Role of Silicon in Plasmalemma H^+-ATPase Hydrolytic and Pumping Activity in Maize (*Zea mays* L.)

Hafiz Faiq Siddique Gul Bakhat

A thesis submitted for the requirement of the **doctoral degree in agriculture** from Faculty of Agricultural Sciences, Nutritional Sciences, and Environmental Management, Justus Liebig University Giessen

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To my Parents

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Table of contents

Table of contents iii

1 Introduction 1

1.1 Silicon in soil-plant system 1

1.2 Role of silicon in plant growth 4

1.3 Plasma membrane H^+-ATPase, nutrient uptake, and plant growth 6

2 Materials and Methods 8

2.1 Optimal level of silicon for maize (*Zea mays* L. cv. Amadeo) growth in nutrient solution under controlled conditions 8

2.1.1 Plant cultivation 8

2.1.2 Plant height and leaf-area measurement 8

2.1.3 Plant fresh and dry mass measurement 9

2.1.4 Cation analysis 9

2.1.5 Silicon analysis 9

2.2 Effect of Si on plasma membrane H^+-ATPase hydrolytic and pumping activity isolated from maize root and shoot 11

2.2.1 Plant cultivation 11

2.2.2 Isolation of plasma membrane vesicles 11

2.2.3 Protein quantification 15

2.2.4 Hydrolytic activity of plasma membrane ATPase 16

2.2.5 Measurement of H^+ pumping activity 17

2.2.6 *In vitro* effect Si on H^+-ATPase hydrolytic and pumping activity 18

2.3 Immunodetection of plasma membrane H^+-ATPase protein 18

2.4 Effect of Si nutrition on the transcription of plasma membrane H^+-ATPase isoforms.. 22

2.4.1 Spectrophotometric determination of RNA 22

2.4.2 Determination of RNA integrity 23

2.4.3 Synthesis of cDNA 23

2.4.4 Real-time PCR analysis of H^+-ATPase isoforms..... 24

2.5 Effect of Si nutrition on apoplastic pH of maize shoot 27

2.6 Statistical analysis 28

2.7 Chemicals 29

3 Results 31

3.1 Optimization of silicon concentration in nutrient solution for maize growth 31

3.1.1 Effects of various concentrations of silicon on fresh and dry mass of maize shoot and root……...31

3.1.2 Effects of various concentrations of silicon on maize plant height and leaf area of young growing leaves 32

3.1.3 Effects of various concentrations of silicon on cation concentrations in maize plants……...33

3.1.4 Effects of various concentrations of silicon in nutrient solution on silicon concentration in young maize shoot……37

3.2 Role of silicon in plasma membrane H^+-ATPase characteristics..... 39

3.2.1 Effects of silicon supply in nutrient solution on the purity of plasma membrane vesicles isolated from maize shoots and roots 39

3.2.2 Effect of silicon nutrition on plasma membrane H^+-ATPase hydrolytic activity isolated from roots and shoots 40

3.2.3 Effect of silicon nutrition on H^+ transport activity of plasma membrane ATPase isolated from roots and shoots 41

3.2.4 Effects of silicon supply in nutrient solution on the kinetic characteristics of plasma membrane H^+-ATPase isolated from maize shoot 43

3.2.5 Effects of silicon supply in nutrient solution on the activation energy of plasma membrane ATPase 45

3.2.6 *In vitro* effect of silicon on plasma membrane ATPase hydrolytic activity 45

3.2.7 *In vitro* effect of silicon on plasma membrane H^+- ATPase transport activity 47

3.2.8 *In vitro* effect of silicon on plasma membrane ATPase kinetic characteristics and activation energy 48

3.2.9 Effects of silicon supply in nutrient solution on plasma membrane H^+-ATPase concentration in the plasma membrane vesicles isolated from maize shoots and roots 50

3.2.10 Relative expression of mRNA of plasma membrane H^+-ATPase enzyme isoforms 50

3.3 Effect of silicon supply in nutrient solution on apoplastic pH in growing shoot tissues . 53

4 Discussion 56

4.1. Exogenously supplied silicon in nutrient solution improves maize growth 56

4.2. Effect of silicon on plasma membrane ATPase in maize shoots and roots 61

4.2.1 Effect of silicon supply in nutrient solution on the purity of plasma isolated from maize shoots and roots 62

4.2.2 Effect of silicon on plasma membrane ATPases in maize shoots 63

4.2.3 Effect of silicon supply in nutrient solution on plasma membrane ATPases in maize roots 67

4.3 Effect of silicon supply in nutrient solution on apoplastic pH in maize leaves 68

5 Summary 71

6 Zusammenfassung 73

7 References 75

1 Introduction

1.1 Silicon in soil-plant system

Silicon (Si) is the second most abundant element in lithosphere and together with oxygen it makes up about 50-70% of the earth crust (Ma and Yamaji, 2008). Silicon is taken up from the soil solution by plant roots as uncharged monomeric silicic acid. The concentration of monosilicic acid ranges from 0.1 - 0.6 mM in the soil solution (Epstein, 1999; Datnoff et al., 2001). Sesquioxides and soil pH are the major driving forces that determine the silicic acid concentration in the soil solution (Jones and Handreck 1963). Silicic acid is freely available in the soil solution at a pH below 4, however, its availability decreases when the pH rises from 4 to 10 not least due to the conversion into silicate salts at a pH above 10. Silicon deficiency is one of the limiting factors of crop production especially in highly weathered and organic soils (Alvarez and Datnoff, 2001). Intensive cereal cultivation may also deplete the plant-available Si, which results in low yields e.g. of rice and sugar cane (Epstein, 1994; Ma, 2005). In order to guarantee high yields of these crops, Si fertilization is a very common practice in Japan, South Korea, Brazil, and Florida (Datnoff et al., 2001).

The Si requirements of some specific plant species such as rice and sugar cane are several fold higher compared to other macro nutrients such as N, P, and Ca (Rodrigues and Datnoff, 2005). Silicon concentrations range from 0.1 - 10% in the shoot on a dry weight basis (Epstein, 1994; Ma and Yamaji, 2008). Silicon is the only element that does not have any toxic effect on plants even when accumulated in higher concentrations (Richmond and Susman, 2003). There is a great variation in Si concentrations among various plant species and genotypes within a species compared to any other element (Winslow et al., 1997; Epstein, 2009). On the basis of Si concentrations, plants are classified as accumulators (wet land grasses), intermediates (dry land grasses), and excluders (dicots) (Hodson et al., 2005; Ma and Yamaji, 2006). Variations in Si concentrations in plants depend mainly on Si-uptake and the xylem loading ability of the roots (Ma et al., 2001). Even though the plant plasma membrane may be permeable to uncharged monomeric silicic acid via diffusion, the permeability coefficient calculated for silicic acid

across the membrane is only 10^{-10} m s^{-1} (Raven, 2001). Therefore, the experimentally measured Si contents in plants that belong to the family of *Poaceae* cannot be simply explained by the permeability coefficient (Tamai and Ma, 2003).

Mitani and Ma (2005) reported that the radial transport of Si from the soil solution to the xylem involves both a passive as well as a transporter-mediated active transport. This conclusion is also supported by the observations that Si uptake was partially inhibited when plants were exposed to metabolic inhibitors such as 2,4-dinitrophenol, potassium cyanide (Tamai and Ma, 2003; Mitani and Ma, 2005; Rains et al., 2006; Liang et al., 2006a; Nikolic et al., 2007) or low temperature (Liang et al., 2006a). Transport of Si from the rooting medium to cortical cells involves a low affinity silicic acid transporter with a K_m of 0.15 mM. Kinetic studies for Si uptake in rice, cucumber, and tomato showed that the density of the transporters in the plasma membrane was responsible for the variation in Si uptake, with rice having the highest and tomato the lowest density (Mitani and Ma, 2005). Recently, genes involved in Si uptake and distribution (*Lsi1, Lsi2, and Lsi6*) were identified in rice, barley, maize, and cucumber (Ma et al., 2011). The transporters Lsi1 and Lsi6 belonging to the aquaporin family are Si-influx transporters and are mainly involved in the distribution of Si in root and shoot tissues (Mitani et al., 2011). On the other hand, Lsi2 is a putative anion transporter and is mainly expressed in the endodermis of roots (Ma et al., 2007). It has been reported that the transport activity of Lsi2 is proton driven (Ma et al., 2007) and it works as Si/H^+ antiport (Figure 1). In this context, it was shown that a decrease of pH of an external solution led to an increase of silicic acid in the bathing medium of preloaded *Xenopus levis* oocytes, and exclusion of Si was inhibited by a decrease in temperature and in the presence of protonophores such as 2, 4-dinitrophenol (DNP), carbonylcyanide 3-chlorophenylhydrazone (CCCP) and carbonylcyanide p-(trifluoromethoxy)- phenylhydrazone (Ma et al., 2007). The transport of Si from stele to xylem may also require some transporters however transporters involved in xylem loading are yet to be identified.

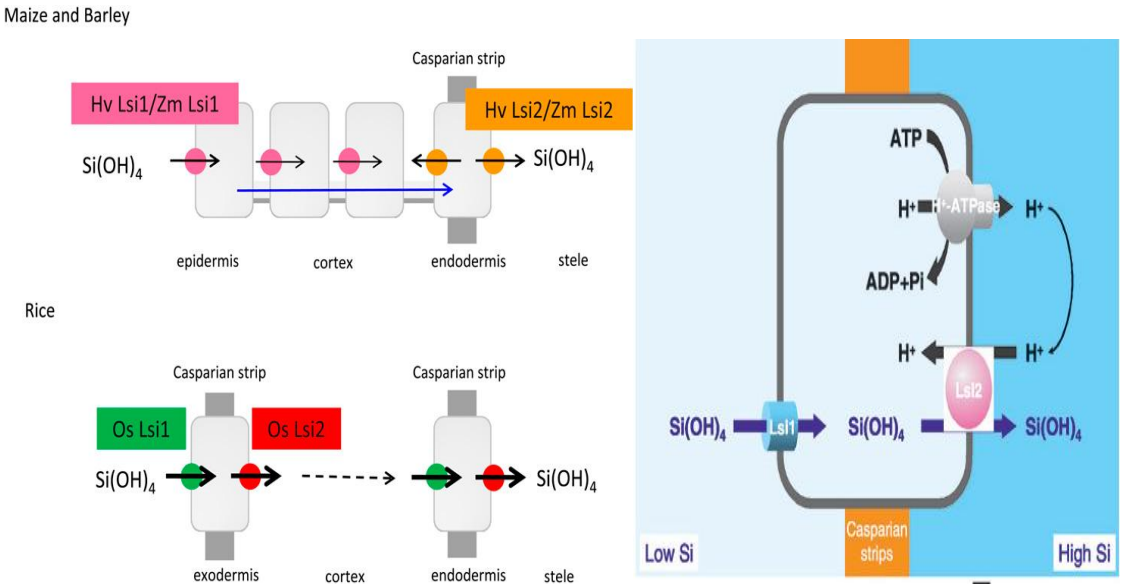

Figure 1. Uptake and transport systems of Si in rice, maize and barley. Silicon is taken up via an influx transporter (*LSi1*) in the form of uncharged monomeric silicic acid from rooting medium and passed through the endodermis to xylem by another transporter (*LSi2*) (Left). Functioning of the *LSi2* and speculated involvement of plasma membrane H^+-ATPase (Right). (Ma and Yamaji, 2008; Mitani et al., 2009b)

In plants, Si is transported as uncharged monomeric silicic acid from roots to shoot via the xylem stream. In xylem, the concentration of silicic acid in Si-accumulating species exceeds 2 mM. In aqueous solutions, the silicic acid in concentrations above 2 mM tends to polymerize into polymeric silicic acid. However, higher concentrations of monomeric silicic acid (> 2 mM) were observed in xylem sap of rice (Mitani et al., 2005) and wheat (Casey et al., 2004). Higher concentrations of silicic acid in xylem sap are transient and once the Si is unloaded from the xylem tissues into the leaf sheaths and leaf blades, it starts to polymerize into amorphous silica. The polymerized silica is deposited into cell wall, cell lumen, intra-cellular spaces, and trichomes (Cooke and Leishman, 2011), and thus increases the tissue strength. Reinforcement of the cell-wall by silica deposition impedes penetration of insect herbivores (Reynolds et al., 2009) and fungi (Wiese et al., 2005).

1.2 Role of silicon in plant growth

Almost 150 years ago, Julius Sachs raised few questions about the role of Si in plant biology as "whether silicic acid is an indispensable substance for those plants that contain silica, whether it takes part in the nutritional processes, and what is the relationship that exists between the uptake of silicic acid and the life of the plant?" (Lewin and Reimann, 1969). These questions are still valid because the role of Si as a plant nutrient and its function in plant physiology is quite contradictory since under normal growing conditions plant response to Si is inconsistent. Studies carried out with minus Si may not provide the true picture as the Si is ubiquitous in soil-plant systems and it is almost impossible to create conditions free of Si (Epstein, 1994; 1999). However, role of Si in plant metabolism may also depend on the requirement of Si for the specific plant species. It is evident from the studies carried out at molecular level to define the role of Si in plant biology. Silicon treatment under normal growing conditions changed the expression of only two out of 40,000 transcripts in *Arabidopsis* (Fauteux et al., 2006). In contrast, transcriptome analysis of wheat and rice revealed that Si treatment changed the expression of 47 and 221 genes (Chain et al., 2009; Brunings et al., 2009). Beside these studies, there are still evidences that suggests that Si increases plant growth by changing metabolic processes such as photosynthesis, composition of the cell wall and its extensibility, and by changing the physiological availability of mineral nutrients (Adatia and Besford 1986; Marschner et al., 1990; Hattori et al., 2003; Zhu et al., 2004; Guo et al., 2006; Vaculík et al., 2009). Silicon deficient plants are structurally weak and more prone to various environmental stresses and are experimental artifacts (Epstein, 1999). Moreover, plants that require higher amounts of Si (rice and horse tail) showed a "weeping willow" habit of growth, developed leaf necrosis, and were of reduced fertility (Lewin and Reimann, 1969). Furthermore, plant devoid of Si showed reduced leaf and root lengths due to a decrease in cell wall extensibility in young growing zones (Hossain et al., 2002; Hattori et al., 2003). On the other hand, bulk deposition of Si into mature regions of roots strengthens the cell wall (Hattori et al., 2003). Silicon not only changes the properties of the cell wall by its deposition but also changes other constituents of the cell wall (Agarie et al., 1998). Some of the Si located in the cell wall forms ester-like derivatives of silicic acid, acting as a bridge in the structural organization of the polyuronides (Schwarz,

1973). Despite of all these facts, the underlying mechanisms of Si-induced increase in plant growth under normal growing conditions are not fully understood yet.

