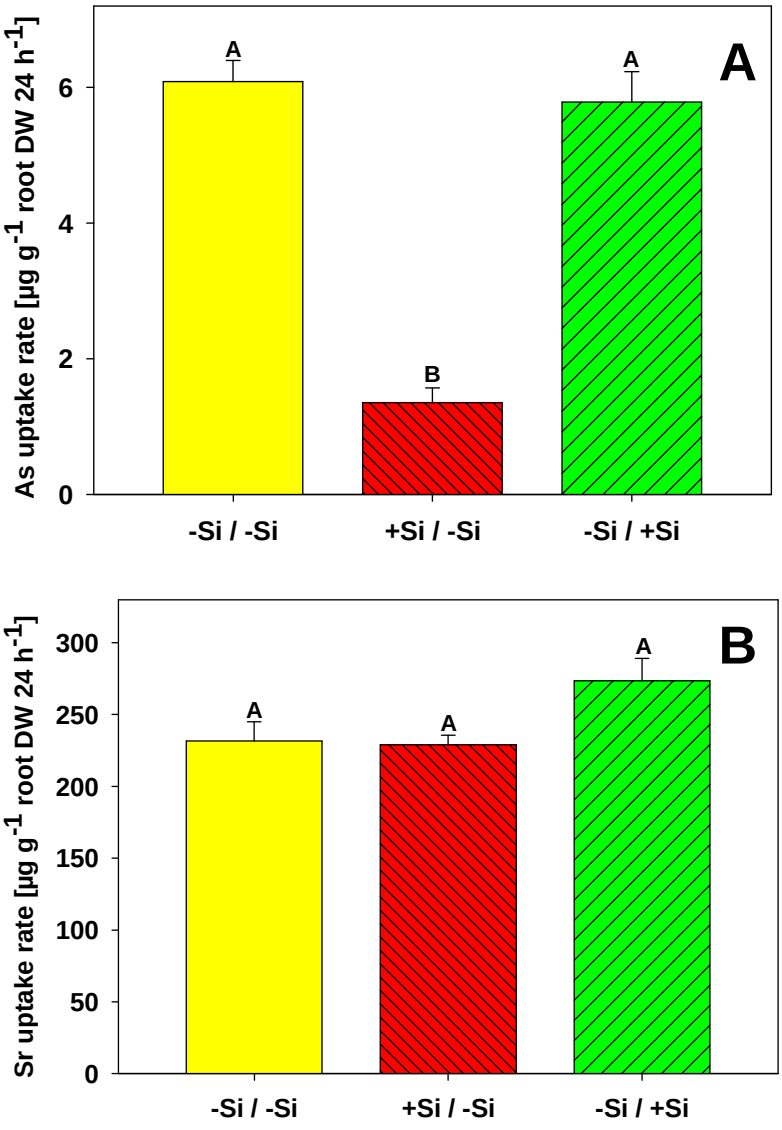

Fig. 2: As (A) and Sr (B) uptake rate of rice plants as affected by Si supply during the preculture for 18 days and by Si supply of whole plants for 48 h. Different letters indicate significant differences between Si treatments. Bars are SE, n=4.

To detect the effect of the Si treatments on the As transporters Lsi1 and Lsi2, the transcript levels of both genes coding for the transporters were measured in two root zones after the preculture and after 48 hours of differential Si supply. Continuous Si supply during the preculture decreased the transcript levels of *Lsi1* and *Lsi2* in both root zones, 2-6 cm (Fig. 3A, C) and 6-10 cm drt (Fig. 4A, C). Also after 48 hours of Si supply (-Si/+Si), the transcription of *Lsi1* and *Lsi2* was already reduced, while the transcript levels in both root zones of Si deprived plants (+Si/-Si) did not recover within 48 hours (Fig. 3B, D, 4B, D).

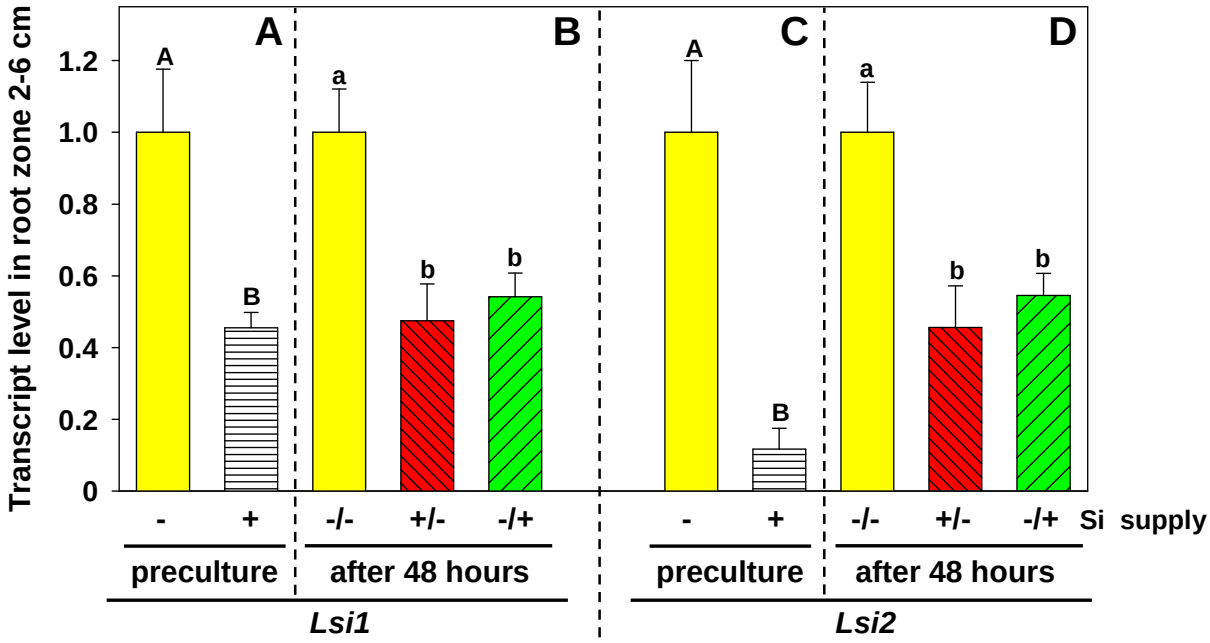

Fig. 3: Transcript levels of *Lsi1* (A, B) and *Lsi2* (C, D) in root zone 2-6 cm behind the root tip as affected by the Si supply during the preculture (A, C) and after 48 hours of differential Si supply. Different capital and small letters indicate significant differences between the Si treatments during the preculture and after 48 hours of differential Si supply, respectively. Bars are SE, n=3.

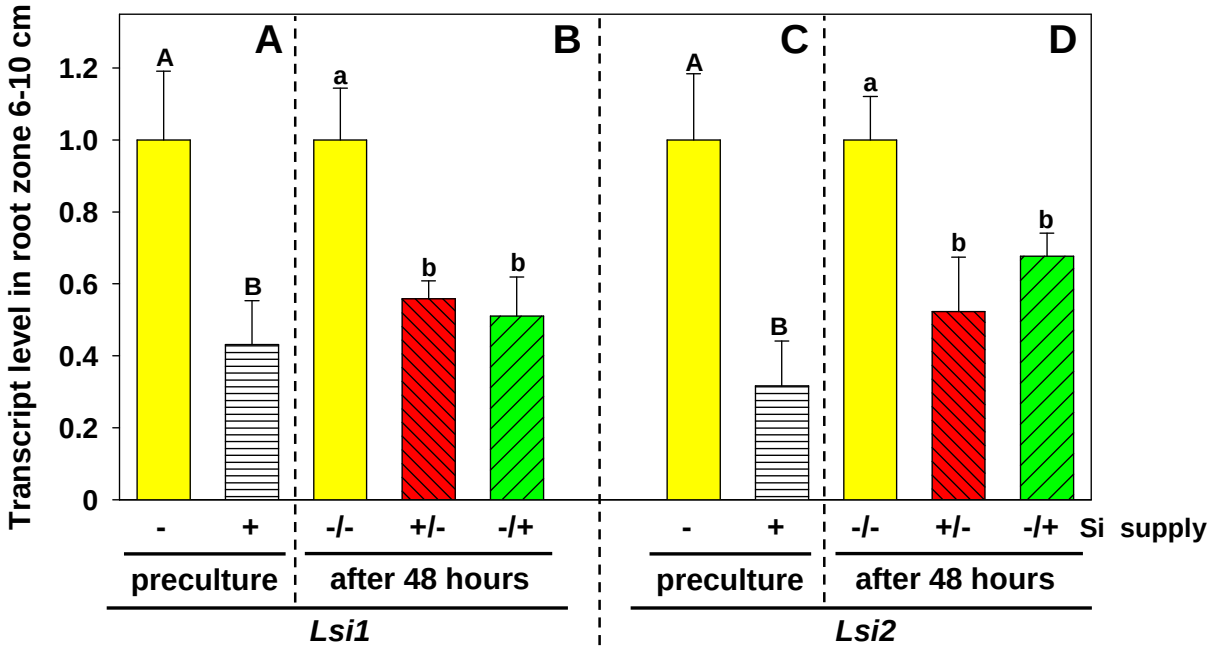

Fig. 4: Transcript levels of *Lsi1* (A, B) and *Lsi2* (C, D) in root zone 6-10 cm behind the root tip as affected by the Si supply during the preculture (A, C) and after 48 hours of differential Si supply. Different capital and small letters indicate significant differences between the Si treatments during the preculture and after 48 hours of differential Si supply, respectively. Bars are SE, n=3.

Arsenic and strontium uptake rates of excised roots

The As uptake rate of excised roots mounted into rhizotrons was not affected by short term Si supply for 4 hours, while Si supply during the preculture halved the As uptake rate compared to the control treatment (-Si/-Si) (Fig. 5A). In contrast, the Sr uptake rates were not influenced by the Si treatments (Fig. 5B). The effects of the Si treatments on the As and Sr uptake rates were the same in both root zones, although the As and Sr uptake rates in the root zone 6-10 cm drt were two times higher than in 2-6 cm drt.