On the other hand, a broad spectrum of Si in plant physiology is presented under various biotic and abiotic stresses (Lewin and Reimann, 1969; Epstein, 1999, 1994; Wiese et al., 2005; Liang et al., 2007; Cai et al., 2009; Reynolds et al., 2009). Under unfavorable environmental conditions, the redox balance of the cell is disturbed which leads to an accumulation of reactive oxygen species (Van Breusegem and Dat, 2006). The reactive oxygen species disturb metabolic/physiological processes of the plant by damaging membranes, enzymes, DNA, and other cellular components. Plants supplied with Si showed enhanced resistance to environmental stresses due to the activation of enzymatic and non-enzymatic anti-oxidant systems (Liang et al., 2006a; Saqib et al., 2008). Moreover, silica deposition beneath the cuticle layer forms a double silica-cuticle layer (Currie and Perry, 2007) that acts as a mechanical barrier, and thus prevents the penetration of pathogens (Liang et al., 2005). Under various biotic stresses, Si induces systemic stress signals such as salicylic acid and jasmonic acid, which play key roles in plant defense (Fauteux et al., 2005). Additionally, Si-treated plants had higher concentrations of antifungal compounds e.g. phenolics (Rémus-Borel et al., 2005), diterpenoid phytoalexins (Fawe et al., 1998; Rodrigues et al., 2004), and pathogenesis-related proteins (Chérif et al., 1994; Liang et al., 2005).

Silicon deposition does not only increase the plant resistance against biotic stresses but also contributes to the induction of resistance against abiotic stresses. The endodermis of roots which contains silica deposits is known to reduce the apoplastic bypass flow, thus creating the hindrances in apoplastic movement of toxic cations (Yeo et al., 1999; Gong et al., 2006; Saqib et al., 2008; Shi et al., 2005; Guo et al., 2005; Cocker et al., 1998a). Furthermore, apoplastic Si deposition changes the binding properties of the cell wall (Horst et al., 1999) thus increasing the adsorption capacity for toxic mineral ions (Iwasaki et al., 2002; Rogalla and Römheld, 2002; Saqib et al., 2008). In addition, compared to untreated plants Si treatment resulted in a release of phenolic compounds such as catechin and quercetin from roots (Kidd et al., 2001) that contribute to reduce the metal toxicity.

1.3 Plasma membrane H^+-ATPase, nutrient uptake, and plant growth

The plasma membrane is formed by a lipid bilayer which surrounds the cytosol and thus represents a barrier between the cells and their surrounding environment. Any nutrient from the soil solution entering the plant has to pass this barrier either at the exodermis or endodermis or in between in order to cross the Casparian band (Sondergaard et al., 2004). Nutrients that occur in higher concentrations in the plant cell compared to the outer medium require uptake against a concentration gradient. It is well known that the uphill movement of a solute is a metabolically energy requiring process. However, solute/nutrient movement is rather secondary active, driven by the electrochemical H^+ gradient. Membrane-bound plasma membrane H^+-ATPases extrude H^+ out of the cytosol into the apoplast. Due to this process a negative electrical potential on the inside of the membrane is formed and high concentrations of H^+ are found outside the membrane. The apoplastic transport of solutes/nutrients from one cell to another occurs through specific proteins called channels, carriers, and pumps. The proton motive force created by the plasma membrane H^+-ATPase activates transport proteins involved in the uptake and transport of mineral nutrients. It has been demonstrated in a number of studies that the plasma membrane H^+-ATPase is involved in the uptake of various nutrients such as nitrogen (Glass et al., 1992; Schubert and Yan, 1997; Santi et al., 2003), phosphorus (Yan et al., 2002), sulfur (Leustek and Saito, 1999), potassium (Schachtman and Schroeder, 1994), and iron (Schmidt, 2003; Dell'Orto et al., 2000). Although there is evidence that Si uptake and translocation may require metabolic energy but there is a lack of evidence of the interaction between Si uptake and plasma membrane H^+-ATPase.

In addition to nutrient uptake and transport, the plasma membrane H^+-ATPase is also involved in cell elongation and expansion growth. The energy-driven transport of H^+ out of the cytosol can affect plant growth in multiple ways. The electrochemical H^+ gradient activates voltage-dependent K inward-rectifying channels (Perrot-Rechenmann, 2010). The uptake of K in-turn contributes to the generation of an increased turgor pressure which enables the wall to expand. Furthermore, in accordance with the acid-growth theory a lower apoplastic pH is the major requirement for expansion growth (Hager, 2003). The acid-growth hypothesis is supported

by the observations that auxin-induced increased plasma membrane H^+-ATPase activity increases the cell-wall extensibility (Hager et al., 1991). A higher activity of H^+ in the apoplast may also activate cell-wall proteins such as expansins (Cosgrove and Li, 1993; Cosgrove, 2000) contributing to increase the cell wall extensibility by rearrangement of the load-bearing bonds (Keller and Cosgrove 1995; Purugganan et al., 1997).

Similarly, there are several reports showing that Si inclusion in rooting medium increased plant growth. However, the physiological processes behind the Si-induced beneficial effects on plant growth are not fully understood yet. Hossain et al. (2002) showed that Si-induced expansion growth was due to an increase in individual cell size not due to the total number of cells in rice leaves. Increased cell size may be a result of increased water uptake into the cell and increased turgor pressure. However, studies showed that Si had no effect on solute or water potential of the plant (Pei et al., 2010; Chen et al., 2011). The other possibility is that Si may increase cell-wall extensibility which resulted in expansion growth. As described earlier plasma membrane H^+-ATPase is involved in expansion growth due to the cell-wall acidification. It is likely to assume that the Si-induced stimulation of plant growth is caused by changes in plasma membrane H^+-ATPase activity.

To elucidate the role of Si on maize growth and its contribution in the control of plasma membrane H^+-ATPase in roots and shoots the following experiments were performed:

1. Exogenously supplied Si to the nutrient medium improves maize (*Zea mays* L cv. Amadeo) growth under normal growth conditions.
2. Changes in plasma membrane H^+-ATPase are responsible for the Si-induced maize growth.
3. Silicon nutrition increases maize shoot growth by inducing apoplastic acidification.

2 Materials and Methods

2.1 Optimal level of silicon for maize (*Zea mays* L. cv. Amadeo) growth in nutrient solution under controlled conditions

2.1.1 Plant cultivation

Seeds of maize hybrid (*Zea mays* L. cv. Amadeo) were imbibed in 1 mM $CaSO_4$ solution for 24 h. The seeds were germinated in foam layered with filter papers moistened with 1 mM $CaSO_4$ in dark. At d 6, seedlings were transferred to a climate chamber with following environmental conditions: humidity 50%, day/night cycle 16/8 h at 26/18^oC, and a light intensity of 150 W m^{-2} (Philips Master HPI-T Plus, 400 W). Next day plants were transferred to half-strength nutrient solution in 4.5 L pots each with three plants. The composition of full-strength nutrient solution was in mM, 2.5 $Ca(NO_3)_2$, 0.2 KH_2PO_4, 1.0 K_2SO_4, 0.6 $MgSO_4$, 5.0 $CaCl_2$; in µM, 1.0 H_3BO_3, 2.0 $MnSO_4$, 0.5 $ZnSO_4$, 0.3 $CuSO_4$, 0.005 $(NH_4)_6Mo_7O_{24}$, 200 Fe-EDTA. After two days, plants were supplied with full strength nutrient solution. With the start of full-strength nutrient solution, plants were also supplied with seven silicon (Si) concentrations in nutrient solution. The Si concentrations were in mM, 0, 0.4, 0.8, 1.2, 1.6, 2.0, and 3.0. To avoid the effects of alkalization and sodium from Na_2SiO_3 on plant growth, the solution was neutralized with HCl and Na was replenished in nutrient solution with NaCl. Plant cultivation was extended to 21 d.

2.1.2 Plant height and leaf-area measurement

To investigate the effects of various concentrations of Si supply on maize expansion growth, plant height and leaf area of different leaves were measured using a ruler. Plant height was measured as a distance between plant base to youngest leaf tip. For leaf area calculation, length of each leaf was multiplied with its width and divided by two.

2.1.3 Plant fresh and dry mass measurement

After 2 weeks of Si treatment plants were harvested and separated into shoots and roots. Plant roots were thoroughly washed with deionized water thrice and blotted dry with tissue paper. After determination of fresh weights, plant shoots and roots were dried at 78°C till up to a constant weight and then the dry weights were determined.

2.1.4 Cation analysis

The ground plants samples were digested by the method given by Rosopulo et al. (1976). For this 500 mg of ground dry material were put into digestion flask containing 10 mL of digestion mixture (HNO_3, $HClO_4$ and H_2SO_4 with a ratio of 40:4:1). 1 mL tri-chloro-ethylene (C_2HCl_3) was added in the digestion mixture to avoid the foaming of plant material. The digestion tubes were put in an aluminum block and heated for 8h at rising temperature up to 200 °C. After the digestion 5 mL HNO_3 were added, briefly boiled, and filtered in 50 mL measuring flasks. The cations were determined by the atomic absorption spectrophotometer (SpectrAA 220FS, Varian).

2.1.5 Silicon analysis

For the quantification of Si, the powdered material was digested in a microwave-digestion system. 200 mg plant material were filled into a microwave Teflon® digestion tube and 3 mL of 65% HNO_3 were added. After 5 min, 2 mL 30% H_2O_2 were added. The tubes were capped and assembled in a microwave rotor. The rotor was placed in the microwave and conditions were adjusted as described below (Table 1) and acid digestion step was conducted. After acid digestion, tubes were cooled down by placing the rotor in ice for 1 h. In the second step, alkali digestion was carried out by adding 10 mL 10% NaOH. The tubes were fitted to the rotor and the microwave conditions were adjusted as in the below-given Table 1. After alkali digestion step, tubes were cooled by placing the rotor in ice bath and the digested material was filtered through Wattman filter paper 42 and sample diluted to 100 mL. The second step is important as it solubilizes the amorphous Si. Operating conditions of the microwave were adjusted as described by Haysom and Ostatek-Boczynski (2006).

Table 1: Operating parameters of microwave-assisted digestion system for plant material

Digestion step	Microwave setting
Acid digestion	500 W to 1000 W over 5 min
	Maintained at 1000W for 10 min
	Cool for 15 min at Fan setting 2
Alkali dissolution	500 W to 1000 W over 5 min
	Maintained at 1000 W for 10 min
	Cool for 15 min at Fan setting 2

The filtrate was used to determine Si by three different methods; color method, atomic absorption spectroscopy and inductively coupled plasma spectroscopy.

The reagents for color method were as follows;

Sample	100 µL
H_2O bidest	1.15 mL
HCl (0.26 N)	160 µL
$(NH_4)_6Mo_7O_{24}$ (10%)	80 µL
Tartaric acid (10%)	160 µL
Reducing agent*	80 µL

Color development was completed within 1 h and A_{600} was measured spectrophotometerically (Carry 4 Bio, Varian Australia Pty Ltd., Australia). To determine Si by atomic absorption spectrophotometer (SpectrAA 220FS, Varian) using a N_2O/acetylene flame and inductively coupled plasma mass spectrometry (ICP-MS) the same filtered material was used.

*The reducing agent was prepared by dissolving 250 mg Na_2SO_3, 125 mg 1-amino-2-naphthol-4-sulfonic acid, and 7.5 g $NaHSO_3$ in 50 mL of bidest water.

2.2 Effect of Si on plasma membrane H^+-ATPase hydrolytic and pumping activity isolated from maize root and shoot

2.2.1 Plant cultivation

Seeds of *Zea mays* L. cv. Amadeo were soaked in aerated 1 mM $CaSO_4$ for 1 d and germinated in dark between two layers of filter paper. On d 5, seedlings were transferred to a climate chamber with a light intensity approximately 150 W m^{-2} (Philips Master HPI-T Plus, 400 W), a day/night cycle of 16 h/8 h at 26°C/18°C and a relative humidity of 50%. Next morning, seedlings were transferred to 50 L plastic containers (70 plants per container) with half-strength nutrient solution. After 2 d, plants were transferred to full-strength nutrient solution and Si treatment was started. Nutrient solution was renewed after each second day. The full-strength nutrient solution was as in Table 2.

Table 2: The full-strength nutrient solution composition

Substrate	Final concentration	Substrate	Final concentration
$Ca(NO_3)_2$	2.5 mM	H_3BO_3	1.0 µM
K_2SO_4	1.0 mM	$MnSO_4$	0.2 µM
KH_2PO_4	0.2 mM	$ZnSO_4$	0.5 µM
$MgSO_4$	0.6 mM	$CuSO_4$	0.3 µM
$CaCl_2$	5 mM	$(NH_4)_6Mo_7O_{24}$	0.035 µM
Na_2SiO_3	1 mM	Fe-EDTA	200 µM

2.2.2 Isolation of plasma membrane vesicles

To determine the effect of Si nutrition on plasma membrane H^+-ATPase in maize plant, plasma membrane vesicles were isolated from roots and shoots after 2 weeks of Si treatment. The young developing roots and shoots were cut and washed thrice with deionized water and then immersed in cold deionized water. Microsomal membrane fractions for roots and shoots were prepared as described by Yan et al. (1998, 2002) and Zörb et al. (2005) respectively. Roots

were cut and ground in ice-cold homogenization buffer with a mortar and pestle, while shoot material was ground with a grinder. The homogenization buffer has the following composition:

250 mM sucrose

2 mM EGTA

10% (v/v) glycerol

0.5% (w/v) bovine serum albumin

2 mM dithiothreitol

1 mM phenylmethylsulfonyl fluoride

5 mM 2-mercaptoethanol

50 mM 1, 3-bis (tris[hydroxymethyl]methylamino) propane (BTP) was nadjusted to pH 7.8 with MES. For the shoots, 250 mM KI was also added to the homogenization buffer.

The homogenization buffer has to maintain the activity of plasma membrane-bound enzymes and prevent their degradation during the isolation process (Dey et al., 1997). The homogenization buffer contains high concentration of sucrose that maintains the integrity of cell organelles. It also contains EGTA that acts as a chelating agent for divalent cations that are required for the activity of proteases. Phenylmethylsulfonyl fluoride (PMSF) inclusion in buffer also controls the activity of some proteases (Schaller and DeWitt, 1995). Addition of glycerol inhibits the phosphatidic acid phosphatase while fatty-acid free bovine serum albumin (BSA) has dual functions as it is an effective scavenger of the quinones which can polymerize with the proteins. Some of the quinones are already present in plant tissues while some may also be produced from phenols during homogenization. In addition, BSA also binds with free fatty acids that inhibit the ATPases (Dey et al., 1997). Dithiothreitol and mercaptoethanol are disulfide reducing agents that keep thiol groups in reduced state which are important for the enzyme activity. The pH of buffer

solution is adjusted near the physiological pH of the cytoplasm that keeps some lypolytic enzymes under control (Dey et al., 1997).

The homogenization buffer was used at a ratio 4 mL g^{-1} fresh weight. The homogenate was filtered through two layers of Miracloth (Calbiochem-Novabiochem, San Diego) and centrifuged in a swinging bucket rotor at 11,500 *g* (AH 629 rotor, 36 ml, Sorvall Products, Newtown, CT) for 10 min at 0°C to remove the insoluble plant debris and organelles. The supernatants were centrifuged at 87,000 *g* for 35 min to pellet the microsomal membranes.

The pellets were re-suspended in phosphate buffer, which contained:

250 mM Sucrose

3 mM KCl

5 mM KH_2PO_4 (pH 7.8)

The microsomal membranes were fractionated using two-phase partitioning in aqueous dextran T-500 (Sigma) and polyethylene glycol (Sigma) according to the method of Larsson (1985). Phase separations were carried out in a series of 32-g phase systems that contained:

6.1 and 6.3% (w/w) dextran T-500

6.1 and 6.3% (w/w) PEG 3350 (for shoots and roots, respectively)

250 mM Sucrose

3 mM KCl

5 mM KH_2PO_4 (pH 7.8)

Stock solutions of polymers were prepared with concentrations of 20% and 40% (w/w) for dextran and polyethylene glycol, respectively. The concentration of dextran stock solution

was determined with optical rotation (Larsson, 1985). The phase stock was weighed and diluted to 6.1 and 6.3% (w/w, each polymer) with phase buffer to a final weight of 32 g (Tube B and C). Polymers in "start tubes (Tube A)" were, however, diluted to 26 g. Five grams of re-suspended microsomal membranes (in phase buffer) were added to the upper phase of each start tube. The tubes were sealed with Parafilm (American National Can, Greenwich, CT) and mixed by inversion (30 times). Phase separation was achieved at 4°C by centrifugation at 720 *g* (Sorvall AH-629 rotor, 36 mL) for 23 min followed by second and third phases with centrifugation times 15 and 10 min, respectively.

The upper phases obtained after three separations were diluted with phosphate buffer and centrifuged at 135,560 *g* for 50 min. The pellets were washed with re-suspension buffer and pelleted again.

The re-suspension buffer contained;

250 mM Sucrose

3 mM KCl

5 mM BTP/MES, pH 7.8

1 mM DTT

The pellets were re-suspended in re-suspension buffer, divided into aliquots, and immediately stored in liquid nitrogen.

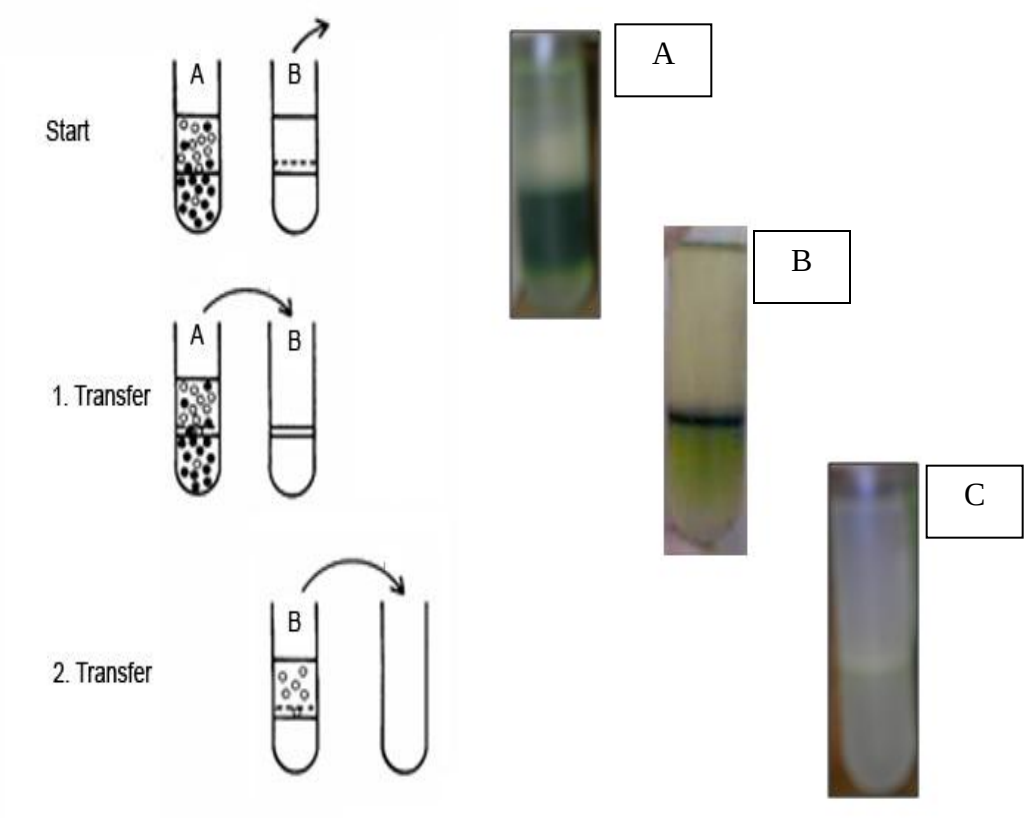

Figure 2: Schematic diagram of the isolation of plasma membrane vesicles. Re-suspended microsomal membranes were mixed with two-phase system and centrifuged at 720 *g* (A). The 90% of the upper phase was removed from tube A and mixed with lower phase of tube B and centrifuged. The second step was repeated again in tube C to finally get plasmalemma vesicles free of intracellular membranes.

2.2.3 Protein quantification

Protein was quantified according to the method of Bradford (1976) and the reagent composition was as described below:

0.01% (w/v) Coomassie Brilliant Blue G-250

4.7% (w/v) alcohol

8.5% (w/v) phosphoric acid

15-20 µL membrane protein were mixed with 3.5 mL reagent and incubated for 40 min at room temperature. The absorbance was measured at 595 nm using a spectrophotometer (Carry 4 Bio, Varian Australia Pty Ltd., Australia). Bovine serum albumin was used as standard.

2.2.4 Hydrolytic activity of plasma membrane ATPase

ATPase hydrolytic activity was determined by measuring the released P_i amount after 30 min of hydrolysis reaction.

The 0.5 mL reaction medium was composed of;

30 mM BTP/MES

10 mM $MgSO_4$

50 mM KCl

50 mM KNO_3

1 mM Na_2MoO_4

1 mM NaN_3

0.02% (w/v) Brij 58

5 mM disodium-ATP

Reaction was initiated by the addition of 30 µL membrane vesicles with 2 to 3 µg protein, proceeded for 30 min at 30°C, and stopped with 1 mL of stopping reagent [4% (v/v) concentrated HNO_3, 5% (w/v) SDS, and 0.7% (w/v) $(NH_4)_2MoO_4$)] followed immediately by 100 µL of 10% (w/v) ascorbic acid. After 15 min, 1.45 mL of arsenite-citrate reagent [2% (w/v) sodium citrate, 2% (w/v) sodium m-arsenite, and 2% (w/v) glacial acetic acid] were added to prevent the measurement of P_i liberated because of ATPase activity from ATP hydrolysis under

acidic conditions (Baginski et al., 1967). Color development was completed after 30 min and A_{820} was measured by means of a spectrophotometer (Carry 4 Bio, Varian Australia Pty Ltd., Australia). ATPase activity was calculated as phosphate liberated in excess of boiled-membrane control.

The kinetic characteristics of plasma membrane H^+-ATPase were studied in the presence of an ATP regenerating system that included 5 units of pyruvate kinase (Sigma) and 5 mM phosphoenolpyruvate (PEP) (Boehringer Mannheim/Roche, Basel; Sekler and Pick, 1993). V_{max} and K_m were determined by means of Dynafit (Kuzmic, 1996). Activation energy of ATPase was calculated, using the Arrhenius equation, from Vmax values determined at 25°C and 30°C.

2.2.5 Measurement of H^+ pumping activity

The formation of a pH gradient across the plasma membrane *inside-out* vesicles was measured as the quenching of A_{492} by acridine orange (AO). The change of the quenching was continuously monitored by means of a spectrophotometer (Carry 4 Bio, Varian Australia Pty Ltd., Mulgrave, Victoria, Australia).

The assay mixture contained:

5 mM BTP/MES (pH 6.5)

12 μM AO

50 mM KCl

250 mM Sucrose

1 mM NaN_3

1 mM Na_2MoO_4

50 mM KNO_3

0.05% (w/v) Brij 58

50 μg membrane protein in a final volume of 1.5 mL

Brij *58* was used to create *inside-out* vesicles (Johansson et al., 1995). After equilibration of the membrane vesicles with the reaction medium, the reaction was initiated by the addition of Mg-ATP (mixture of $MgSO_4$ and disodium-ATP, adjusted to pH 6.5 with BTP) to give a final concentration of 5 mM. The reaction temperature was 25°C. Quenching was stopped by adding 300 μM Na_3VO_4 and passive efflux of H^+ was calculated after 1 min. The H^+ gradient was completely collapsed by the addition of 10 μL 755 μM gramicidine, an H^+ ionophore.

2.2.6 *In vitro* effect Si on H^+-ATPase hydrolytic and pumping activity

To investigate the direct effects of Si on the H^+-ATPase, the enzyme activity was determined in the presence of Si in the assay medium. Monosilicic acid was prepared as described by Jugdaohsingh et al. (2000). For monomeric silicic acid preparation, a stock solution of oligomeric silicic acid (10 mM) from sodium silicate was prepared. The stock of sodium silicate solution was neutralized to pH 7.0 with HCl. The neutralized solution was incubated at room temperature for 24 h. Monomeric silicic acid (2 mM) was prepared by diluting the oligomeric solution and incubating it at room temperature for 7 d. Hydrolytic and pumping activity were determined as described in section 2.2.4 and 2.2.5 except the stop reagent used for hydrolytic activity measurements. Stopping reagent composition was [4% (v/v) concentrated HNO_3, 5% (w/v) SDS, 0.7% (w/v) $(NH_4)_2MoO_4$), and 0.25% oxalic acid]. Addition of oxalic acid to color-forming reagent prevents the formation of the silico-molybdenum complex and eliminates the possibility of overestimation of P_i released by the enzyme activity.

2.3 Immunodetection of plasma membrane H^+-ATPase protein

Plasma membrane H^+-ATPase protein from vesicles isolated by two-phase partioning was separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the system of Laemmli (1970). The method relies on electrophoretic mobility of protein

that results in separation of protein according to molecular mass. Plasmalemma vesicles (10 µg for root and 8 µg for shoot) were solubilized in SDS-loading buffer containing:

0.125 mM Tris-HCl, pH 7.4

10% (w/v) SDS

10% (v/v) glycerol

0.2 M dithiothreitol

0.002% (w/v) bromocresol blue

5 mM phenylmethylsulfonyl fluoride

0.05% (w/v) trasylol

After mixing and shaking at room temperature, the samples and standard marker (Sigma) of proteins were loaded on a discontinuous SDS polyacrylamide gel which contains stacking gel (6% w/v) acrylamide and separating gel (10% w/v) acrylamide. The stacking and separation gels have the following composition:

Stacking gel

1.25 mL H_2O bidest.

0.625 mL 0.5 M Tris-HCl, pH 6.8; 0.4% SDS

0.5 mL Acrylamide solution

10 µL 10% APS (w/v)

10 µL TEMED

Separation gel

1.5 mL H_2O bidest.

1.5 mL 1.5 M Tris-HCl, pH 8.8; 0.4% SDS

3 mL Acrylamide solution

50 µL 10% APS (w/v)

5 µL TEMED

For western blotting, the gels were incubated in blotting buffer for 15 min at room temperature. The buffer was prepared as under:

0.025 M Tris base

0.192 M glycine and,

10% methanol with a pH of 8.3.

After incubation, the separated membrane proteins were electrophoretically transferred to polyvinylidene diflouride (PVDF) membrane filters (0.2 µm, Pall) as described by Zörb et al. (2005) using a semi-dry blotting system with a buffer containing 10 mM 3-cyclonexylamino-1-propane sulfonic acid (pH 11, adjusted with NaOH) and 20% (v/v) methanol for 1 h at room temperature and at a current intensity of 0.8 mA cm^{-2}. After transfer, the membrane filter was washed with H_2O bidest and incubated for 2 h in blocking buffer which was prepared by adding 2.5g of milk-powder in 50 mL of Tris-bufferd saline (TBS). The TBS solution was prepared as follows;

1 mM tris-HCl (pH 8.0)

15 mM NaCl

After 2 h, the blocking buffer attached to the membrane was removed by washing it three times with TBS-T. For the identification and quantification of plasma membrane H^+-ATPase, the PVDF membrane filter with plasma membrane proteins was incubated with a polyclonal antibody specific for the central part of plant H^+-ATPase (amino acids 340-650 of AHA_2). The anti-serum was diluted 1:3,000 in TBS-T buffer that has 1 mM Tris-HCl (pH adjusted to 8.0 with NaOH), 15 mM NaCl, and 0.1% [v/v] Tween 20) and incubation was carried out for 1 h at room temperature followed by incubation at 4°C overnight. Next day, the membrane was rinsed to remove the antibody with TBS-T twice for 10 min. After washing, the membrane was incubated with secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG, Sigma) for 2 h and was rinsed in TBS-T. After rinsing, the filter was incubated for 5 min in a buffer containing 100 mM Tris-HCl (pH 9.5, adjusted with NaOH), 100 mM NaCl, and 5 mM $MgCl_2$. After several washing steps in TBS-T or TBS buffers, filters were incubated for 5 min in AP buffer and Western Blots were developed using a buffer containing the substrates 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro blue tetrazolium (NBT). The buffer solutions were prepared as described below:

<u>AP-buffer</u>

100 mM Tris-HCl, pH 9.5

100 mM NaCl

5 mM $MgCl_2$

<u>Developing buffer</u>

66 µL NBT (100 mg NBT in 1.9 mL 70% (v/v) Dimethylformamid)

32 µL BCIP (100 mg BCIP in 1.9 mL Dimethylformamid)

10 mL AP-buffer

For quantification of plasma membrane H^+-ATPase, the blots were scanned, and the H^+-ATPase immuno-reactive bands were quantified densitometrically (software TINA, Raytest Isotopenmessgeräte, Straubenhardt, Germany).

2.4 Effect of Si nutrition on the transcription of plasma membrane H^+-ATPase isoforms

To determine the effect of Si supply in nutrient solution on plasma membrane H^+-ATPase transcription; plants were grown with and without 1 mM Si supply. Young growing leaves and roots were harvested and shock-frozen in liquid nitrogen. The frozen root and shoot samples were ground with mortar and pestle in liquid nitrogen to avoid the degradation of proteins. The ground samples were stored at -80°C.

TRIzol reagent was used to isolate the total RNA. This phenol-based reagent contains guanidine isothiocyanate and phenol. Guanidine isothiocyanate denatures RNAses and proteins while the phenol dissolves the proteins. Lipids are dissolved in chloroform which is added after the homogenization of samples. 100 mg of plant tissue were mixed with 1 mL of reagent to denature and homogenize the sample tissues and nucleoproteins. Samples were incubated for 5 min at room temperature and were mixed with 200 µL of chloroform. After 3 min, the samples were centrifuged for 15 min at 4°C (12,000 *g*) and separated into three phases; upper colorless phase, an inter-phase and a lower phenol-chloroform phase (organic phase). The upper colorless aquous phase contains RNA while DNA and protein remain in the inter and organic phase, respectively. The upper phase was transferred to another Eppendorf tube and mixed with 0.5 mL of isopropanol and incubated at room temperature for 10 min. The RNA sample was pelleted by centrifugation at 4°C (12,000 *g*) for 10 min. The supernatant was removed and the RNA pellet was washed with 75% ethanol to remove the isopropanol. RNA was pelleted again by centrifugation for 5 min at 4°C (7500 *g*). The supernatant was removed and the pellets were air-dried to evaporate the residual ethanol. The samples were dissolved in 40 µL of DEPC-water and incubated at 60°C for 10 min. The RNA samples were shock-frozen in liquid nitrogen and stored at -80°C for further analysis.

2.4.1 Spectrophotometric determination of RNA

A NanoDrop spectrophotometer (ND 1000, Thermo Scientific) was used to quantify the RNA amount from the samples. Before measurements, the lower and upper pedestals of NanoDrop were cleaned with deionized water and 2 µL of RNA sample were put on lower

pedestal. The upper optical pedestal was lowered and the measurements were taken using an absorbance maximum at 260 nm. After each reading, the both pedestals were cleaned using dry tissue paper. The purity of RNA samples can be accessed by measuring the ratio of absorbance/optical density at 260 nm and 280 nm (OD260/OD280). Sample with high purity have OD260/OD280 of 2.0 or more than 2.0. A ratio below 2.0 indicates that sample is contaminated with protein or phenol and should not be used for further analysis.