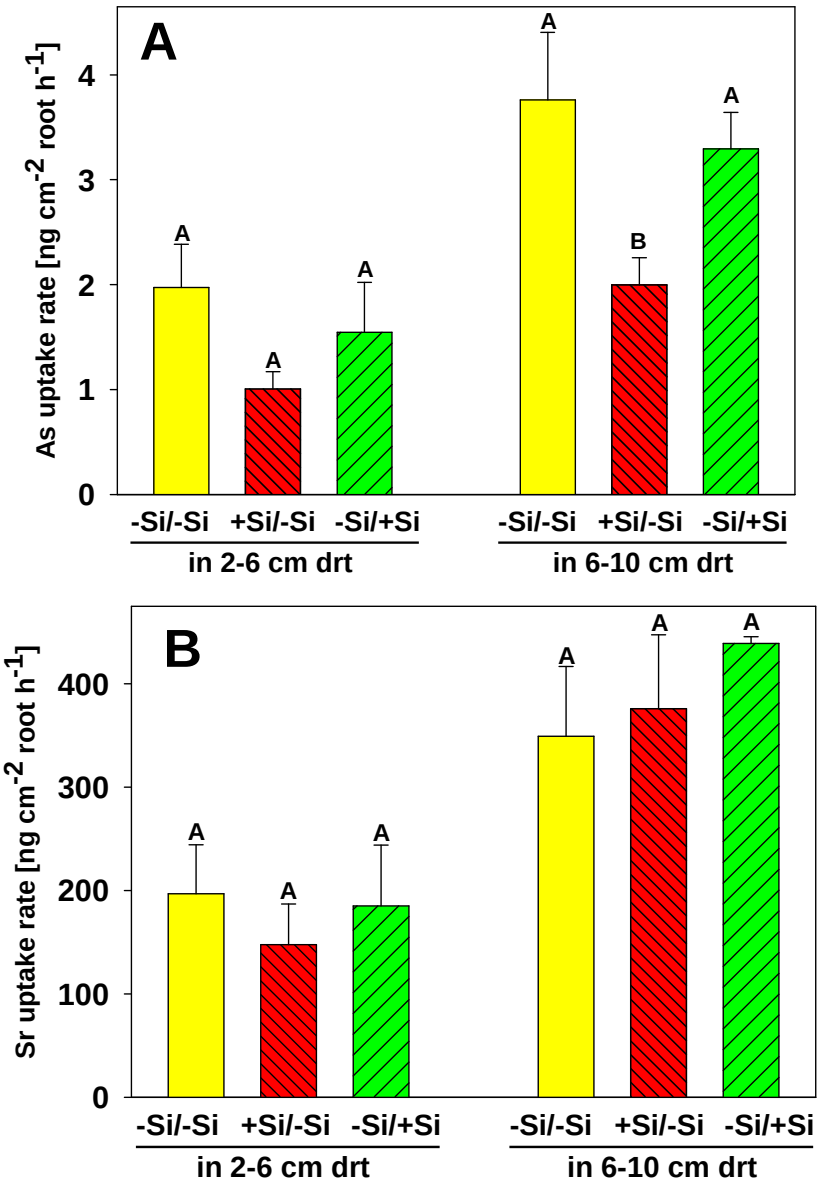

Fig. 5: As (A) and Sr (B) uptake rate of rice plants in root zones 2-6 and 6-10 cm distance from the root tip (drt) as affected by Si supply during the preculture for 18 days and by short term Si supply of excised roots for 4 h. Different letters indicate significant differences between Si treatments. Bars are SE, n=4.

The effect of the Si treatments on the transcript level of *Lsi1* and *Lsi2* in excised roots was similar to the experiment with whole plants, since continuous Si supply decreased the transcription of *Lsi1* and *Lsi2* in 2-6 and 6-10 cm drt (Fig. 6A, C, 7A, C). Moreover, the transcript levels in excised roots had not been recovered after 4 hours of Si deprivation (Fig. 6B, D, 7B, D). On the other hand, short term Si supply of excised roots clearly decreased the transcript levels of both genes in 2-6 cm drt (Fig. 6B, D) and the trend was similar in 6-10 cm drt (Fig. 7 B, D).

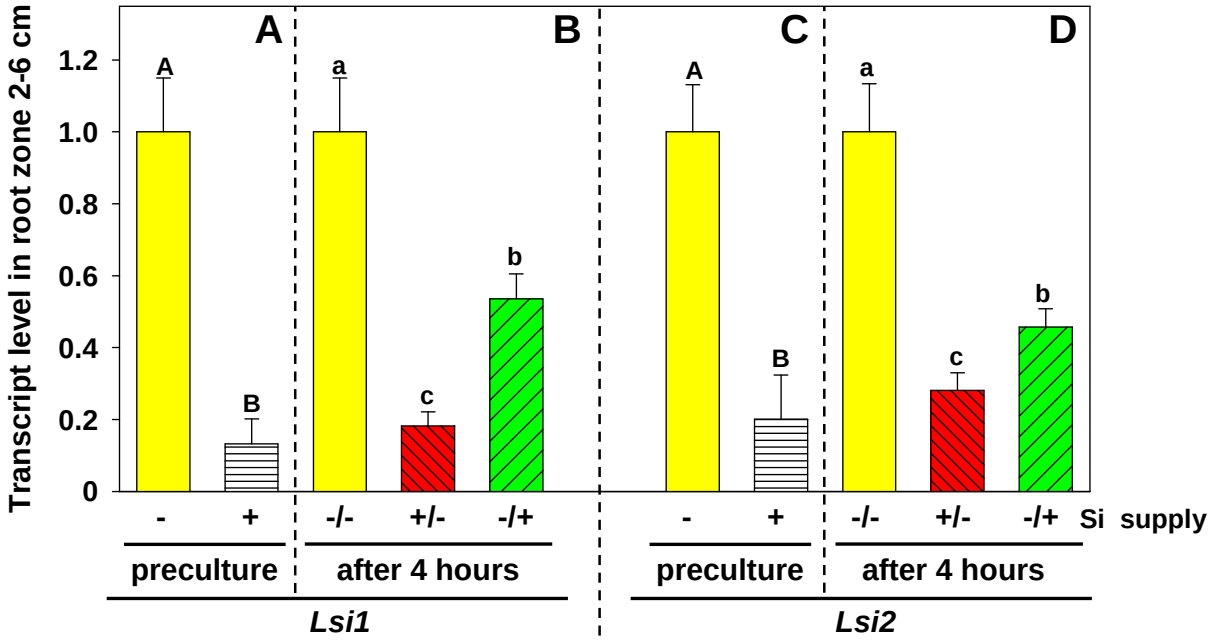

Fig. 6: Transcript levels of *Lsi1* (A, B) and *Lsi2* (C, D) in root zone 2-6 cm behind the root tip as affected by the Si supply during the preculture (A, C) and after 4 hours of differential Si supply of excised roots. Different capital and small letters indicate significant differences between the Si treatments during the preculture and after 4 hours of differential Si supply, respectively. Bars are SE, n=3.

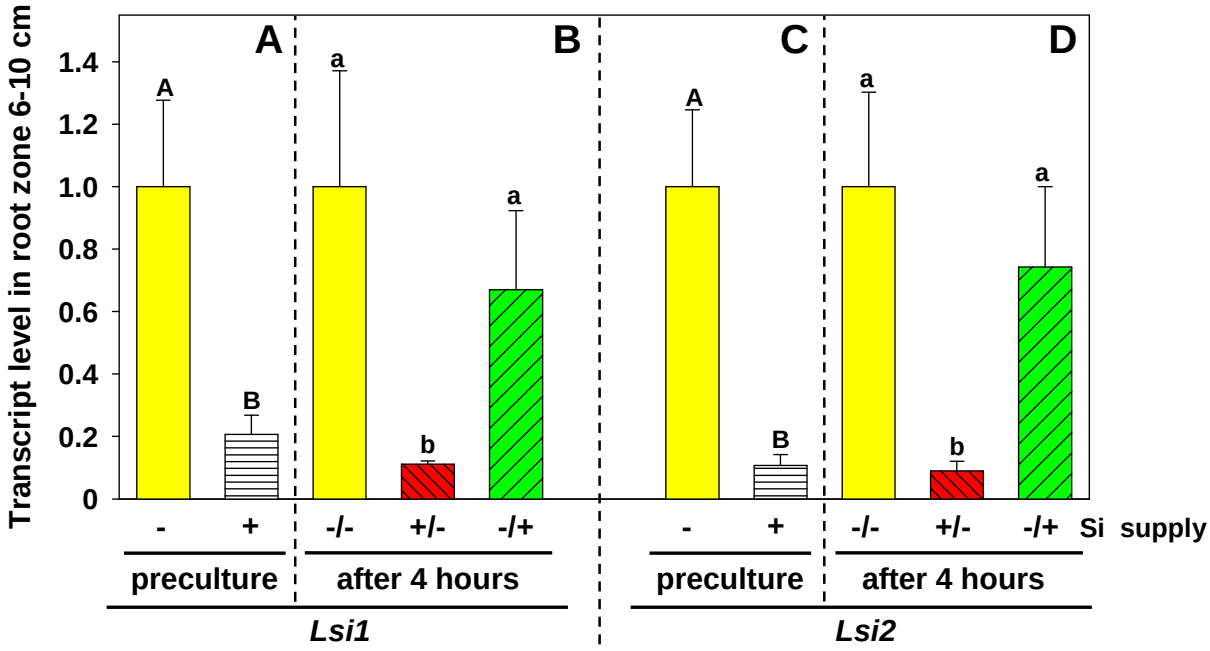

Fig. 7: Transcript levels of *Lsi1* (A, B) and *Lsi2* (C, D) in root zone 6-10 cm behind the root tip as affected by the Si supply during the preculture (A, C) and after 4 hours of differential Si supply of excised roots. Different capital and small letters indicate significant differences between the Si treatments during the preculture and after 4 hours of differential Si supply, respectively. Bars are SE, n=3.

Silicon depositions in the roots of rice, maize, and onion

The distribution of Si was analyzed with LA-ICP-MS and the position of the ablation spots was set on one hand to cover the different cell layers of the root and on the other hand, to keep the integrity of the root. As a consequence of the latter, the spot density in the rice root had to be lower than in maize and onion because the cross section of the rice root was relatively labile.

The Si concentration in the adventitious rice root was highest in the outer part of the root comprising the exodermis and the sclerenchyma, while the Si concentration around the central cylinder was low (Fig. 8A, B). High Si concentrations were found also punctual in the cortex tissue.