2.4.2 Determination of RNA integrity

RNA integrity is an important factor that determines the accuracy of mRNA transcript measurements obtained with real-time PCR (qPCR). As mRNA is only 1-2 % of the total RNA so total RNA on agarose gel can be used as a rough indicator of the mRNA stability. The integrity of the RNA can be checked by the band intensity. Ribosomal bands should appear as sharp bands on the stained gel. If the bands appear like a smear it is likely that the RNA was degraded during preparation.

The gel was prepared by adding 1 g of agarose in 100 mL of TBE buffer (Tris-borate-EDTA solution). For the dissolution of agarose, slurry was heated in a microwave for 3 min. The solution was allowed to cool down (60°C) and to prevent uneven cooling the solution was stirred occasionally. Ethidium bromide (6 µL) was mixed in agarose solution and poured onto the gel tray and allowed for 1 h to solidify. Prior to loading of RNA sample to the gel, 3 µg of RNA sample were mixed with RNA loading buffer and the gel was run at 120 V for 1 h. The buffer used for electrophoresis purpose contained 400 mM Tris-borate and 10 mM EDTA (pH 8.0), dissolved in bidest. H_2O. RNA bound with ethidium bromide shows increased fluorescence compared to the unbound dye in the background. The gel was illuminated under UV light and analyzed to check the integrity of RNA.

2.4.3 Synthesis of cDNA

Complementary DNA (cDNA) is a DNA copy synthesized from mRNA. Most eukaryotic mRNA contains a 3‘sequence of polyadenylic acid (poly-(A) tail), which is different from other

RNAs (rRNA, tRNA and prokaryotic RNAs). Actually, the poly-(A)tail represents multiple adenosine monophosphates bases. Reverse transcriptase using poly-(A)tail as a starting point hybridizes synthetic oligonucleotide (oligo-dT primer) and synthesizes a complementary DNA on the mRNA template. This method is quite convenient to synthesize cDNA using total RNA.

The cDNA was synthesized using Verso^{TM} cDNA kit (Thermo Scientific). RNA samples containing 4 µg each were heated at 70°C for 5 min to degrade the secondary structure of RNAs. After heating, the samples were cooled down and mixed with 10 µL of reaction mixture that contained:

4 µL 5×cDNA synthesis buffer (Proprietary buffer that improves reverse transcription)

2 µL dNTP mix (Deoxynucleotide triphosphates, the building-blocks for a new DNA strand)

1 µL RNA primer (Oligo-dT)

1 µL RT enhancer (remove the contaminating DNA and to degrade the double-stranded DNA during reverse transcription)

1 µL Verso enzyme mix

1 µL DEPC water

The process of reverse transcription was started by placing the samples in the PCR machine. The samples mixed with reaction mixture were incubated at 42°C for 40 min. The reaction was stopped by raising the temperature to 95°C for 2 min to inactivate the RT enhancer and reverse transcriptase. The newly synthesized cDNA was stored at -20°C.

2.4.4 Real-time PCR analysis of H^+-ATPase isoforms

Polymerase chain reaction (PCR) is recognized as a rapid, sensitive, and specific tool for the analysis of transcription of specific genes. The repeated thermal cycling of DNA melting and

enzymatic reaction, PCR produces thousands to millions copies of a particular DNA sequence by an exponential amplification. The reliability of the technique is very high because this technique uses house-keeping genes whose expression usually does not change under various experimental conditions.

To investigate the effect of Si on expression of plasma membrane H^+-ATPase isoforms, cDNA from shoot and roots of maize was used as a template for real-time PCR. Use of SYBR Green (a fluorescent dye which binds with double stranded DNA) provides an opportunity to detect small amounts of DNA. Primers for the reference and target genes as reported by Santi et al. (2003) and Zörb et al. (2005) are as under:

Actin	Forward Primer	GAGCTCCGTGTTTCGCCTGA	J0238
172 bp	Reverse Primer	CAGTTGTTCGCCCACTAGCG	J0238
MHA1	Forward Primer	TTTGGAAGTTTGACTTCCCA	U09989
215 bp	Reverse Primer	AAGAAGTCGGTCTTGTACGC	U09989
MHA3	Forward Primer	GAGAACAAGACCGCCTTCAC	AJ441084.1
436 bp	Reverse Primer	AAGACGGGTACCCAACCATA	AJ441084.1
MHA4	Forward Primer	TCTGGCTCTACAGCATCGTG	AJ539534
230 bp	Reverse Primer	CTTGTCGTGGAACAGCGTGC	AJ539534
MHAfam	Forward Primer	ATCGTCAGCCAGGCTCTGAT	*
231 bp	Reverse Primer	CGAAGCGGATGAAGAACTTG	

*designed on the basis of homologue regions of all possible *MHA* isoforms (family-specific primers) of maize

SYBR Green mix from Sigma was used in all assays. Real-time PCR assays for all specific genes were performed on the Rotor-Gene 2000/3000 real-Time Amplification Thermal Cycling System. For 10 µL of each reaction the following solutions were mixed:

5 µL SYBR Green Mix

0.2 µL primer pair (10 µM)

2.8 µL sterile water

2 µL diluted cDNA (1: 20)

The program was designed as follows:

Cycle 1 Initial denaturation at 94°C for 2 min

Cycle 2-35 Denaturation at 94°C for 15 s

Primer specific annealing at 50-60°C for 30 s

Primer elongation at 72°C for 30 s

Cycle 36 Melt curve analysis at 72-99°C

The gene expression was quantified using the equation of Pfaffl (2001). It does not quantify the absolute amounts of the transcripts but a relative change in the target gene in comparison to a reference gene. The ratio of relative expression gives the physiological changes in gene expression. Actin was used as a reference gene which is supposed not to be influenced by Si treatment. The following equation shows Pfaffl´s model of relative expression:

$$\text{ratio} = \frac{(E_{target})^{\Delta Ct\ target\ (control\text{-}treated)}}{(E_{ref})^{\Delta Ct\ ref\ (control\text{-}treated)}}$$

Where,

ΔCt_{target} = difference in the Ct values for the target gene between control and treated samples

ΔCt_{ref} = difference in the Ct values for the reference gene between control and treated samples

E_{target} = Real-time PCR amplification efficiency of the target gene.

E_{ref} = Real-time PCR amplification efficiency of the reference gene.

Real-time PCR amplification efficiencies ($E= 10^{[-1/slope]}$) were calculated by making a series of dilution of cDNA (1 : 5, 1 : 10, 1 : 20, 1 : 40, 1 : 80). Specificities of the primers for target and reference genes were confirmed by making the melt curve analysis.

2.5 Effect of Si nutrition on apoplastic pH of maize shoot

Fluorescence microscopy provides an opportunity to measure the apoplastic concentration of different ions (Mühling and Läuchli, 2000). This method has an advantage over the others as it is a non-destructive method. To measure the apoplastic pH by ratio image technique, the fluorescent dye fluorescein isothiocyanate (FITC)-dextran (MW = 10,000, Sigma-Aldrich, Munich, Germany) was used. For this purpose, the second-youngest leaf from maize plants grown with and without Si supply was cut and washed with deionized water. Apopalst of the leaf segments was infiltrated with 50 µM FITC-dextran. The infiltrated leaf was washed with deionized water to remove the adhering dye. The leaf segments were cut to appropriate size and put under a microscope (Leica DM IRB, Solms, Germany) connected to a highly sensitive CCD camera (CoolSNAP, Photometrics, Tucson, Arizona, USA) with upside down and covered with cover slip to measure the fluorescence emission intensity. The calibration curve was drawn by using the dye standards of different pH ranging from 5.5 to 7.0.

The images taken from inverted microscope were processed on computer and calculated with Meta Fluor imaging system (Visitron, Puchheim, Germany) software (Meta Series, Version 6.2). The adaxial side of the leaf was excited at 440 nm/490 nm for pH by using the 20× objective (Leica pH 1; 20×/0.40). Ratios were converted to pH values using an *in vivo* calibrated pH curve. The fluorescence of the whole image, resulting from the intercellular spaces (apoplast) of the epidermal and stomatal cells of the intact leaf was determined and used for ratio imaging.

2.6 Statistical analysis

The experimental data were subjected to analysis of variance using SPSS. Multiple comparisons separating means in homogenous subgroups were done using post hoc Tukey test. For all analyses, a P-value of less than 5% was interpreted as statistically significant. The significant difference between two treatments was determined using Microsoft Excel (2007) t-test.

2.7 Chemicals

Agar (Agar Agar Kobe I): Serva 11392

Ammonium-Molybdate ($(NH_4)_6Mo_7O_{24}$): 82.3 %; Sigma

AO = Acridine Orange (3, 6-Bis[Dimethylamine] Acridine-Base): ca. 95% purity; Sigma

L(+)-Ascorbic acid: min. 99.7% purity (idodometrisch); Merck

Brij 58 (Polyoxyethylen-20-cetyl ether): Sigma

BSA (Bovines serum albumin): fatty acid free ≥ 96% Albumin; Sigma

BTP (1, 3-Bis [tris (hydroxymethyl) methylamino] propane: min. 99 % purity (Titration); Sigma

Coomassie Brilliant Blue G-250: Calbiochem Corp., La Jolla

Dextran T 500: Average molecular weight = 485 000 g/mol; Sigma

Di-potassium hydrogen phosphate (K_2HPO_4): p.a.; Merck

DTT (DL-Dithiothreitol): 99 % purity (Titration); Sigma

EGTA (Ethylene glycol-bis (ß-aminoethylether) N,N,N`,N-Tetraacitic acid): 97 % purity; Sigma

Glycerin: 99 % purity; Sigma

Gramicidine D: from *Bacillus brevis*, 1080 µg Gramicidin mg^{-1}; Sigma

2-Mercaptoethanol: min. 99% purity (GC); Serva

MES (2-[N-Morpholino]ethanesulfonic acid): Free acid, min. 99.5% purity (Titration).; Serva

Magnesium sulfate ($MgSO_4$): p.a.; Fluka

Na_2ATP (adenosin 5´-Triphosphate, Na_2-Salz): 98 % purity; Merck

Oxalic acid

PEG 3350 (Polyethylene glycol): Average molecular weight = 3350 g/mol; Sigma

PEP (Phosphoenol pyruvate): Boehringer Mannheim GmbH

PMSF (Phenylmethylsulfonylfluoride): > 99 % purity (GC); Sigma

Potassium chloride (KCl): p.a.; Fluka

Potassium di-hydrogen phosphate (KH_2PO_4): p.a.; Fluka

Potassium iodide (KI): ≥ 99.5% purity (argentometrische Titration); Fluka

Potassium nitrate (KNO_3): p.a.; Merck

Potassium sulfate (K_2SO_4): p.a.; Fluka

Pyruvate Kinase: 1 000 U; Sigma

D (+)-Sucrose: For biochemical use; Merck

SDS (Sodium dodecylsulfate): 99 % purity; Sigma

Sodium azide (NaN_3): p.a.; Merck

Sodium citrate-Dihydrate: p.a.; Merck

Sodium-metaarsenite ($NaAsO_2$): min.99 % purity; Sigma

Sodium molybdate (Na_2MoO_4): p.a.; Merck

Sodium orthovanadate (Na_3VO_4): min. 95 % purity; Sigma

Sodium silicate (Na_2SiO_3); Merck

3 Results

3.1 Optimization of silicon concentration in nutrient solution for maize growth

3.1.1 Effects of various concentrations of silicon on fresh and dry mass of maize shoot and root

The results show that various concentrations of silicon (Na_2SiO_3 neutralized with HCl) in nutrient solution increased shoot and root biomass (Figure 3). Plants supplied with 0.8 and 1.2 mM Si produced higher biomass as compared to the plants grown without Si supply. Further increase of Si concentration in nutrient solution (> 1.2 mM) did not increase fresh biomass. A similar trend was observed for shoot and root dry biomass. Application of 0.8, 1.2, and 2 mM Si significantly increased the shoot and root dry mass in comparison to control treatment.

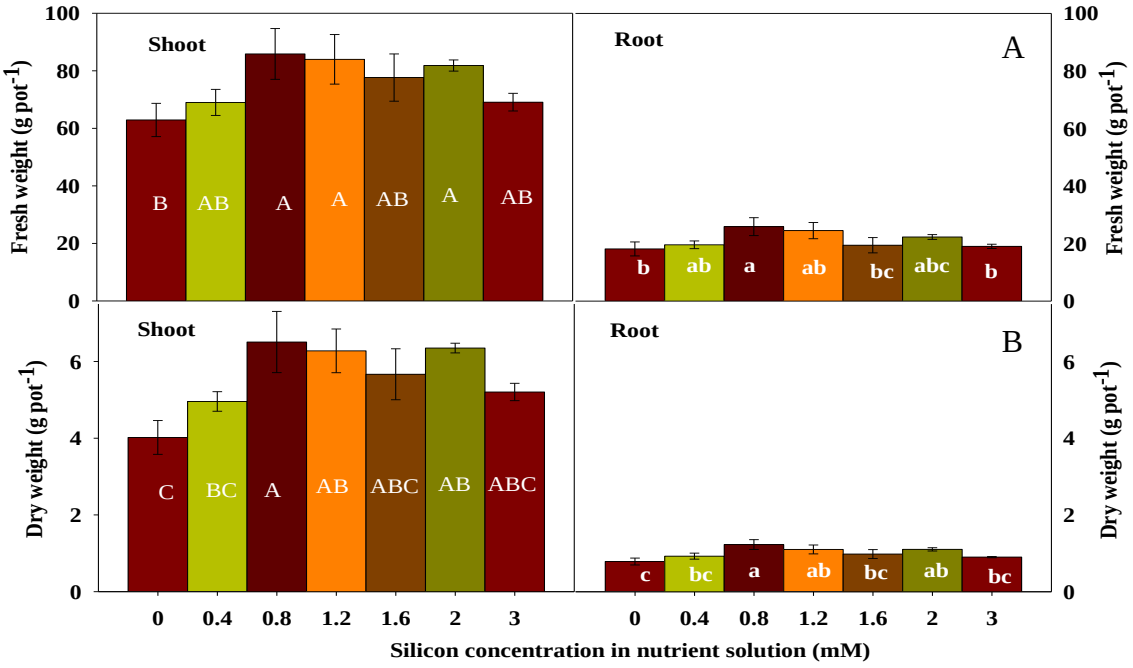

Figure 3: Effect of various concentrations of Si in nutrient solution on plant shoots and roots fresh (A) and dry mass (B) of maize plants. The plants were grown in nutrient solution for 21 d. Values are the means of four replicates ± SE. Columns with different letters indicate significant differences at 5% level.

3.1.2 Effects of various concentrations of silicon on maize plant height and leaf area of young growing leaves

Silicon addition to the nutrient solution produced beneficial effects on the expansion growth. Application of 1.2 mM Si significantly increased plant height in comparison to the control treatment. Older leaves (leaf no. 2, 3, 4 and 5) did not show any effect of Si on expansion growth (data not shown). The length of the sixth and seventh leaf increased gradually with the increase of Si concentration in the nutrient medium. Maximum length was achieved at 0.8 mM for the sixth and 1.2 mM Si for the seventh leaves (Figure 5). Data show that above the optimal concentration, further increase of Si in nutrient solution did not have any effect on expansion growth.

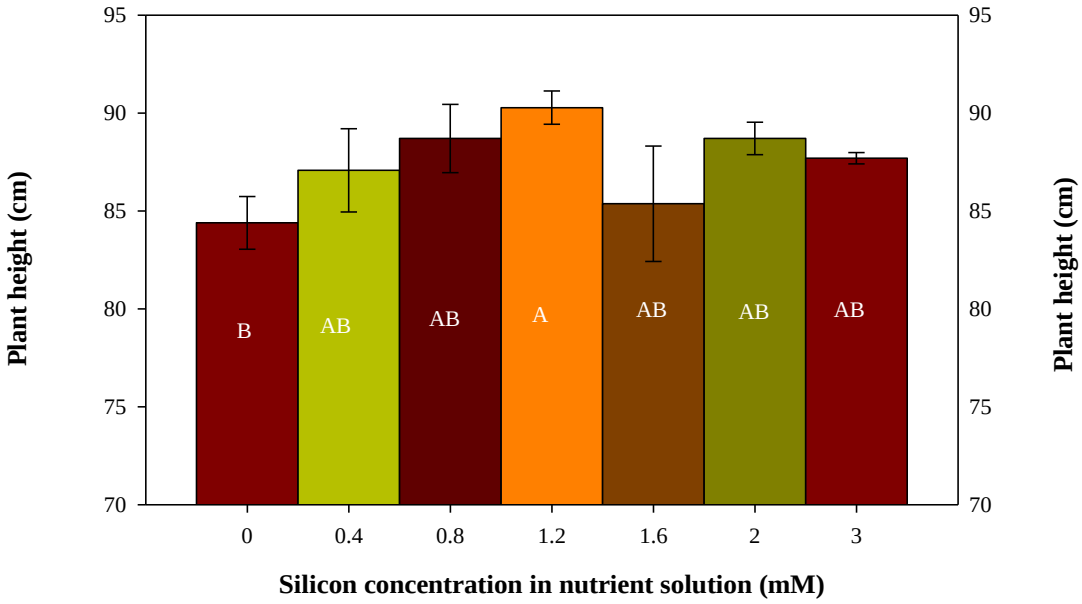

Figure 4: Effect of various concentrations of Si in nutrient solution on plant height. Values are the means of four replicates ± SE. Columns with different letters indicate significant difference at 5% level.

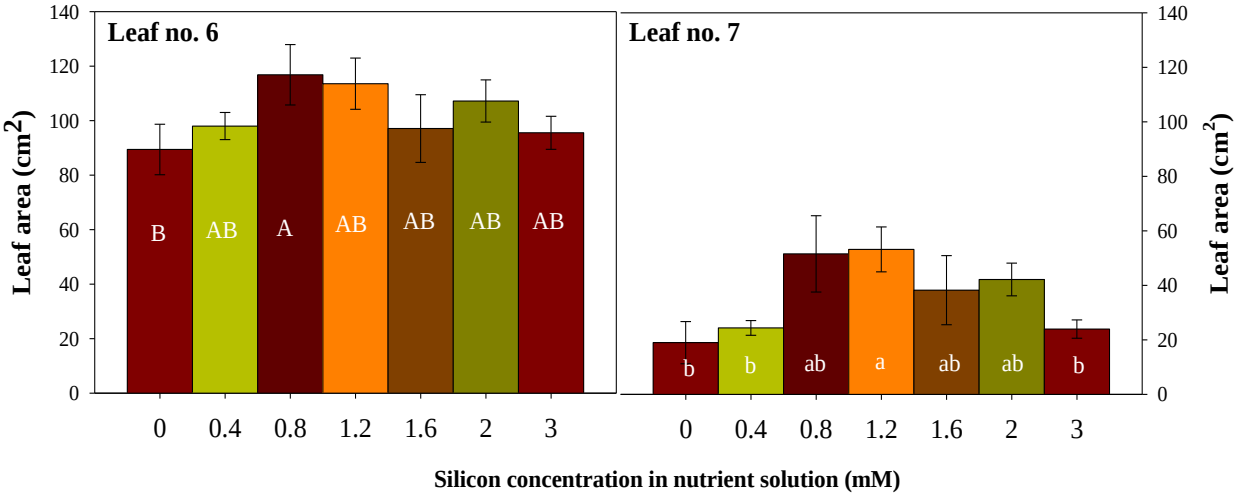

Figure 5: Effect of various concentrations of Si in nutrient solution on leaf area of leaf no. 6 and 7 of maize plants. Values are the means of four replicates ± SE. Columns with different letters indicate significant difference at 5% level.

3.1.3 Effects of various concentrations of silicon on cation concentrations in maize plants

In response to Si application in nutrient solution, changes in cation concentrations were observed in various studies (Liang et al., 1999; Miao et al., 2010). In present study, an increase of Si concentration in nutrient solution caused a slight and gradual decrease of Ca concentration in young as well as in old shoot, but no significant differences were observed in root tissues (Figure 6 A). Furthermore, the results for Mg concentration show the same trend as Ca in young shoot. In old shoot and root the Mg concentration remained unchanged (Figure 6 B).

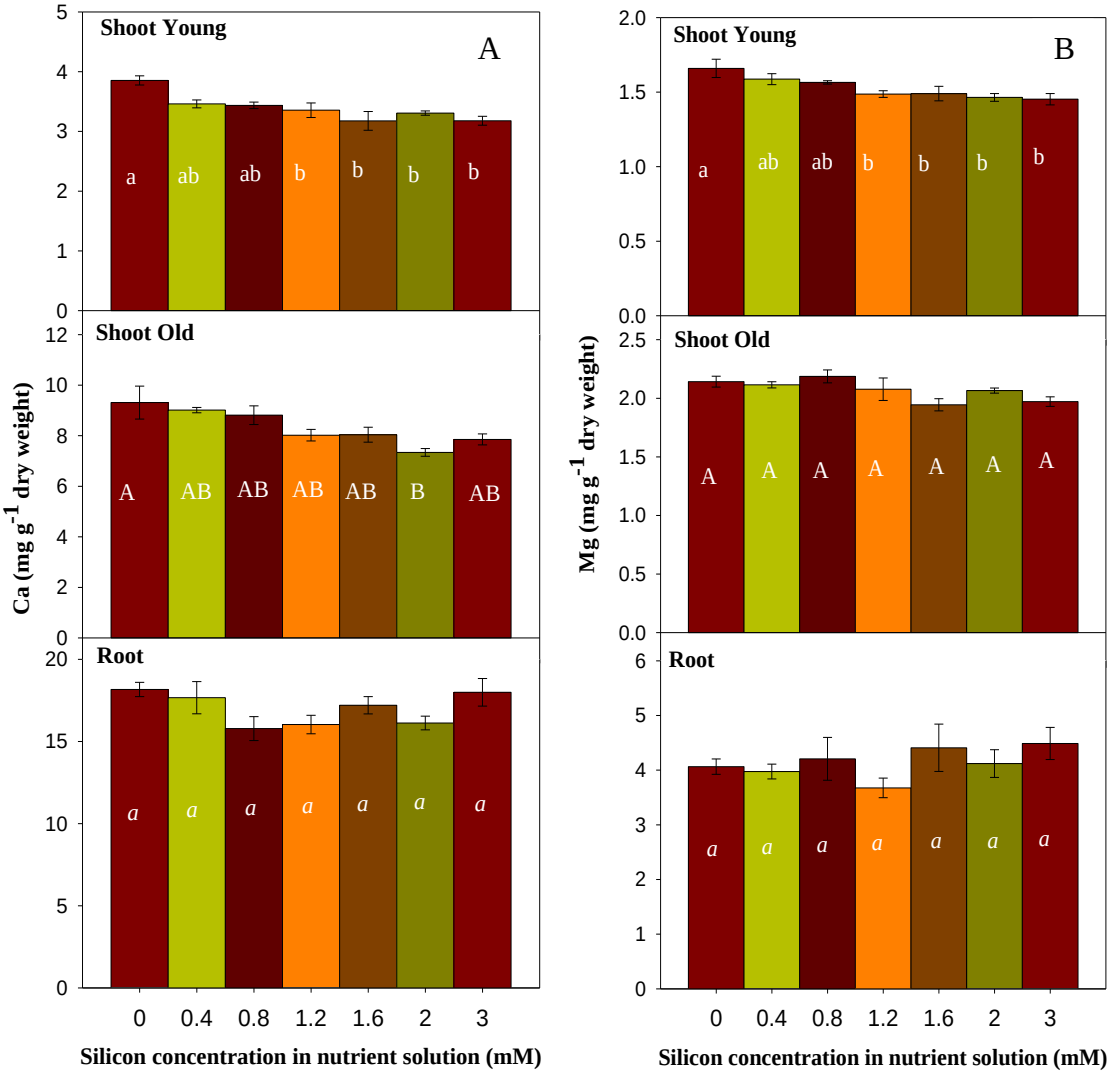

Figure 6: Effect of various concentrations of Si in nutrient solution on shoot (young, old) and root Ca (A) and Mg concentrations (B). Values are the means of four replicates ± SE. Columns with different letters indicate significant difference at 5% level.

Additionally, the results show that the increased level of Si in nutrient solution had no effect on the K concentrations in young shoots and roots but slightly decrease the K in old shoots. A more consistent response was observed for Na concentration in all three plant parts. Increased Si supply in the root medium gradually decreased the Na concentrations (Figure 7 B) in shoot as well as in root.

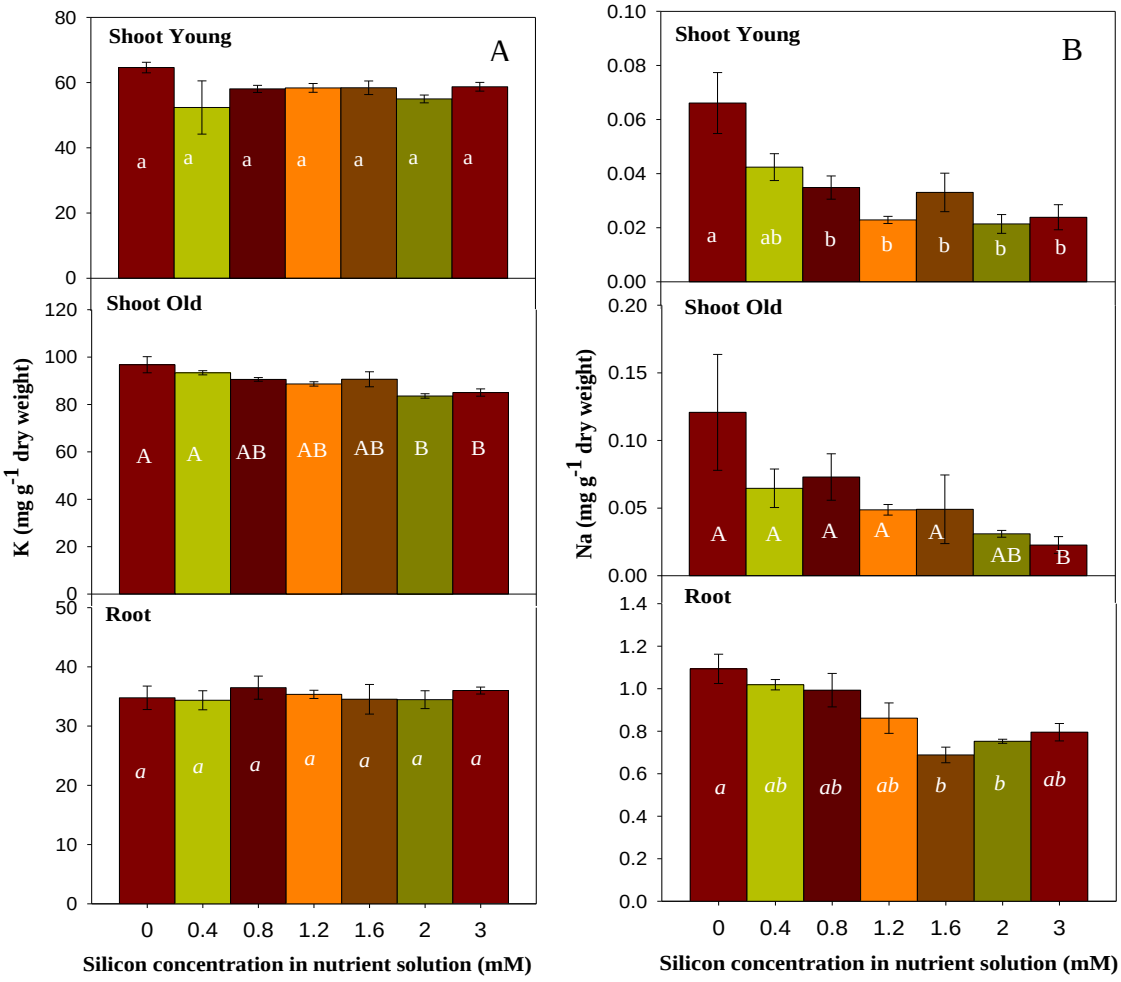

Figure 7: Effect of various concentrations of Si in nutrient solution on shoot (young, old) and root K (A) and Na concentrations (B). Values are the means of four replicates ± SE. Columns with different letters indicate significant difference at 5% level.

Silicon application in nutrient solution differentially affected the metal cations in different plant tissues. In general, higher levels of Si showed an antagonistic effect on metal ion concentrations. Increased concentration of Si in the nutrient solution gradually decreased the Cu Mn and Zn concentrations in young and old tissues while there was no effect of Si on Fe concentration in shoots.