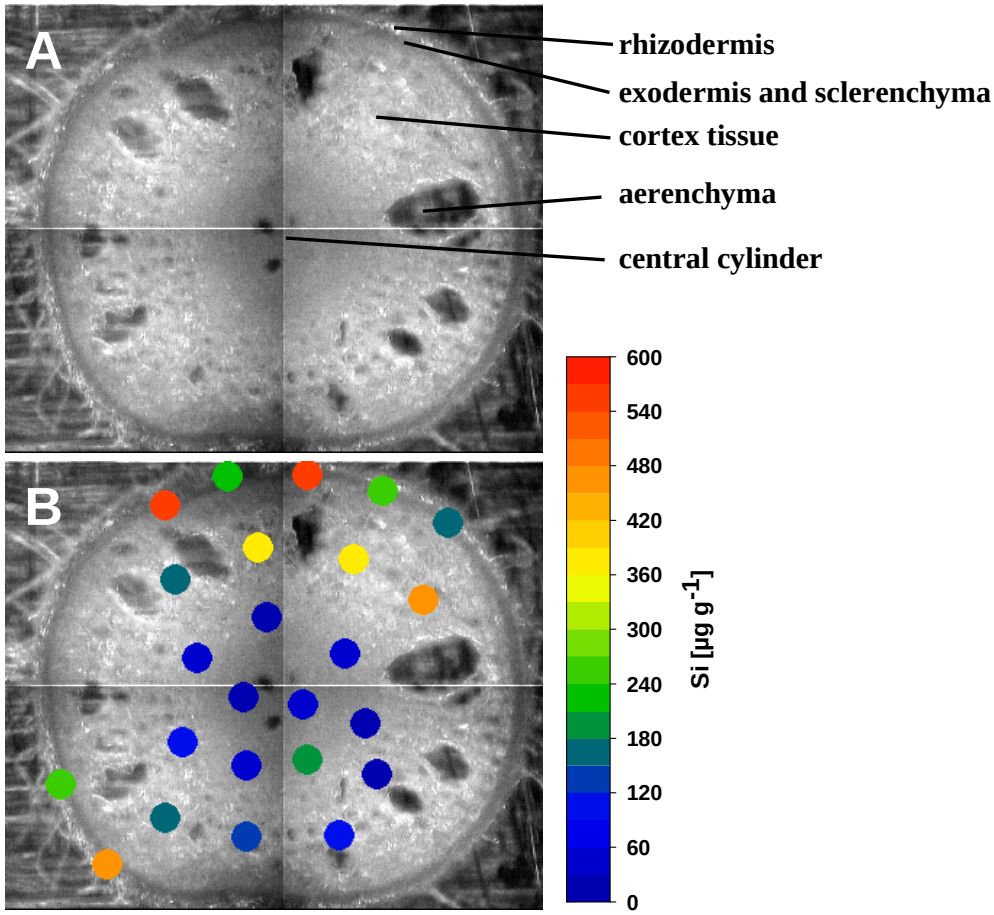

Fig. 8: Cross section of an adventitious rice root in 4-6 cm drt (A) and corresponding Si concentration as measured by LA-ICP-MS (B). The diameter of the ablation spots is 75 µm. The Si distribution is characteristic for three rice roots analyzed.

These findings were supported by SEM-EDX analysis, where the Si signals are illustrated as white dots. In the adventitious rice root, Si signals were detected in the outer part of the root comprising the exodermis and the sclerenchyma (Fig. 9A, B).

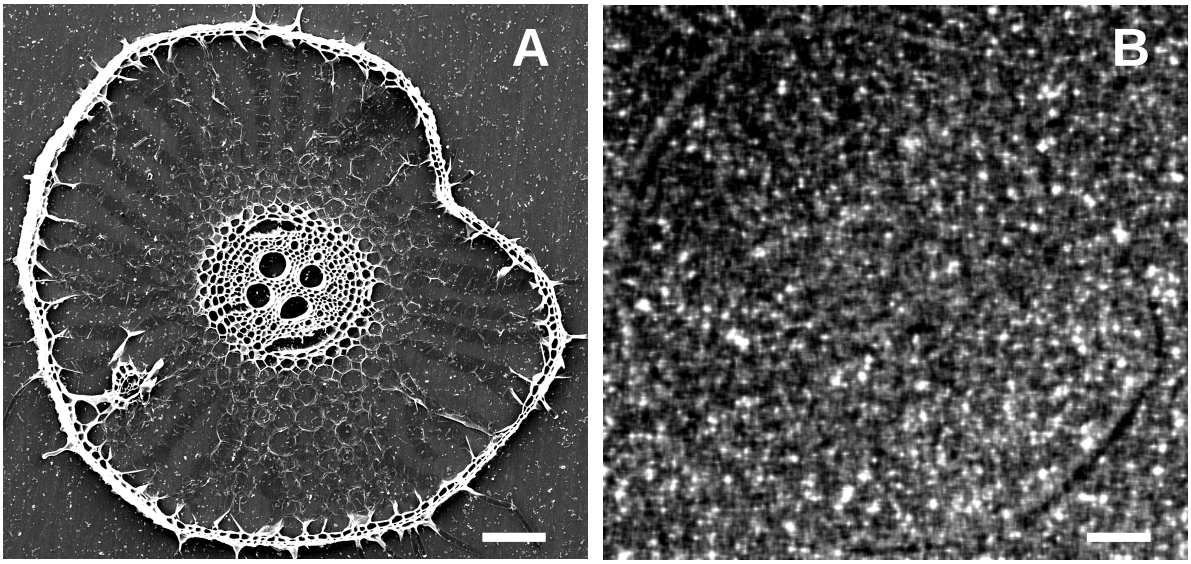

Fig. 9: Scanning electron micrograph (A) of a cross section of rice root in 4-6 cm drt and corresponding EDX analysis mapping of the silicon signal (B). Silicon is indicated as white dots. White bars indicate 100 µm.

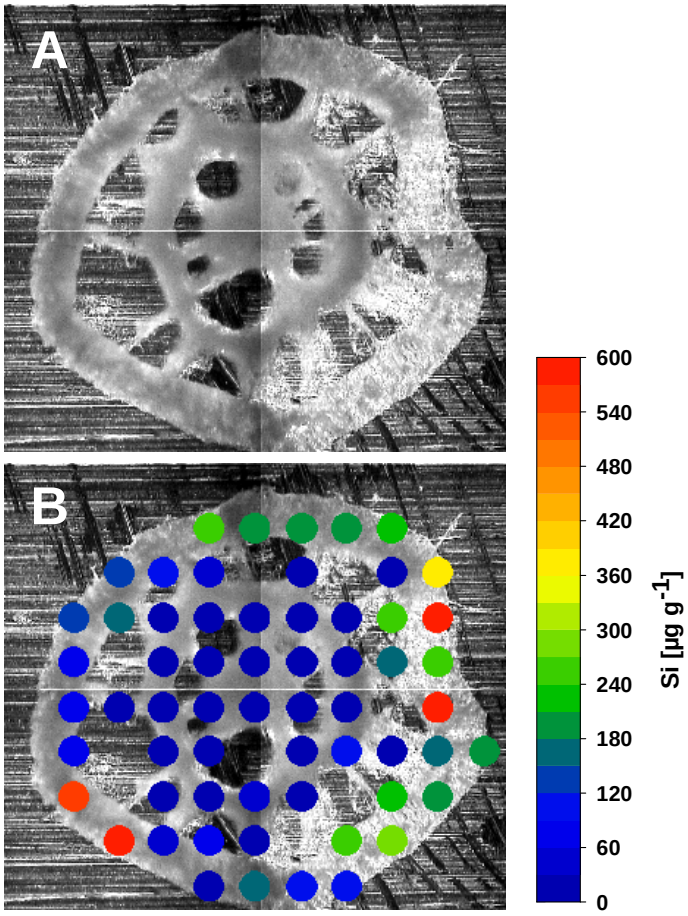

Fig. 10: Cross section of a maize root in 6-8 cm drt (A) and corresponding Si concentration as measured by LA-ICP-MS (B). The diameter of the ablation spots is 75 µm. The Si distribution is characteristic for three maize roots analyzed.

In the maize root, Si depositions were found nearly exclusively in the outer part of the root, where the exodermis is located (Fig. 10A, B). Some areas of the cortex tissue were collapsed after the air-drying of the cross section.

The distribution of Si in the onion root was similar to maize, since the highest Si concentrations were found in the outer part of the root, while the Si concentration in the cortex cells, the endodermis, and the central cylinder was low (Fig. 11A, B).

The distribution of Si in the maize and onion root could not be recognized by SEM-EDX analysis because the background signal superposed the Si signals of the root.

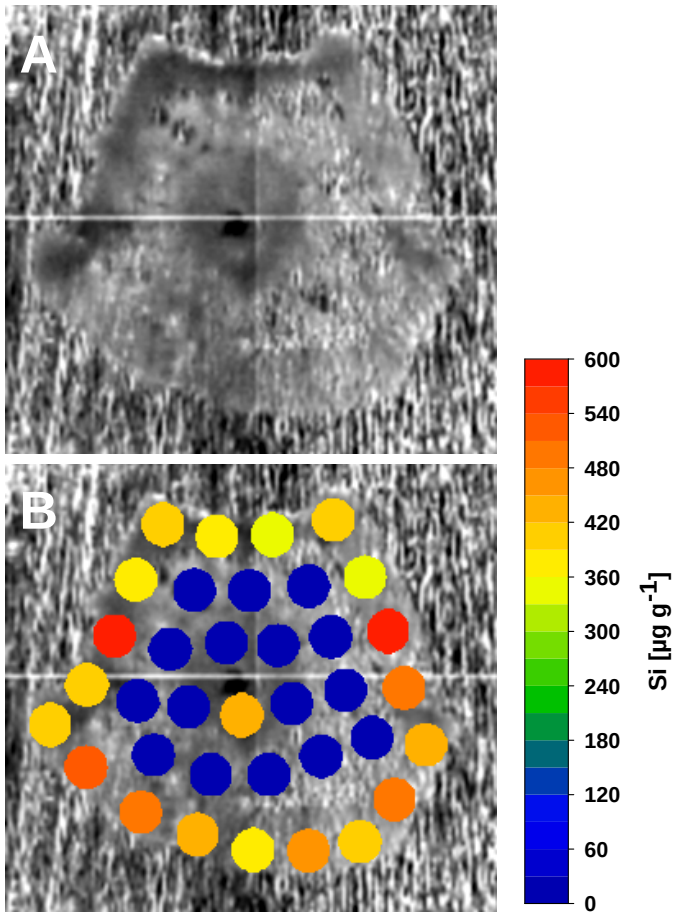

Fig. 11: Cross section of an onion root in 4-6 cm drt (A) and corresponding Si concentration as measured by LA-ICP-MS (B). The diameter of the ablation spots is 50 µm. The Si distribution is characteristic for three onion roots analyzed.

Discussion

Effect of silicon supply on arsenite uptake

In the rhizotron experiment with excised roots, the uptake rate of As and Sr per surface was nearly twice as high in 6-10 cm drt compared to the younger root zone (Fig. 5A, B). This can be attributed to the development of lateral roots which emerged at around 6 cm drt and led to a nearly halved surface:length ratio in the old root zone compared to 2-6 cm drt.

The As and Sr uptake rates were determined in two different experimental systems because both have advantages and disadvantages. The nutrient solution experiment allowed the usage of whole plants but needed to be carried out for 48 hours to accumulate enough As in the plant for analytical measurements. On the other hand, different root zones can be treated with As in the rhizotrons, but the experimental conditions are quite artificial since excised roots are used. However, in both systems a non-toxic concentration of As(III) was used (Hoffmann and Schenk, 2011) avoiding possible toxicity-related side effects.

Finally, the results of both experiments were similar and clearly demonstrated that the As uptake rate of rice roots was not affected by a short term Si supply (Fig. 2A, 5A). These findings contradict the hypothesis, that a reduced As concentration in rice plants by Si supply is due to a competitive inhibition of the As(III) uptake by silicic acid (Guo *et al*., 2009; Li *et al*., 2009b; Zhao *et al*., 2009). In contrast, the As uptake rate was clearly reduced by a continuous Si supply for a long time (Fig. 2A, 5A).

A continuous Si supply was shown to increase the casparian band formation in the exodermis of rice roots (Fleck *et al*., 2011), what could be observed in this study, too (data not shown). The casparian bands might limit the apoplastic flow (Krishnamurthy *et al*., 2009) and thus, the uptake rate of the apoplastic tracer Sr was measured. However, the Si treatments did not affect the Sr uptake rate (Fig. 2B, 5B) and thus, an inhibition of the apoplastic movement was not the reason for the reduced As uptake.

Rather, the uptake of As(III) might be limited by a reduced abundance of the As(III) transporters Lsi1 and Lsi2, since Si supply for three days was shown to decrease the transcription of *Lsi1* and *Lsi2* (Ma *et al*., 2006, 2007). This could be observed in this study too, since the transcript levels of *Lsi1* and *Lsi2* were decreased by continuous Si supply during the preculture (Fig. 3A, C, 4A, C, 6A, C, 7A, C).

However, the transcript levels were reduced also by a short term Si supply both of whole plants for 48 hours (Fig. 3B, D, 4B, D) and of excised roots for only 4 hours (Fig. 6B, D, 7B, D). A reason for this contrast to the literature might be that Ma *et al*. (2006, 2007) used rice

seedlings without adventitious roots by and in addition, it is not clear what root sections were examined.

The decreased transcript level of *Lsi1* and *Lsi2* already after short term Si supply did not result in a reduced As uptake rate and this can be explained by the abundance of the transporter proteins that were not yet considerably decreased. An altered transcription leads to altered protein abundance depending mainly on protein synthesis rate and protein degradation rate. In a study with 84 *Arabidopsis* cell culture proteins the degradation rate ranged from less than 2 to 116 % day^{-1} (Li *et al*., 2012). Among these proteins, there were two anion channels that had degradation rates of 17 and 19 % day^{-1}. Assuming a mean degradation rate of 18 % day^{-1} for Lsi2, which is an anion transporter, the amount of protein left after 48 hours would still be 67 % of the initial amount. Moreover, this value is still too low considering that the protein synthesis rate was not 0. Thus, the decreased transcript rate most likely did not considerably decrease the transporter protein abundance within 48 hours to limit the As(III) uptake.

The transcript level after short term Si deprivation (+Si/-Si) was still lower compared to the control, presumably because the reaction upon Si deprivation was delayed since the Si in the root firstly had to be depleted.

Silicon depositions in the roots of rice, maize, and onion

Si supply increased the formation of the casparian bands in the exodermis of rice, maize, and onion roots (Chapter II), which could be observed also in this study (data not shown). Si might chemically interact with cell wall components and aromatic compounds to promote casparian band formation, and if so, Si should be deposited in the exodermis. Therefore, the deposition of Si was investigated in those root zones, where the promotion of casparian band formation was strongest, which was in 4-6 cm drt of rice and onion roots and in 6-8 cm drt of maize roots (Chapter II).

Silicon depositions were found in the outer part of the adventitious rice root, where the exodermis and the sclerenchyma are located, and the results were the same in LA-ICP-MS as well as in SEM-EDX analysis (Fig. 8A, B, 9A, B). This is in line with reports from Gong *et al*. (2006), who found silica depositions in the exodermis of rice roots. Moreover, Moore *et al*. (2011) reported that silica was deposited in the cell walls of sclerenchyma and exodermis in rice roots. However, in both studies silica deposits were found also in the endodermis, what is in contrast to this study, where Si deposits in the endodermis could be observed neither in the LA-ICP-MS nor in the SEM-EDX analysis. However, Gong *et al*. (2006) used seminal rice

roots and in the study of Moore *et* al. (2011), it is not clear what kind of rice roots were used and which root zones were investigated.

The cortex tissue of the maize root was partially collapsed and compressed probably as a result of the air-drying (Fig. 10A). However, the compressed cortex tissue could still be analyzed using LA-ICP-MS, because the calculation of the Si concentration is based on the ratio of the ^{28}Si:^{13}C signals and thus, is independent from the amount of tissue ablated by the laser. Si deposits in the maize root were found in the outer part of the root, where the exodermis is located, while the cortex tissue and the central cylinder contained only very small concentrations of Si (Fig. 10A, B).

In onion roots, Si depositions were found also nearly exclusively along the exodermis (Fig. 11A, B), which was not reported before.

Si might promote the formation of casparian bands by inducing the precipitation of aromatic compounds in the cell walls of exodermis and endodermis or by cross-linking aromatic compounds with the cell wall. Silicon supply promoted the formation of casparian bands in the exodermis of rice plants (Fleck *et al*., 2011) and also in roots of other plants including maize and onion (Chapter II, p. 56). In rice, the transcription of genes related to the synthesis of suberin and lignin, which are components of casparian bands, was increased by Si supply (Fleck *et al*., 2011). However, whether Si directly affects transcription or whether this is a secondary effect remained unclear.

The mode of action of Si could rather be chemical, since Si was shown to form complexes with catechol as six-coordinated Si (Barnum, 1970, 1972). Moreover, silica deposition was induced by lignin and by callose and Si was found along with the latter in cell walls of horsetail (*Equisetum arvense*) (Fang and Ma, 2006, Currie and Perry, 2009; Law and Exley, 2011).

The combination of both, the reactivity of Si with aromatic compounds and the deposition of Si in the exodermis of rice, maize, and onion supports the hypothesis, that Si chemically promotes the casparian band formation. Assuming this hypothesis proving true, Si would be effective on a broad range of plant species.

General Discussion

Arsenic dynamics in flooded soils

Rice plants can accumulate large amounts of arsenic (As) in the shoot and high As levels in the rice grain expose a serious health risk for human beings (Kile *et al*., 2007; Su *et al*., 2009). A reason for the high As concentration in the rice plant is the high availability of arsenite, As(III), in the soil solution of flooded soils, where paddy rice is grown. Another reason for the high As levels in rice is the highly efficient uptake of As(III) by the rice root. Silicon (Si) was shown to reduce As concentrations in straw and husk of rice plants, while the As concentration in the grain was decreased less (Li *et al.*, 2009b). However, the underlying mechanisms remained unclear. Thus, the effect of Si application to three paddy rice soils on soil solution conditions as well as As concentration and speciation in rice was investigated (Chapter III).

After the soils were flooded, the redox potential decreased and caused radical chemical changes in the soil solution. The Fe concentration in the soil solution rapidly increased up to a maximum and decreased afterwards and the As concentration followed a similar pattern. This is because arsenate, As(V), the predominant As species in aerobic soils, is bound to Fe-(hydr)oxides and released into the soil solution when the (hydr)oxides are reduced at a low redox potential (Fig. 4) (Onken and Hossner, 1996; Bogdan and Schenk, 2008; Yamaguchi *et al*., 2011). The subsequent decrease of the Fe and As concentration is due to the very low redox potential, where sulfate reduction occurs leading to the precipitation of iron sulfide and As(V) sulfide (Inskeep *et al*., 2001; Bostick *et al*., 2004). Si application to soils increased the concentration of Si, Fe, and As in the soil solution, while the redox potential was not altered. The observed increase of As concentrations was also reported in literature (Li *et al*., 2009b) and can be explained by a substitution of As(V) by silicic acid since both compete for the same adsorption sites (Waltham and Eick, 2002). At a low redox potential, As(V) is reduced to As(III), which exhibits the predominant As form in the soil solution of flooded soils like reported in several studies (Takahashi *et al*., 2004; Bogdan and Schenk, 2008; Xu *et al*., 2008; Li *et al*., 2009b).