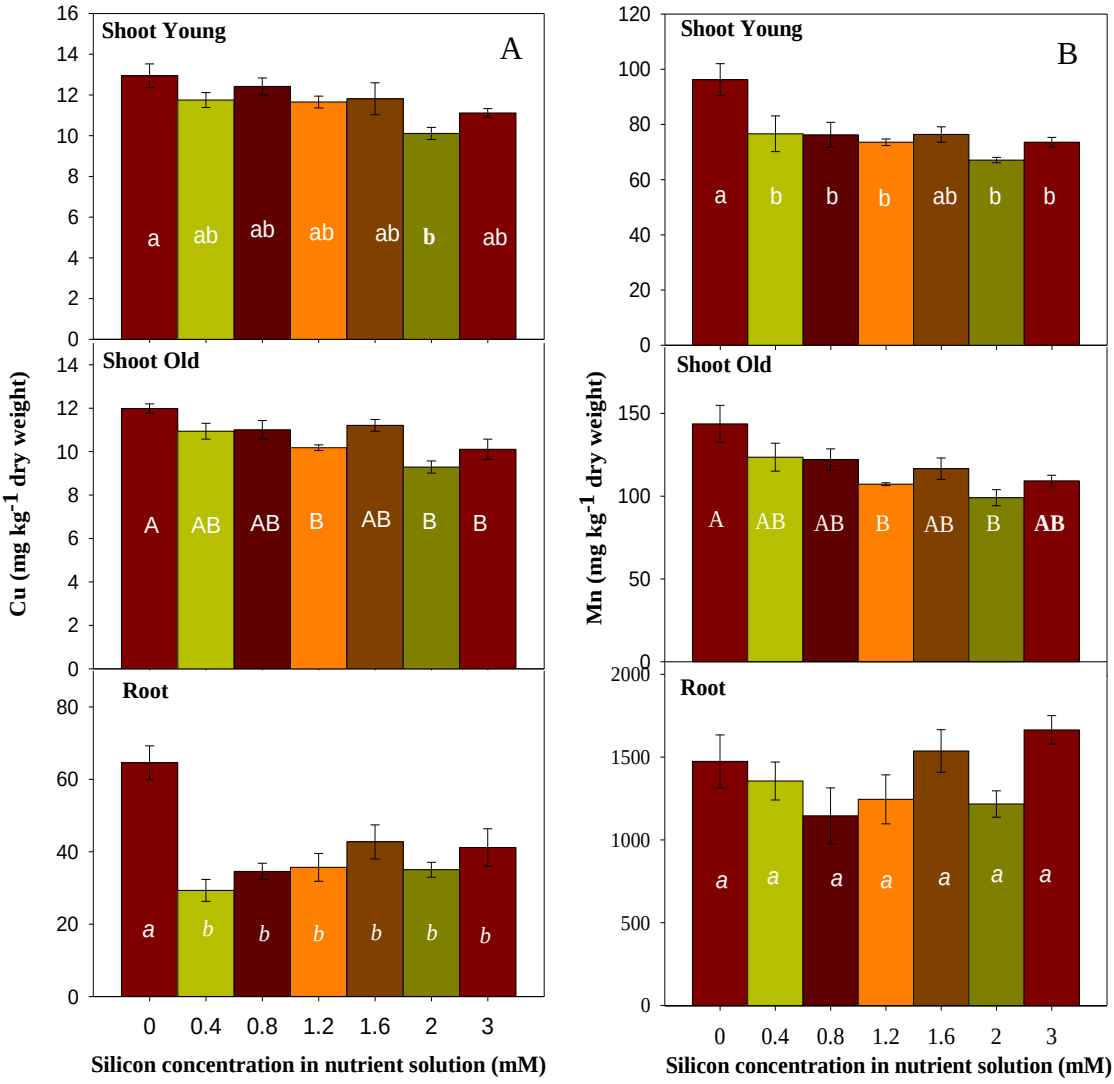

Figure 8: Effect of various concentrations of Si in nutrient solution on shoot (young, old) and root Cu (A) and Mn concentrations (B). Values are the means of four replicates ± SE. Columns with different letters indicate significant difference at 5% level.

Silicon application in the root medium significantly reduced the Cu concentration in roots with respect to the control treatment. The decrease in Cu concentration in root tissues was independent of Si level in nutrient solution. Additionally, a significant gradual decrease in Fe and Zn concentrations by increasing the Si concentration in nutrient medium were observed (Figure 9).

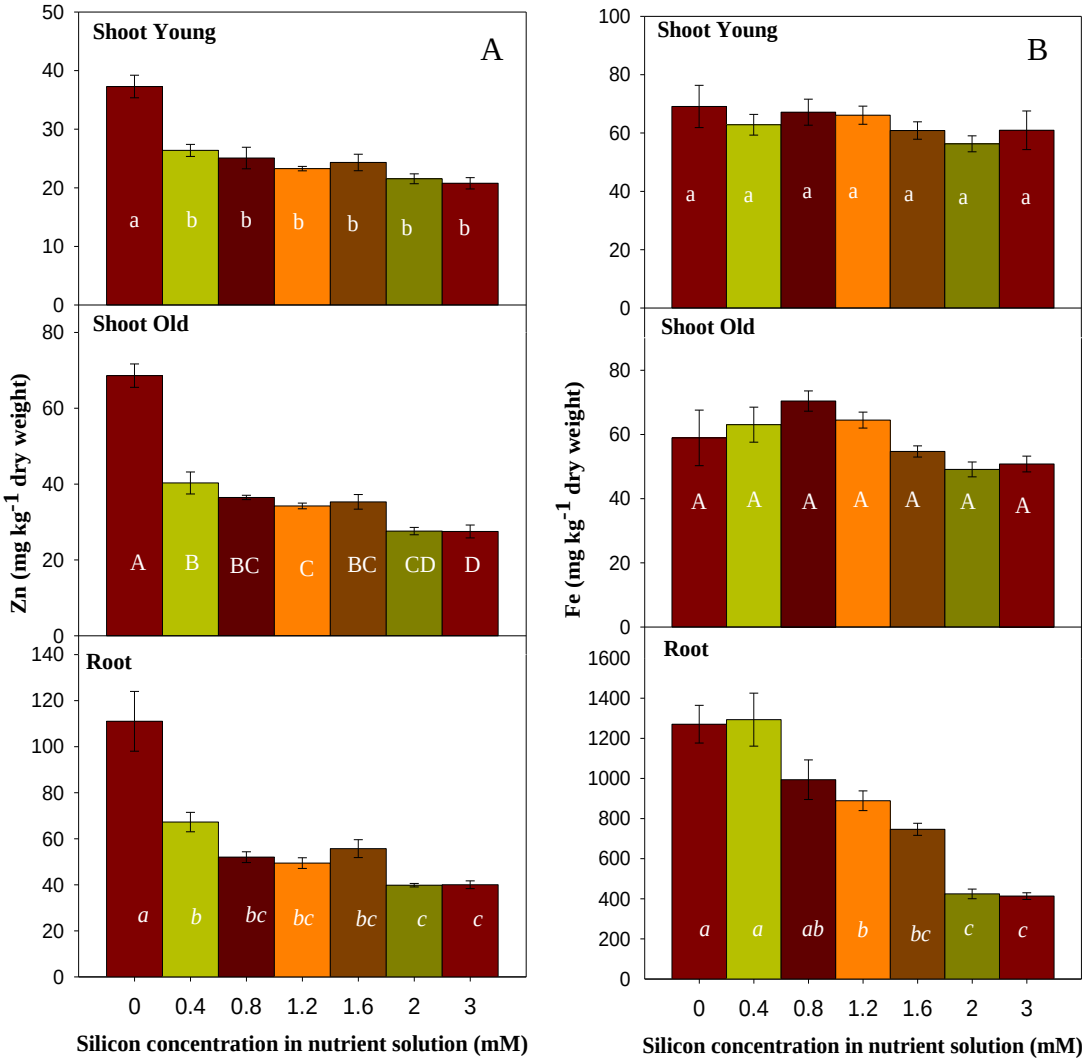

Figure 9: Effect of various concentrations of Si in nutrient solution on shoot (young, old) and root Zn (A) and Fe concentrations (B). Values are the means of four replicates ± SE. Columns with different letters indicate significant difference at 5% level.

3.1.4 Effects of various concentrations of silicon in nutrient solution on silicon concentration in young maize shoot

In general, the results showed that Si treatments in nutrient solution increased the Si concentrations in plant tissues in comparison to the control treatment. However, plants grown without any exogenous Si supply in the root medium also contained substantial amounts of Si

(0.38 mg g^{-1} DM) in shoot tissues. Here, it is worth to mention that the water used for plant growth was not totally free of Si and it contained ≅0.3 µM Si. The data show that the increased level of Si in the nutrient solution resulted in a gradual significant increase of Si concentration in young shoots, irrespective of the method used for measurements. Both AAS and ICP produced comparable results though the sensitivity of the two methods is highly different. ICP is much more sensitive than the atomic absorption spectrophotometer and can determine very low levels of Si in sample solutions. The results for the color method also showed a similar increasing trend as shown by the other two methods. However, Si concentrations determined with the color method were higher in comparison to AAS and ICP. This might be due to the unspecificity of the method for Si. The color produced by a silico-molybednum complex gives the same blue color as that of phospho-molybdenum. Overestimation of Si by the color method might be due to the presence of phosphate in sample solutions.

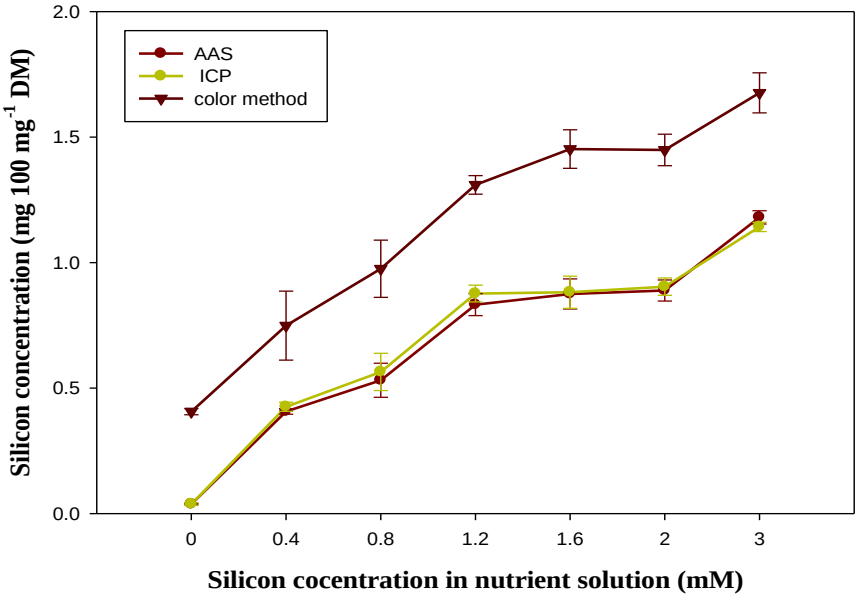

Figure 10: Effect of various concentrations of Si in nutrient solution on Si concentration in maize young shoots. Plant material was digested with the help of a microwave digester and filtered. The filtrate was used to determine Si concentration with three different methods. Each data point indicates the mean of four replicates ± SE.

3.2 Role of silicon in plasma membrane H^+-ATPase characteristics

3.2.1 Effects of silicon supply in nutrient solution on the purity of plasma membrane vesicles isolated from maize shoots and roots

The results in Table 3 show the effect of Si supply in nutrient solution on the relative ATPase activities of the membranes isolated from roots and shoots of maize plants by two-phase partioning (Yan et al., 2002). The inhibitor-sensitive activity was calculated by subtracting the activity of vesicles fraction in the presence of specific inhibitors from the activity without any inhibitor. The plasmalemma vesicles isolated from roots and shoots showed a non-significant small increase in hydrolytic activity in the presence of 50 mM KNO_3. The increase in activity might be due to additional 50 mM K in the assay medium. Presence of 1 mM molybedate, a specific inhibitor of unspecific acid phosphatases also showed some inhibitory effect on membrane fractions. The results show that about 87-91% of the ATPase activities were sensitive to 0.1 mM vanadate (Table 3). Considering the results, all assays were performed in the presence of 1 mM azide, 50 mM nitrate, and 1 mM molybdate to inhibit the activities of unspecific phosphatases, mitochondrial and tonoplast ATPases.

Table 3: Purity of plasmalemma vesicles isolated from maize roots and shoots. Plants were grown with and without 1 mM Si supply in nutrient solution for 23 days. Specific inhibitors were used as markers of tonoplast (nitrate), mitochondrial (azide), acid phosphatases (molybdate), and plasma membrane (vanadate) origin. Assays were performed in 30 mM BTP-MES buffer adjusted to pH 6.5 containing 100 mM KCl, 5 mM $MgSO_4$, and 5 mM Na_2-ATP. Values are the means of at least five replicates ± SE

Treatment	**Relative ATPase activities** (μmol P_i mg^{-1} protein min^{-1})			
	Shoot (Control)	**Shoot (1 mM Si)**	**Root (Control)**	**Root (1 mM Si)**
No inhibitor	0.42 ± 0.06 (100%)	0.43 ± 0.02 (100%)	0.60 ± 0.08 (100%)	0.57 ± 0.07 (100%)
Nitrate	0.46 ± 0.05 (110%)	0.48 ± 0.02 (111%)	0.63 ± 0.10 (104%)	0.57 ± 0.09 (96.20%)
Azide	0.43 ± 0.07 (102%)	0.44 ± 0.02 (103%)	0.62 ± 0.09 (102%)	0.58 ± 0.08 (98%)
Molybdate	0.37 ± 0.05 (89%)	0.41 ± 0.02 (95%)	0.58 ± 0.08 (97%)	0.53 ± 0.08 (90%)
Vanadate	0.05 ± 0.01 (11%)	0.05 ± 0.00 (12%)	0.08 ± 0.02 (13%)	0.06 ± 0.02 (9%)
Vanadate + molybdate	0.04 ± 0.01 (9%)	0.05 ± 0.00 (11%)	0.08 ± 0.01 (14%)	0.06 ± 0.01 (10%)

3.2.2 Effect of silicon nutrition on plasma membrane H^+-ATPase hydrolytic activity isolated from roots and shoots

To examine the effect of Si supply in nutrient solution on plasma membrane H^+-ATPase, hydrolytic activity of ATPase was measured. Hydrolytic activity measured at 25 and 30°C show that there was no effect of Si supply in nutrient solution on the plasma membrane ATPases (Figure 11). There was a strong effect of changing the assay temperature from 30 to 25°C on the hydrolytic activity of the enzyme. One degree decline in temperature resulted in 9-10% and 6-7% decrease in hydrolytic activity of plasma membrane H^+-ATPase from shoots and roots, respectively.

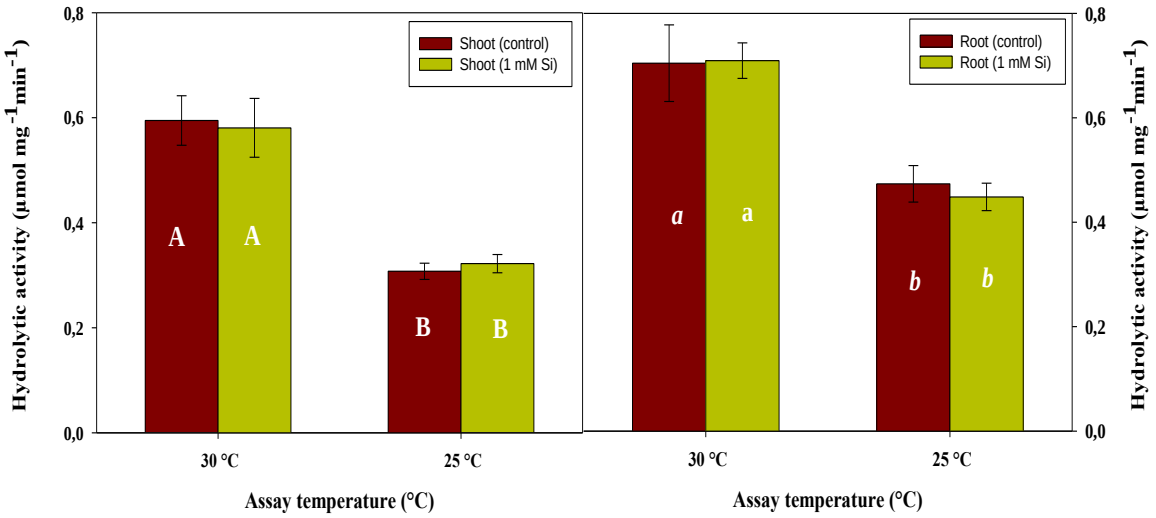

Figure 11: Hydrolytic activity of the plasma membrane H^+-ATPase derived from maize shoots (Left) and roots (Right). Plants were grown with and without 1 mM Si supply in nutrient solution under controlled environmental conditions. Plasma membrane vesicles were isolated and purified from the young, developing shoots and roots by two-phase partioning. Plasma membrane ATPases activities were analyzed in 30 mM BTP-MES buffer (pH 6.5) in the presence of 1 mM molybdate, 1 mM azide, and 50 mM nitrate at 25 and 30°C. An ATP-regenerating system (5 mM PEP and 5 units of pyruvate kinase) was used to keep constant ATP concentrations (5 mM) in the assay medium. Values represent means ± SE of four independent experiments.