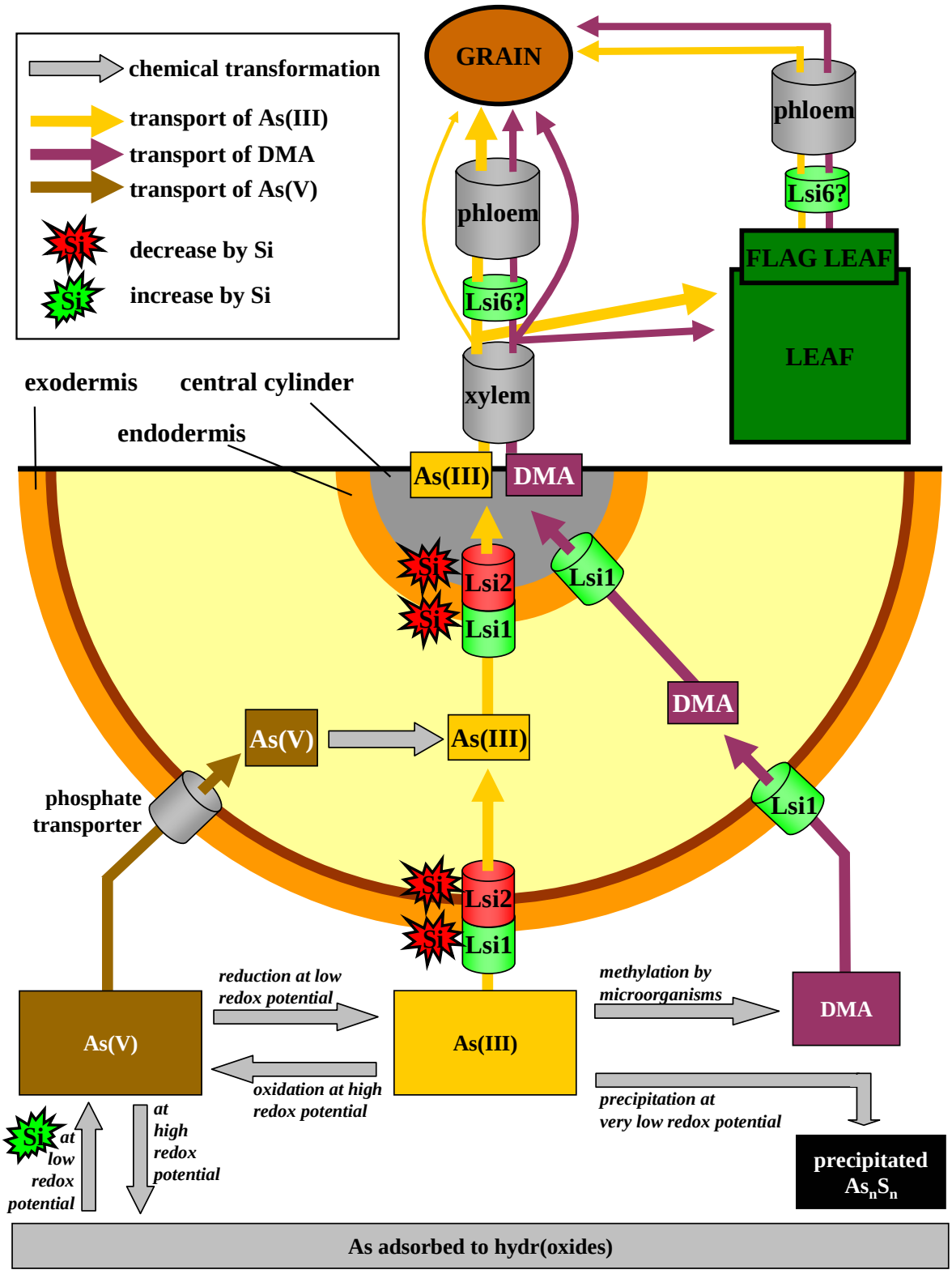

Fig. 4: Schematic overview of As dynamics in soil solution and As uptake as affected by Si. As(III): arsenite; As(V): arsenate; DMA: dimethylarsinic acid.

Uptake and translocation of As in rice

As(III) is taken up into the rice root by the transporters Lsi1 and Lsi2 that also facilitate the transport of As(III) towards the xylem, while DMA is transported only by Lsi1 (Fig. 4). In the xylem, As(III) and DMA are translocated to the shoot and the As concentration in rice was highest in the flag leaf, followed by straw, husk, brown rice and polished rice, which is in accordance with other studies (Abedin *et al*., 2002a; Liu *et al*., 2006; Rahman *et al*., 2007). The grain As concentration was an order of magnitude lower than in straw, which is also in line with literature (Bogdan and Schenk, 2008; Xu *et al*., 2008). The main As species in the polished rice was As(III), followed by DMA and As(V).

As(III) is transported into the grain almost exclusively via the phloem (Carey *et al*., 2010; Zhao *et al*., 2012), which may be loaded with As(III) either in the leaves, particularly the flag leaf, or in the stem nodes by xylem-to-phloem transfer. Whether As(III) is transported from the flag leaf to the grain is not certain, since there are contrary reports in literature (Carey *et al*., 2011; Zhao *et al*., 2012). On the other side, intensive xylem-to-phloem transfer occurs in the stem nodes as shown for potassium and amino acids (Marschner, 2012). Moreover, cadmium coming from the root via the xylem is transferred from xylem to phloem in the nodes of rice plants before it is transported to the grain (Fujimaki *et al*., 2010).

The Si transporter Lsi6, a homolog of Lsi1, might be involved in the xylem-to-phloem transfer in the nodes (Fig. 4), since Lsi6 is supposed to unload Si from the xylem in the rice node (Yamaji and Ma, 2009). In addition, Lsi6 facilitated the transport of As(III) when expressed in yeast and in *Xenopus* oocytes (Bienert *et al*., 2008; Ma *et al*., 2008).

Effect of Si on As uptake

Although the levels of As(III) in the soil solution were increased by Si, the As concentration in the flag leaf, straw, and husk were decreased by Si application to the soils (Chapter III), what is in agreement with previous studies (Guo *et al*., 2005; Li *et al*., 2009b). In the polished rice, concentration of As(III) was reduced by Si, while DMA and As(V) were not affected by Si supply and hence, the decreased As concentration in polished rice was caused solely by a reduced As(III) concentration. The decreased As(III) concentration in the rice grain and the rice leaves is very likely the consequence of a limited As(III) uptake by the rice root. A decreased uptake of As(III) by Si supply might be either due to a competitive inhibition of As(III) by silicic acid (Guo *et al*., 2009; Li *et al*., 2009b; Zhao *et al*., 2009) or a decreased expression of *Lsi1* and *Lsi2* (Ma *et al*., 2006, 2007). For Lsi1, a competitive inhibition of the As(III) uptake by silicic acid could not be verified in experiments with yeast cells and

Xenopus oocytes expressing *Lsi1* (Bienert *et al*., 2008; unpublished data of Ma and Mitani in: Li *et al*., 2009a). However, a competitive inhibition of As(III) transport could take place at Lsi2 that is supposed to be crucial for As transport to the shoot by actively mediating the As(III) efflux towards the xylem (Li *et al*., 2009a; Zhao *et al*., 2009).

To clarify how Si decreases the As(III) uptake, rice plants cultivated with different Si treatments were exposed to As(III) and the uptake rates were examined (Chapter IV). A continuous preculture of rice plants with Si resulted in a clearly reduced As(III) uptake rate compared to a -Si control, while the presence of Si during As(III) exposure did not affect the As(III) uptake rate. The results were the same in experiments both with whole plants in nutrient solution as well as with excised adventitious rice roots in rhizotrons. Thus, a competitive inhibition of As(III) uptake by silicic acid could not be verified. Instead, the reduced As(III) uptake was the result of the Si preculture.

A possible reason could be a limited apoplastic flow of As(III), since formation of casparian bands was promoted by Si supply (Chapter I). However, the uptake rate of strontium, which is taken up similar to calcium (Ca) and is an indicator of the apoplastic movement (Storey and Leigh, 2004), was not affected by the Si treatments and hence, a change of the apoplastic flow was not observed in this experiment (Chapter III). Rather, the reduced As(III) uptake rate might be a consequence of a reduced abundance of the transporters, since the transcription of *Lsi1* and *Lsi2* was decreased by the Si preculture, which is in line with literature reports (Ma *et al*., 2006, 2007).

In contrast to As(III), the DMA concentration in polished rice was not affected by Si supply (Chapter III). A reason might be the DMA level in the soil solution that generally is much lower than the As(III) level (Bogdan and Schenk, 2008; Xu *et al*., 2008) and therefore a reduced transporter expression would rather limit the uptake of As(III) than of DMA. Elevated DMA levels in rice grain after Si supply found by Li *et al*. (2009b) could be the result of an enhanced methylation of inorganic As in the soil solution after Si supply, since microorganisms in soil solution are able to synthesize DMA, while plants are not (Lomax *et al*., 2012) and hence, the DMA level in the grain is related to the DMA concentration in the soil solution.

Effect of silicon supply on radial oxygen loss and development of casparian bands in rice

The beneficial effects of Si on the shoot of field grown rice in terms of reducing abiotic and biotic stress and enhancement of yield are known for a long time (Epstein, 1994, 1999; Ma and Yamaji, 2006). However, an effect of Si on rice roots was not described before. In this

study, rice plants cultivated in nutrient solution with Si supply exhibited a reduced radial oxygen loss (ROL) in adventitious roots compared to plants grown without Si supply (Chapter I). The ROL of +Si roots was restricted to the root tip and the first 5 cm of the root, while ROL was observed over the whole length of –Si roots. The diffusion of oxygen can be prevented by a barrier, which is related to densely packed sclerenchyma cells with lignified secondary walls and suberized exodermis with casparian bands (Kotula and Steudle, 2008). Suberin, lignin and casparian bands can be visualized under the UV-microscope after staining free-hand root cross sections with berberine aniline-blue (Brundrett *et al*., 1988). It was shown that Si enhanced the formation of casparian bands in adventitious rice roots, and also the lignification of the sclerenchyma was increased by Si supply (Chapter I). The formation of casparian bands started in 4-6 cm distance from the root tip in +Si roots and this coincided with the restricted ROL in this area. While it is known that both, casparian bands in the exodermis and lignification of the sclerenchyma, reduce ROL, it is not clear how each process contributes to the reduction (Kotula *et al*., 2009a). However, the main components of casparian bands are supposed to be suberin and lignin (Hose *et al*., 2001). Thus, the effect of Si on suberin and lignin synthesis was investigated.

Lignin is mainly built of the three monolignols p-coumaryl, coniferyl and sinapyl alcohol, which are synthesized in the symplast during the phenylpropanoid pathway and then released into the apoplast, where they polymerize to lignin (Boerjan *et al*., 2003; Goujon *et al*., 2003). Similarly, the biopolymer suberin is generated in the apoplast by polymerization of suberin monomers that are synthesized in the symplast before being released into the apoplast. The suberin monomers are composed of fatty acid derivates, glycerol and ferulic acid, the latter being delivered as intermediate from the phenylpropanoid pathway (Franke *et al*., 2005; Franke and Schreiber, 2007).

The rice genome was sequenced completely in 2002 (Goff *et al*., 2002; Yu *et al*., 2002) and thus, candidate genes being potentially involved in suberin and lignin synthesis were known. The transcription of 265 genes as affected by Si supply was investigated by use of a custom-made microarray and the results were confirmed by quantitative Real Time-PCR (Chapter I).

Si increased the transcript level of 12 genes involved in the biosynthesis of monolignols and suberin monomers such as phenylalanine ammonia-lyase (PAL), 4-coumarate-CoA ligase (4CL) and acyltransferase (AT) (Chapter I). Also, the transcription of ABC transporters and peroxidases was enhanced in adventitious roots of rice grown with Si supply. Interestingly, the transcription of genes involved in fatty acid metabolism was not affected by Si supply, suggesting that Si does not affect synthesis of the aliphatic suberin domain. The strongest

impact of Si supply on transcript level was measured for a leucine-rich repeat family protein (LRR), that exhibited a 25-fold higher transcript level in +Si roots than in –Si roots. The LRR proteins belong to the receptor-like kinases (RLK), which are transmembrane proteins with an extracellular domain that is linked via a transmembrane domain to a cytoplasmic serine/threonine protein kinase domain (Shiu *et al*., 2004). Recently, Huang *et al*. (2012) reported about a LRR-RLK that might be an important regulator of genes involved in the development of epidermis, exodermis and sclerenchyma in rice roots.