3.2.3 Effect of silicon nutrition on H^+ transport activity of plasma membrane ATPase isolated from roots and shoots

To investigate the effect of Si supply in nutrient solution on plasmalemma H^+-ATPase transport activity, active and passive transport of H^+ across the membrane vesicles were measured. Active transport can be characterized as initial rate of H^+ pumping, and maximum pH gradient while passive transport was measured as passive efflux of H^+ across the vesicles. The results are summarized in Table 4.

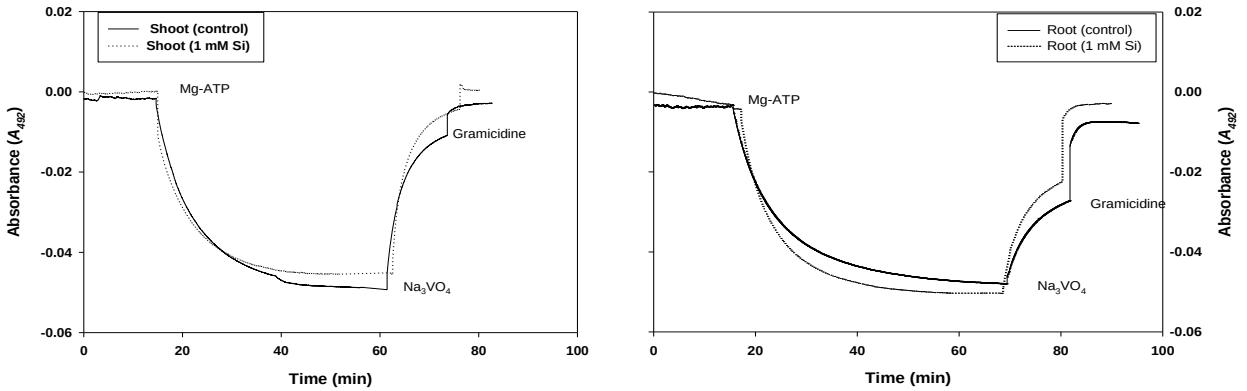

Figure 12: Comparison of active H^+ transport and passive efflux of H^+ from *inside-out* plasma membranes vesicles. Membrane vesicles were isolated from maize shoots (left figure) and roots (right figure) grown with and without 1 mM Si supply in nutrient solution. For the comparison of active H^+ transport, the pH gradient formation across vesicle membranes was monitored by measuring the absorbance (A_{492}) of AO. All the assays were performed at pH 6.5 and the reaction was started by addition of 5 mM Mg-ATP. Almost after 60 min, the reaction was stopped by adding 500 µM vanadate. The resting pH gradient was collapsed by adding 7.5 µM gramicidine. Figures shown are representatives of four replicates.

The formation of pH gradient was monitored as the quenching of acridine orange (AO) in plasma membrane *inside-out* vesicles. The reaction was initiated by the addition of Mg-ATP in reaction medium containing 50 µg plasma membrane vesicles protein. The initial rate of H^+ pumping was measured as AO quenching for the first min after the initiation of reaction (Figure 12). Maximum pH gradient is an equilibrium point where the active influx of H^+ balances with the passive transport of H^+ across the membrane vesicles. At this point there is zero net AO quenching. Plasma membrane vesicles isolated either with and without Si supply did not show any significant difference in active H^+ transport activity across the membrane vesicles isolated either from roots or shoots. The H^+ passive transport across the membrane vesicles was measured by the addition of 500 µM vanadate. Inhibition of plasmalemma H^+-ATPase by vanadate stops the active H^+ pumping and as a result H^+ bound in membrane vesicles release passively and AO absorbance start to increase. The results show that there was no significant effect of Si application on passive H^+ transport across the membrane vesicles.

Table 4: Effect of Si supply in nutrient solution on H^+-transport activity of PM H^+-ATPase across the membrane vesicles derived from maize shoots and roots. Plasma membrane ATPase activity was analyzed in the presence of 1 mM molybdate, 1 mM azide, and 50 mM nitrate at 25°C. Values represent means ± SE of four independent experiments.

Parameters/ Plant part	**pH gradient** (ΔA_{492} mg^{-1} protein)	**Initial rate** (ΔA_{492} mg^{-1} protein min^{-1})	**Passive efflux** (ΔA_{492} mg^{-1} protein min^{-1})
Shoot (control)	0.87 ± 0.08 a	0.13 ± 0.01 a	0.12 ± 0.01 a
Shoot (1mM Si)	0.84 ± 0.14 a	0.11±0.01 a	0.13 ± 0.03 a
Root (control)	0.92 ± 0.12 A	0.10 ± 0.02 A	0.06 ± 0.00 A
Root (1mM Si)	0.81 ± 0.07 A	0.09 ± 0.01 A	0.06 ± 0.01 A

3.2.4 Effects of silicon supply in nutrient solution on the kinetic characteristics of plasma membrane H^+-ATPase isolated from maize shoot

The effect of Si supply in nutrient solution on plasma membrane ATPase kinetic characteristics was determined by measuring the hydrolytic activity. The enzyme activity was measured at various ATP concentrations ranging from 50 – 5000 µM in the presence of an ATP-regeneration system at 25 and 30°C (Sekler and Pick, 1993). The K_m and V_{max} values were calculated using computer software DynaFit 3 (Hanstein et al., 2011). The results show that Si supply in nutrient solution did not have any significant effect on plasmalemma H^+-ATPase V_{max} and K_m values at both temperatures (Table 5).

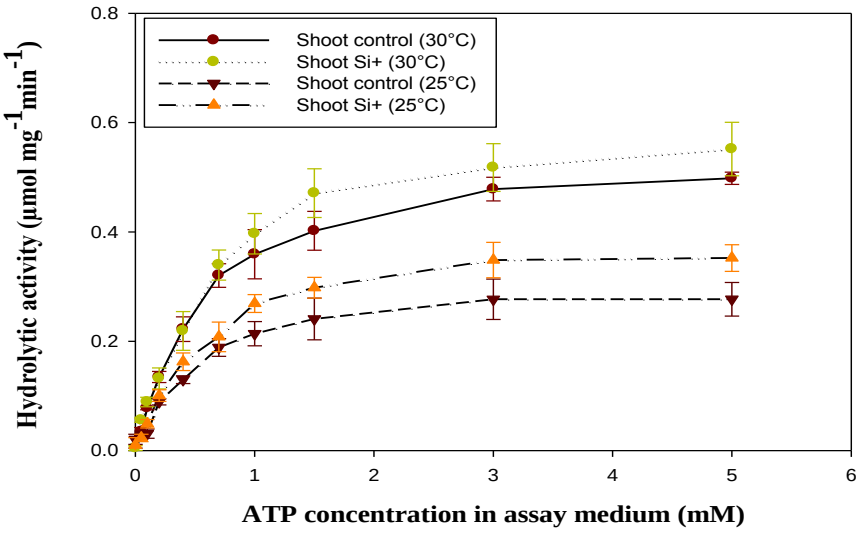

Figure 13: Effect of Si supply in nutrient solution on ATPase activity derived from plasma membrane vesicles obtained from maize leaves. The activity of plasma membrane ATPase was measured at various ATP concentrations ranging from 50 to 5,000 µM. The reaction medium used for ATPase activity contained: 1 mM molybdate, 1 mM azide, 50 mM potassium nitrate and 10 mM $MgSO_4$. Values represent means ± SE of three independent experiments.

Table 5: Effect of 1 mM Si supply in nutrient solution on the kinetic characteristics of plasmalemma ATPase isolated from maize leaves. The enzyme activity was measured at various ATP concentrations ranging from 50 to 5000 µM at 25 and 30°C. An ATP-regenerating system (5 mM PEP and 5 units of pyruvate kinase) was used to keep constant ATP concentrations. The values represent means ± SE of three independent experiments. No significant differences (P < 5%) were observed between the treatments at both temperatures.

Treatment	**Assay temperature**	**K_m** (µM ATP)	**V_{max}** (µmol P_i mg^{-1} min^{-1})
Shoot (control)	25°C	526.1 ± 37.2 a	0.32 ± 0.04 a
Shoot (1 mM Si)	25°C	568.9 ± 75.6 a	0.39 ± 0.01 a
Shoot (control)	30°C	626.2 ± 120.0 A	0.57 ± 0.00 A
Shoot (1 mM Si)	30°C	580.5 ± 55.1 A	0.59 ± 0.02 A

3.2.5 Effects of silicon supply in nutrient solution on the activation energy of plasma membrane ATPase

Activation energy of the plasma membrane ATPase was calculated using V_{max} values calculated at 25 and 30°C. The results show an increase of about 1.8 and 1.5 times in V_{max} by increasing the assay temperature from 25 to 30°C in control and Si-treated plants, respectively. Moreover, the results also show a slight non-significant decrease in activation energy of the enzyme isolated from maize plants supplied with Si in nutrient solution as compared to the membrane vesicles isolated from plants grown without Si supply (Figure 14).

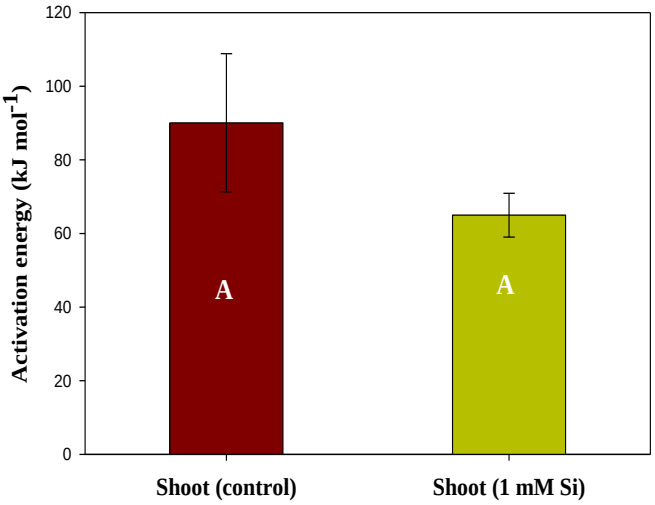

Figure 14: Effect of 1 mM Si supply in nutrient solution on the activation energy plasma membrane ATPase isolated from maize leaves. The activation energy was calculated using V_{max} measured at 25 and 30°C. Values are the means of three replicates ± SE.

3.2.6 *In vitro* effect of silicon on plasma membrane ATPase hydrolytic activity

To determine the direct effect of Si on plasma membrane ATPase, hydrolytic activity of the enzyme was measured by adding silicic acid in reaction medium. The mono-silicic acid was prepared as described in Materials and Methods. Four concentrations of Si were added in the assay medium. Plasma-membrane vesicles isolated from plants supplied with Si in nutrient

solution showed a decrease in hydrolytic activity. The decrease in hydrolytic activity was 14% when 1000 µM Si was added in the assay medium with respect to 0 µM Si (Figure 15). On the other hand, the hydrolytic activities remained un-changed in plasma-membrane vesicles isolated from control shoots, irrespective the concentration of Si in the assay medium.

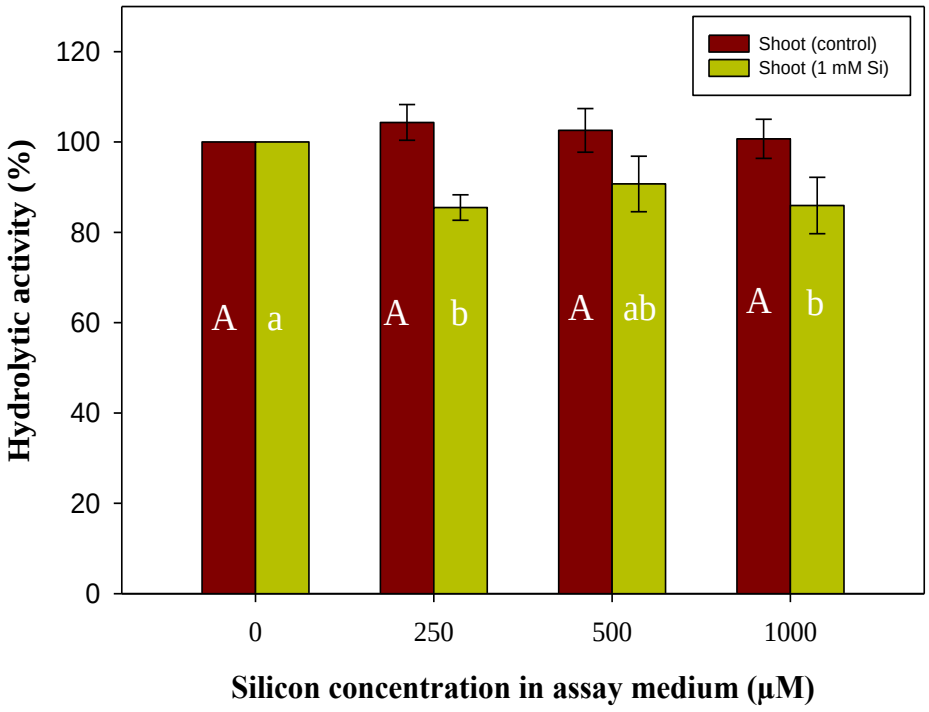

Figure 15: *In vitro* effect of Si on the hydrolytic activity of the plasma membrane ATPase. The activities measured for membrane vesicles without Si in the assay medium were considered as 100%. Plasma-membrane vesicles were isolated from young growing maize shoots grown with and without 1 mM Si supply in nutrient solution. An ATP-regenerating system (5 mM PEP and 5 units of pyruvate kinase) was used to keep constant ATP concentration (5 mM). The values represent means ± SE of four independent experiments. Significant differences ($P < 5\%$) between treatments are indicated by different letters.

Figure 16 shows the *in vitro* effect of Si on plasma membrane ATPase hydrolytic activity of maize roots. A gradual decrease in hydrolytic activity was observed in both treatments but a decrease was less pronounced and non-significant in maize roots grown without Si supply in

nutrient solution. The maximum decrease 23% in hydrolytic activity was observed when 1000 µM of Si were added in the reaction mixture containing membrane vesicles isolated from plants supplied with Si in nutrient solution.