To further characterize the effect of Si on rice roots, Si dose response and Si kinetic experiments were conducted (Chapter II). The formation of casparian bands in rice roots was enhanced with increasing Si supply up to 12 mg L^{-1}. Referring to this value, Si should be effective in most soils, since Si concentration in the soil solution is usually in the range of 3 to 20 mg L^{-1} (Epstein, 1994, 1999) and is even higher under anaerobic and reducing conditions in flooded paddy rice fields (Ponnamperuma, 1984; Bogdan and Schenk, 2008). However, formation of casparian bands is affected also by other environmental factors like aeration or salinity, that have to be considered under field conditions (Colmer, 2003; Schreiber *et al*., 2007; Kotula *et al*., 2009).

The development of casparian bands in rice roots was not affected within 24 h after Si supply, but was increased 48 h after addition of Si to nutrient solution (Chapter II). Beyond this delayed reaction upon Si supply, a direct impact of silicic acid on gene expression seems unlikely, since transcription can be affected very fast by external signals as shown in rice plants, where transcription of several genes was altered within 15 minutes after onset of salt stress (Kawasaki *et al*., 2001).

Effect of silicon supply on development of casparian bands in several plant species

Although plants widely differ in their shoot Si concentration (Epstein, 1999; Chapter II), the roots are quasi constantly exposed to silicic acid in the soil solution and thus, Si might affect development of casparian bands independent from the plant's ability to accumulate Si. So the effect of Si supply on casparian band formation in roots of Si accumulators (rice WT, rice mutant *lsi1*, maize), intermediate type (nug, tradescantia) and Si excluder plants (onion) was studied (Chapter II).

Si supply led to an enhanced development of casparian bands in the exodermis of all plants irrespective of shoot Si concentration. Thus, a signal from shoot-to-root - as known for the regulation of sulfate and phosphate uptake (Rouached *et al*., 2011) - can be excluded for Si, since onion had increased casparian bands by Si supply without accumulating additional Si in

the shoot. Instead, Si presence in the root seems crucial for the fortification of casparian bands. The exact underlying mechanism remains unclear but it can be speculated that Si does not directly affect gene expression, since Si was effective in a range of various plant species with different genetic backgrounds. It seems rather plausible that the mode of action of Si is chemical and that Si might induce the cross-linking or precipitation of suberin, lignin or other aromatic compounds leading to a promoted formation of casparian bands.

In plants, Si is deposited as silica and more than 40 years ago, silica was shown to form complexes with the phenol catechol as six-coordinated Si (Barnum, 1970, 1972). Furthermore, Inanaga *et al*. (1995) suggested that Si might be associated with phenolic acids and leads to cross-linking between lignin and carbohydrates in cell walls of rice. There are also reports that lignin and callose can induce the deposition of silica (Fang and Ma, 2006; Law and Exley, 2011). Currie and Perry (2007) proposed that the surface of silica particles has a negative charge, leading to an interaction of silica with the plant cell wall.

To localize silica depositions in the root, rice, maize, and onion plants were cultivated in nutrient solution containing Si and root cross sections were analyzed using laser ablation-inductively coupled plasma-mass spectroscopy (LA-ICP-MS) (Chapter IV). In all three plant species, Si depositions were detected mainly in the outer part of the root, where the exodermis is located. Moreover, scanning electron microscope energy-dispersive X-ray microanalysis (SEM-EDX) confirmed the deposition of Si in the rice root exodermis and sclerenchyma. This is in line with literature reports, where silica depositions were found in the cell walls of the exodermis (Parry and Soni, 1972; Shi *et al*., 2005; Gong *et al*., 2006) and also in cell walls of the rice sclerenchyma (Moore *et al*., 2011).

In the high Si-accumulator horsetail (*Equisetum arvense*), Si was found in the cell walls along with the hemi-cellulose callose (Currie and Perry, 2009).

Considering these reports and the results from this work (Chapter I, II, IV), it can be hypothesized that Si either promotes cross-linking between the cell wall and aromatic compounds or induces the precipitation of aromatic compounds in the cell walls of the exodermis and endodermis leading to an enhanced development of casparian bands. Furthermore, this mechanism is not restricted to the roots of rice or other Si accumulators but is possible in plant roots in general. Casparian bands in the exodermis and endodermis limit the apoplastic flow and regulate the flux of solutes into the cortex (Hose *et al*., 1999). Also, they expose a barrier to fungal or bacterial colonization of the cortex (Marschner, 2012). Therefore, a promoted development of casparian bands by Si supply can contribute to the beneficial effects of Si on plants (Epstein, 1999; Ma *et al*., 2001).

Outlook

Arsenic and rice

The present work shows that Si limited the As(III) uptake of rice plants most probably by decreasing the protein abundance of the transporters Lsi1 and Lsi2. The As(III) uptake was not decreased by a short time Si supply, although the transcript level of both genes was decreased within hours after Si supply. This is probably because the protein abundance was not yet affected, which could be proved by quantifying the proteins. For this purpose, proteins could be extracted and separated via a two-dimensional gel-electrophoresis and then spots would be scanned followed by mass spectrometric protein analysis. Another possibility exhibits the use of specific antibodies to identify proteins and a subsequent quantification with a reporter enzyme, which is bound to the antibody (Enzyme Linked Immunosorbent Assay, ELISA).

In general, the As(III) uptake might also be limited by casparian bands, since they provide a barrier against apoplastic flux. Thus, the As(III) uptake of rice plants should be studied under salinity or drought stress, because these factors also induce the formation of casparian bands, but likely do not affect the transcription of *Lsi1* and *Lsi2*.

Si reduced the As(III) concentration in the grain, which is loaded with As(III) mostly over the phloem. It is likely that As(III) is transported into the phloem by a xylem-to-phloem transfer, which might be mediated by the transporter Lsi6, since it is capable of transporting As(III) and is located in the node, where xylem-to-phloem transfer (of other solutes) occurs. To clarify the role of Lsi6, the As(III) concentration in the xylem-sap and the phloem-sap between a knockout-mutant of Lsi6 and its corresponding wild type should be investigated.

Casparian bands and candidate genes for suberin and lignin synthesis

Si promoted the formation of casparian bands in the rice root and also in the root of other plant species like maize and onion. The chemical composition of the casparian bands in the different species as affected by Si supply should also be the subject of investigation. This might contribute to understand how Si interacts with the plant root.

Si increased the transcription of genes that are related to suberin and lignin synthesis. The functional characterization of these candidate genes is a very interesting issue, which can be handled by reverse genetic approaches. Possible techniques compass the knockdown of a

gene, for example with virus induced gene silencing, as well as the knockout or the overexpression of a gene by use of mutants like T-DNA insertion lines. Especially the use of overexpression mutants seems promising, since it avoids the problems sometimes occurring with knockout mutants such as lethal phenotypes or masking of the (knocked out) gene's function due to redundant genes.

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Supplementary Data

Chapter I

Table S1: Effect of silicic acid on relative quantity (RQ) of transcripts in root segment 0-2 cm distance from root tip as measured by microarray analysis. Stars represent significance: * for $p < 0.1$, ** for $p < 0.05$ and *** for $p < 0.01$. Genes not significantly regulated are not shown.

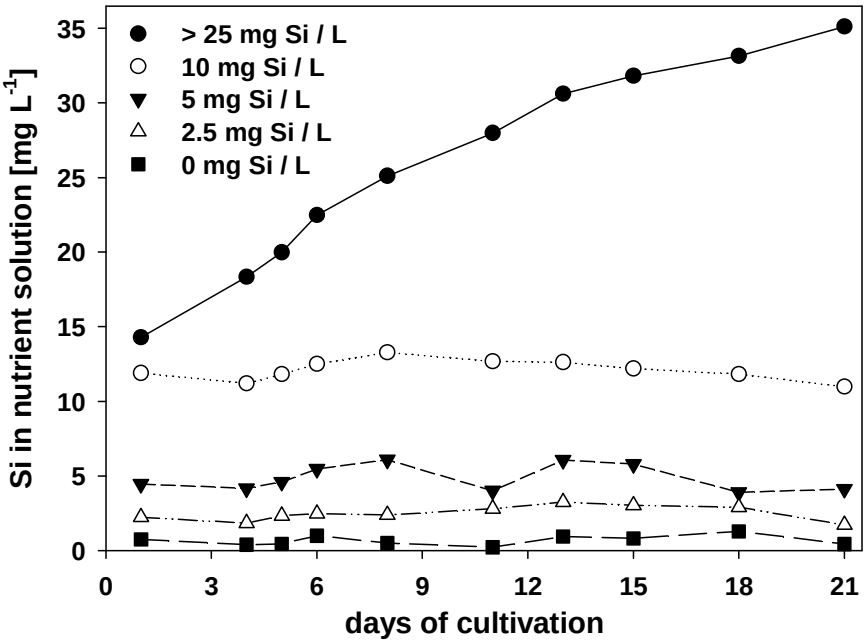
Gene ID	RQ +Si / -Si	Gene Annotation
LOC_Os01g42410	1,4151**	PDR5-like ABC transporter
LOC_Os01g67540	-1,704**	4-coumarate-CoA ligase
LOC_Os05g20100	1,4422**	glycerol-3-phosphate acyltransferase
LOC_Os06g16350	2,4665**	Class III peroxidase
LOC_Os06g39520	-1,3866**	myristoyl-acyl carrier protein thioesterase
LOC_Os07g44560	-1,8572***	4-coumarate-CoA ligase
LOC_Os08g02110	-1,3777**	Class III peroxidase
LOC_Os10g01930	1,813***	Acyltransferase

Table S2: Effect of silicic acid on relative quantity (RQ) of transcripts in root segment 4-6 cm distance from root tip as measured by microarray analysis. Stars represent significance: * for $p < 0.1$, ** for $p < 0.05$ and *** for $p < 0.01$. Genes not significantly regulated are not shown.

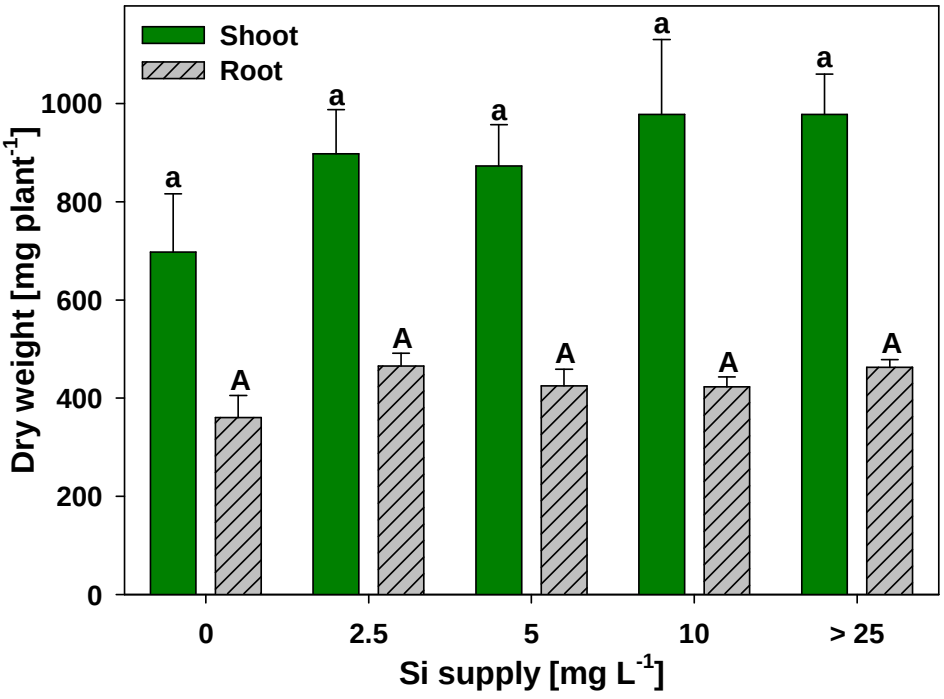
Gene ID	RQ +Si / -Si	Gene Annotation
LOC_Os01g22230	-2,8029**	Class III peroxidase
LOC_Os01g22560	2,1559**	glycerol-3-phosphate acyltransferase
LOC_Os01g24010	1,535**	PDR5-like ABC transporter
LOC_Os01g42410	2,1958**	PDR5-like ABC transporter
LOC_Os02g05630	2,4823**	protein phosphatase 2C, regulator protein
LOC_Os02g06250	-1,7841*	phytosulfokine receptor
LOC_Os02g41680	1,9074**	phenylalanine ammonia-lyase
LOC_Os02g57760	-2,2158**	caffeic acid 3-O-methyltransferase
LOC_Os04g59160	2,3224*	Class III peroxidase
LOC_Os05g20100	2,1526***	glycerol-3-phosphate acyltransferase
LOC_Os06g22080	1,8152**	diacylglycerol O-acyltransferase
LOC_Os06g32990	2,9286**	Class III peroxidase
LOC_Os08g03700	1,943***	glycerol-3-phosphate acyltransferase
LOC_Os09g23540	1,9898***	dehydrogenase
LOC_Os09g32964	-1,5739***	Class III peroxidase
LOC_Os10g30610	2,057**	ABC-2 type transporter family protein
LOC_Os11g14050	1,8668***	leucine-rich repeat (LRR) family protein
LOC_Os12g25870	2,2423**	caffeic acid O-methyltransferase (COMT)
LOC_Os12g32814	1,9379**	PDR-like ABC transporter

Supplementary Data

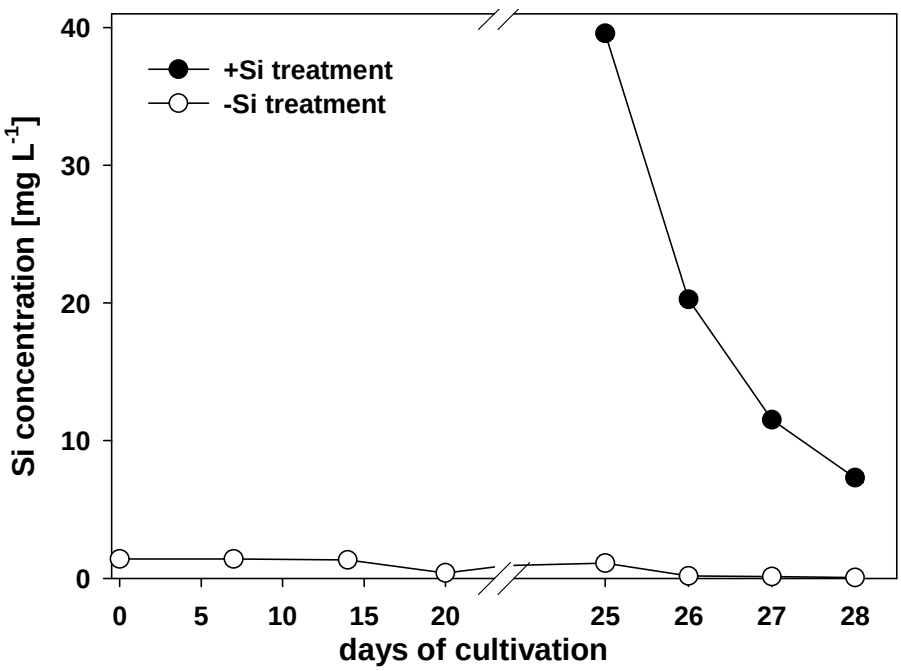
Chapter II



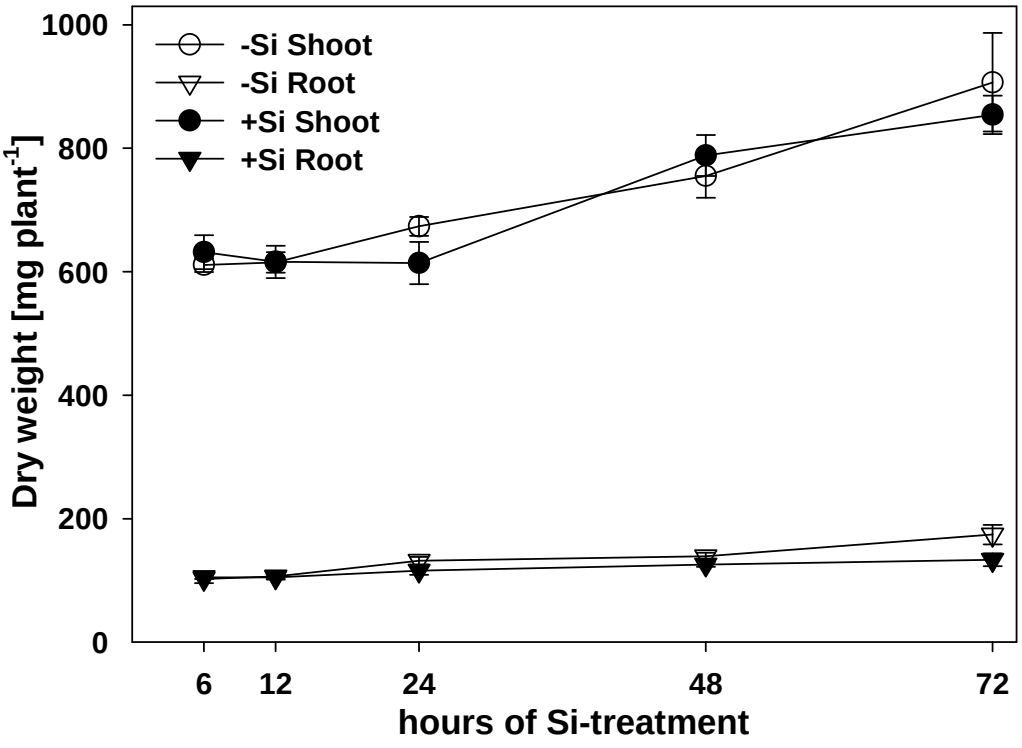
Supplementary Fig. 1: Si concentration in nutrient solution during plant cultivation of the dose response experiment. Data are adapted from Prüß (2010).



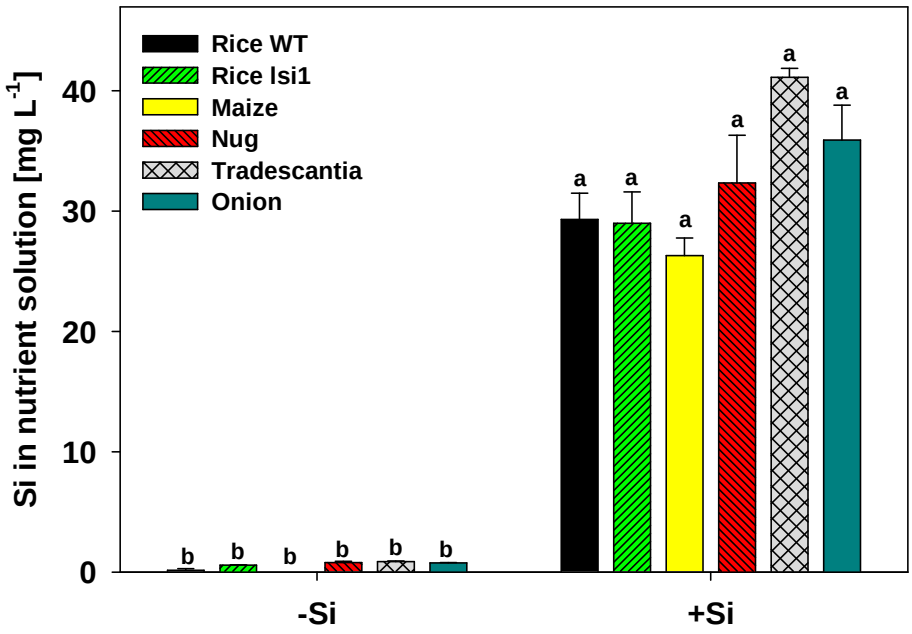
Supplementary Fig. 2: Shoot and root yield of rice plants as affected by Si supply in nutrient solution. Data are mean ± SE, n = 4. Different capital and small letters indicate significant difference between Si treatments in shoot or root; tukey-test with $p < 0.05$. Data are adapted from Prüß (2010).



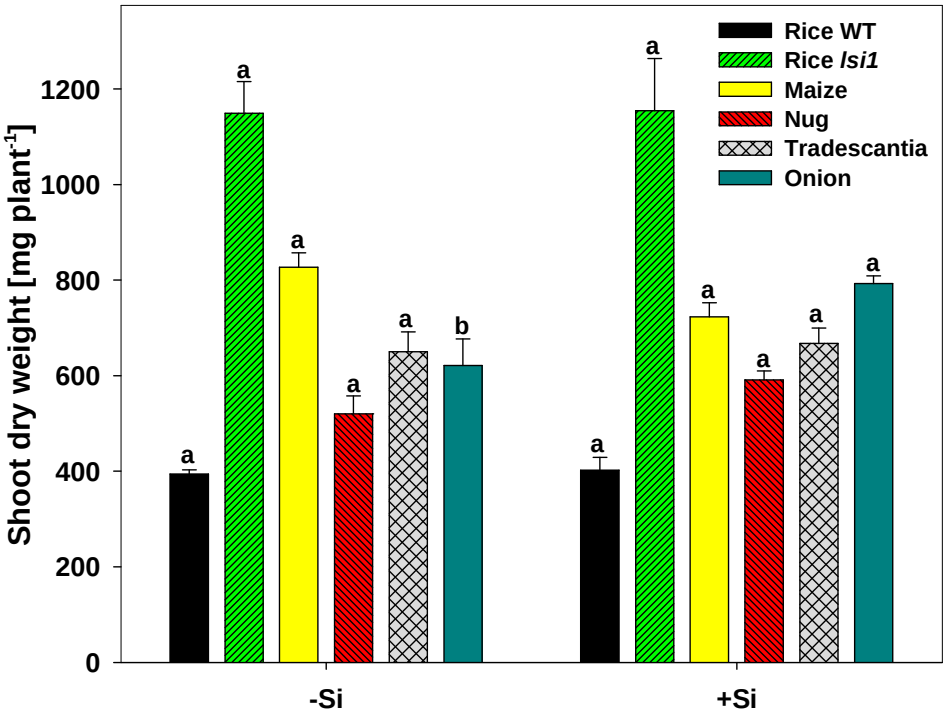
Supplementary Fig. 3: Si concentration in nutrient solution during plant cultivation of the kinetic experiment.



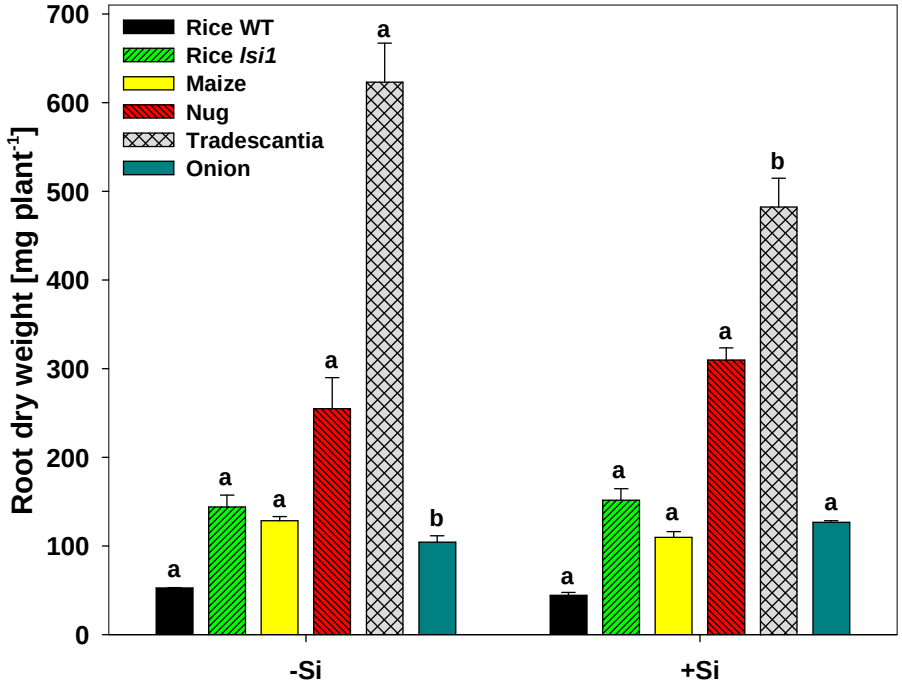
Supplementary Fig. 4: Shoot and root yield of rice plants as affected by short term Si supply in nutrient solution. Data are mean ± SE, n = 4; t-test with $p < 0.05$.



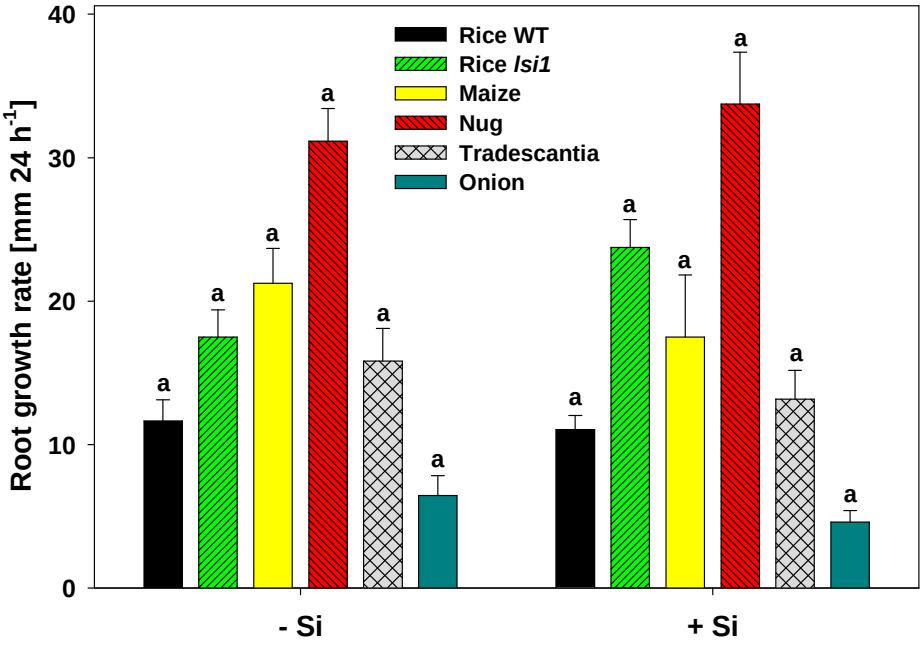
Supplementary Fig. 5: Si concentration in nutrient solution during cultivation of six plant species and cultivars. Data are mean ± SE, n = 4. Different letters indicate significant difference between Si treatments of a species; t-test with $p < 0.05$. Data are adapted from Schulze (2011).



Supplementary Fig. 6: Shoot dry weight of six plant species and cultivars as affected by Si supply in nutrient solution. Data are mean ± SE, n = 4. Different letters indicate significant difference between Si treatments of a species; t-test with $p < 0.05$. Data are adapted from Schulze (2011).



Supplementary Fig. 7: Root dry weight of six plant species and cultivars as affected by Si supply in nutrient solution. Data are mean ± SE, n = 4. Different letters indicate significant difference between Si treatments of a species; t-test with $p < 0.05$. Data are adapted from Schulze (2011).



Supplementary Fig. 8: Root growth rate of six plant species and cultivars as affected by Si supply in nutrient solution. Data are mean ± SE, n = 4. Different letters indicate significant difference between Si treatments of a species; t-test with $p < 0.05$. Data are adapted from Schulze (2011).

Erklärung zur Dissertation

gemäß §6(1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität Hannover
für die Promotion zum Dr. rer. nat.

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel

Silicon effects on arsenic uptake in rice, exodermis development and expression of genes related to suberin and lignin metabolism

selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.
Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

_____

(Unterschrift)
Name: Alexander Fleck

Scientific publications

Fleck AT, Nye T, Repenning C, Stahl F, Zahn M, Schenk MK. 2011. Silicon enhances suberization and lignification in roots of rice (*Oryza sativa*). *Journal of Experimental Botany* **62**, 2001-2011. Oxford University Press.

Author contributions: Determination of radial oxygen loss and histochemical analysis: Nye; microarray experiments: Stahl and Fleck; analysis of microarray raw data: Repenning; molecularbiological consultation.: Zahn; design of the experiments: Schenk and Fleck; all other experimental work and writing of the paper: Fleck.

Poster

Fleck AT, Schenk MK. 2008. Effect of silicon nutrition on uptake of arsenite and arsenate and translocation to shoot of rice. In: Kurzfassung der Poster – Jahrestagung der Deutschen Gesellschaft für Pflanzenernährung 2008. S. 11. Hrsg. BASF Agrarzentrum Limburgerhof, LUFA Speyer

Fleck AT, Nye T, Repenning C, Stahl F, Zahn M, Schenk MK. 2010. Silicon enhances suberization and lignification in roots of rice (*Oryza sativa*). In: Genetics of Plant Mineral Nutrition. Jahrestagung der Deutschen Gesellschaft für Pflanzenernährung. Hannover, Germany. Auszeichnung mit dem 2. Posterpreis der DGP.

Fleck AT, Prüß D, Schenk MK. 2011. Suberization of rice root as affected by silicic acid concentration in the nutrient solution. In: Proceedings of the 11th International Symposium on Metal Ions in Biology and Medicine. Cambridge, United Kingdom.

Oral presentation

Fleck AT, Schenk MK. 2011. Influence of silicon supply on arsenic concentration in straw and grain fractions of rice. In: Proceedings of the 5th International Conference on Silicon in Agriculture. Beijing, China.

Fleck AT, Mattusch J, Schenk MK. 2012. Silicon decreases arsenic level in rice grain by limiting arsenite transport. In: Challenges for Plant Nutrition in Changing Environments. International Workshop and Meeting of the German Society of Plant Nutrition 2012. Bonn, Germany.

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Janna

Lebenslauf

Persönliche Daten

Name:	Alexander Fleck
Geburtsdatum:	14.06.1982
Geburtsort:	Frankfurt am Main
Eltern:	Werner Fleck, Sozialversicherungsfachangestellter
	Angelika Fleck, geb. Link, Sekretärin
Geschwister:	Sandra, Christian und Sebastian
Familienstand:	ledig
Staatsangehörigkeit:	deutsch

Ausbildung

1988 – 1991	Grundschule Fritz-Erler, Wöllstadt
1991 – 1992	Grundschule Astrid-Lindgren, Barsinghausen
1992 – 1994	Orientierungsstufe Ludwig-Windthorst-Schule, Hannover
1994 – 2001	Gymnasium St. Ursula, Hannover
	Abschluss: Abitur
2001 – 2002	Zivildienst: katholische Kirchengemeinde St. Barbara, Barsinghausen
2002 – 2007	Studium der Biologie an der Leibniz Universität Hannover
	Abschluss: Dipl.-Biol.
seit Januar 2008	Wissenschaftlicher Mitarbeiter am Institut für Pflanzenernährung
	der Leibniz Universität Hannover

Hannover, den 18.02.2013