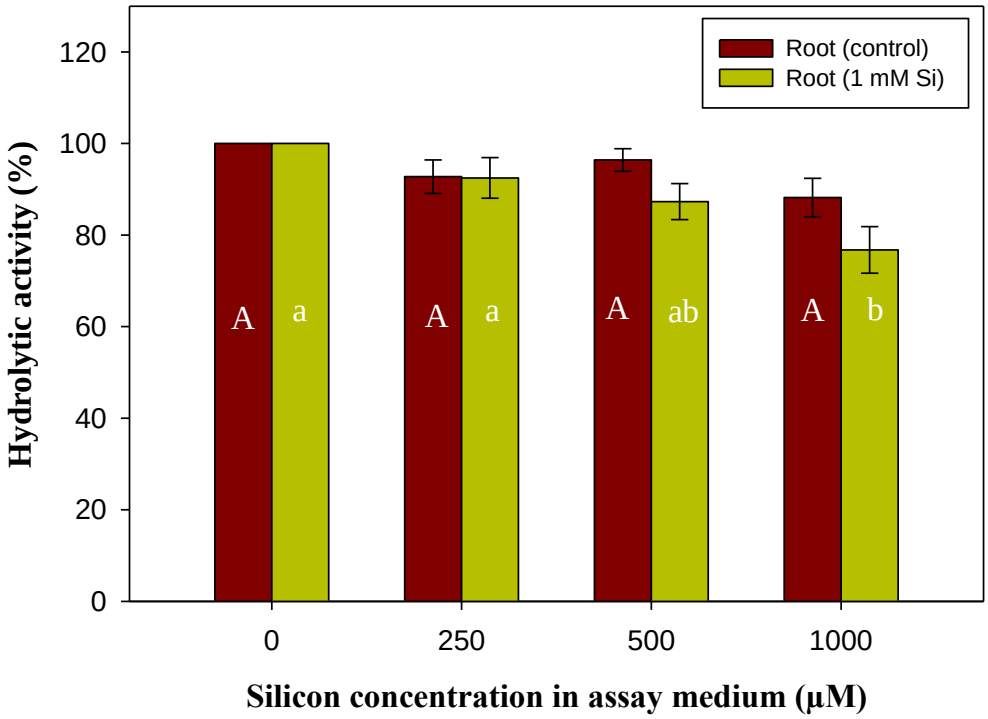

Figure 16: *In vitro* effect of various concentrations of Si on the hydrolytic activity of the plasma membrane ATPase. The activities measured for membrane vesicles without Si in the assay medium were considered as 100%. Plasma membrane vesicles were isolated from young growing maize roots grown with and without 1 mM Si supply in nutrient solution. An ATP-regenerating system (5 mM PEP and 5 units of pyruvate kinase) was used to keep constant ATP concentration (5 mM). The values represent means ± SE of four independent experiments. Significant differences (P < 5%) between treatments are indicated by different letters.

3.2.7 *In vitro* effect of silicon on plasma membrane H^+- ATPase transport activity

The plasma membrane ATPase H^+ transport activity was measured as described in Materials and Methods. The activity measured for membrane vesicles without Si addition in the

assay medium was considered as 100%. The active and passive transport activities of the membrane vesicles isolated from roots remained unchanged (Table 6). Shoot membrane vesicles showed a similar trend as the hydrolytic activity. Though the results were non-significant data clearly show that active transport of H^+ and passive efflux across the membrane was reduced only in membrane vesicles isolated from Si-treated shoots.

Table 6: *In vitro* effect of Si (1000 µM) on relative H^+-transport activities of the plasma membrane ATPase. The activities measured without Si in the assay medium was considered as 100%. Plasma-membrane vesicles were isolated from young growing maize shoots and roots grown with and without 1 mM Si supply in nutrient solution. Values represent means ± SE of four independent experiments.

Si in nutrient solution/ Plant Part	Si in assay medium (µM)	pH gradient (%)	Initial rate (%)	Passive efflux (%)
Shoot (control)	0	100.0	100.0	100.0
	1000	100.9 ± 17.3	105.5 ± 24.2	96.2 ± 21.4
Shoot (1 mM Si)	0	100.0	100.0	100.0
	1000	84.5 ± 7.6	75.0 ± 12.1	87.7 ± 10.5
Root (control)	0	100.0	100.0	100.0
	1000	99.1 ± 12.6	96.5 ± 17.4	86.5 ± 17.8
Root (1 mM Si)	0	100.0	100.0	100.0
	1000	99.9 ± 9.7	101.7 ± 15.1	96.0 ± 16.9

3.2.8 *In vitro* effect of silicon on plasma membrane ATPase kinetic characteristics and activation energy

The membranes vesicles isolated from maize shoot supplied with 1 mM Si in nutrient solution were treated with and without 1000 µM Si (silicic acid) in the assay medium. Enzyme hydrolytic activity was determined at various ATP concentrations in the presence of 1000 µM Si in an assay medium. The results show that the presence of Si in the assay medium significantly reduced the V_{max} while the enzyme affinity towards substrate remained unchanged. Furthermore,

the results show that the presence of Si in the assay medium slightly increased the activation energy from 65 to 77 kJ mol^{-1} of plasma membrane ATPase. A temperature increase from 25 to 30°C resulted in 1.6 and 1.7 times increase in V_{max} of enzyme treated without and with Si in the assay medium.

Table 7: *In vitro* effect of 1000 µM Si (silicic acid) on the kinetic characteristics of plasma membrane ATPase isolated from maize shoots. The plasma membrane vesicles were isolated from maize plants grown with 1mM Si supply in nutrient solution. The enzyme activity was measured at various ATP concentrations ranging from 50 to 5000 µM at 25 and 30°C. An ATP-regenerating system (5 mM PEP and 5 units of pyruvate kinase) was used to keep constant ATP concentrations. The values represent means ± SE of four independent experiments. Significant differences (P < 5%) between treatments are indicated by different letters.

Si in assay medium	**Assay temperature**	K_m (µM ATP)	V_{max} (µmol P_i mg^{-1} min^{-1})
0 µM Si	25°C	412.3 ± 104.3 a	0.34 ± 0.02 a
1000 µM Si	25°C	533.6 ± 146.4 a	0.27 ± 0.01 b
0 µM Si	30°C	373.1 ± 54.8 A	0.52 ± 0.03 A
1000 µM Si	30°C	343.2 ± 35.5 A	0.45 ± 0.01 A

3.2.9 Effects of silicon supply in nutrient solution on plasma membrane H^+-ATPase concentration in the plasma membrane vesicles isolated from maize shoots and roots

To determine the effect of Si supply on plasma membrane H^+-ATPase enzyme concentrations, vesicles isolated from maize shoots and roots were analyzed with western blot. Plasma-membrane proteins were separated with SDS-PAGE on 10% (w/v) acrylamide gel. A polyclonal antibody raised against the central part of plasma membrane H^+-ATPase was used to detect the differences in enzyme concentrations. For a quantitative comparison, the intensity and area of signals was measured by setting control as 100% in four independent experiments. In comparison to control, Si increased the enzyme concentration by 177 ± 28% for shoots, and 116 ± 20% for roots, respectively (Figure 17). However, in interpreting the results caution should be taken as the the antibody may not recognize all the ATPase isoforms (Wu and Seliskar 1998; Sibole et al., 2005). There is evidence showing that the different antibodies raised against H^+-ATPase showed different reactivity to H^+-ATPase (Arend et al., 2004; Sibole et al., 2005).

	Silicon treatment in nutrient solution/Plant part			
	Shoot (control)	**Shoot (1 mM Si)**	**Root (control)**	**Root (1 mM Si)**
100 kDa				
97 kDa	100%	177 ± 28%	100%	116 ± 21%

Figure 17: Immuno-detection of plasma-membrane H^+-ATPase isolated from maize shoots and roots. The plasma-membrane vesicles were isolated from young growing shoots and roots from plants grown with and without 1 mM Si supply in nutrient solution. The values are means four replicates ± SE.

3.2.10 Relative expression of mRNA of plasma membrane H^+-ATPase enzyme isoforms

The possible transcriptional induction of the plasma membrane H^+-ATPase genes by Si supply in nutrient solution was investigated on the basis of purified poly-(A) RNA by using the qPCR technique. The comparative C_t (threshold cycles) method of relative quantification was used to analyze the qPCR data. The C_t values were normalized by comparison with the

endogenous reference gene actin. Data are presented as relative change in expression of different isoforms in Si-treated plants with respect to control (100%).

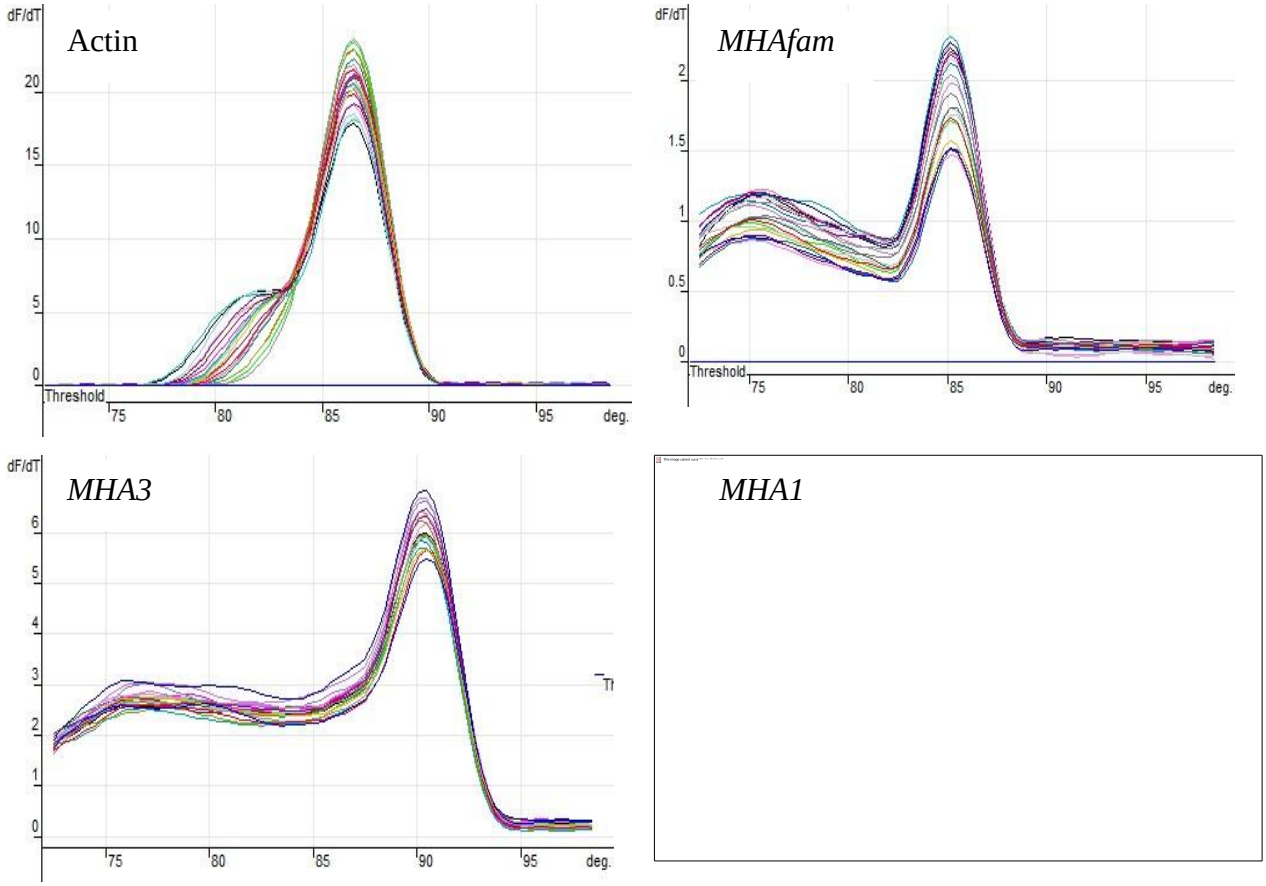

Figure 18: DNA melting profiles shown as PCR products. Melting curves were obtained in a LightCycler using SYBR Green fluorescence (dF/dT) versus temperature (°C). Single peak in the melting curve shows that no contaminating products are present in this reaction. Contaminating DNA or primer dimers would show up as an additional peak separate from the desired amplicon peak.

In general, plants supplied with Si in nutrient solution showed reduced transcription of *MHAfam* gene and *MHA3*. The changes in transcription level of plasma membrane H^+-ATPase mRNA were minute and are not significant in roots tissues (Figure 19 B). However, the transcription of *MHA3* decreased significantly and *MHAfam* also showed a similar decreasing trend in shoot tissues (Figure 19 A). The transcription level of *MHA1* was remained un-changed both in roots and shoots.

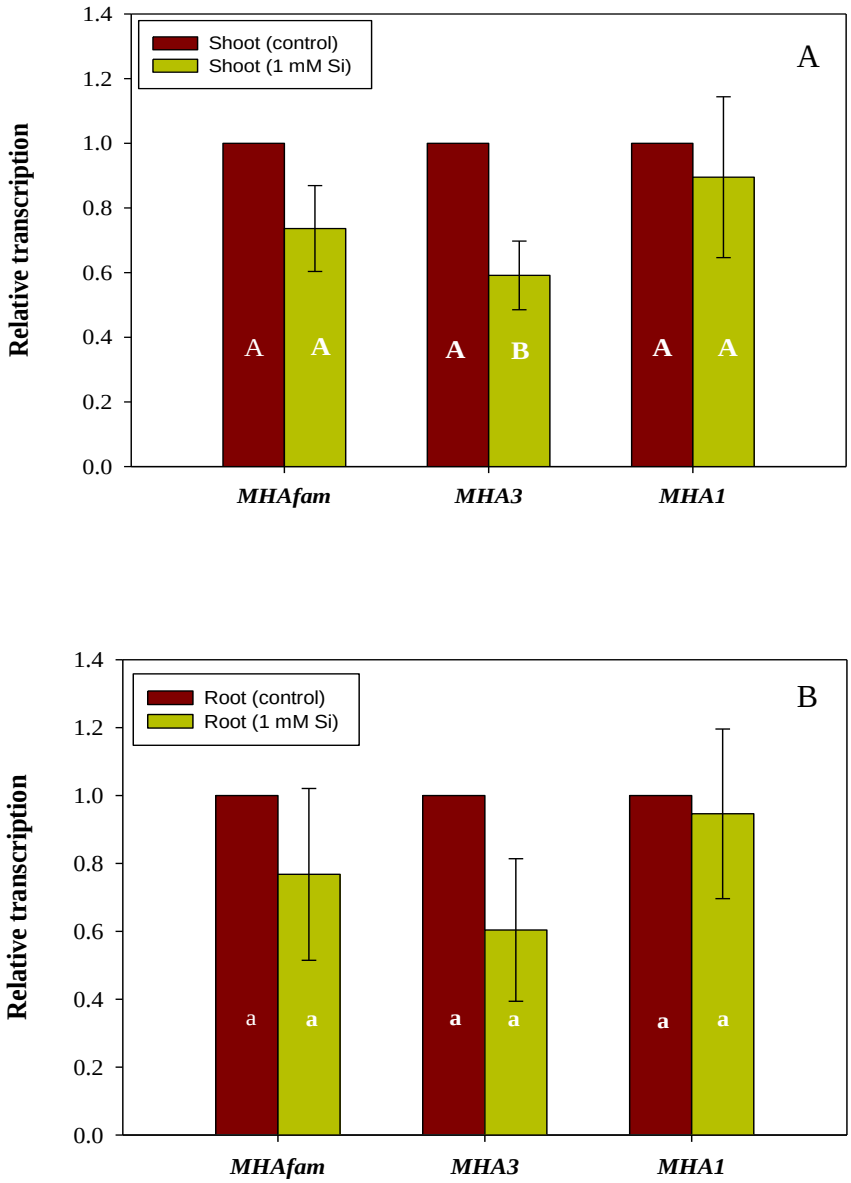

Figure 19: Relative change in transcription of plasma membrane H^+-ATPases (*MHAfam*) and specific isoforms (*MHA1* and *MHA3*) from maize young shoots (A) and roots (B). Plants were grown with and without 1 mM Si supply in nutrient solution for 23 d. RNA was isolated from young shoots and roots and used to prepare cDNA that was further used as a template for real-time PCR. SYBR Green was used as fluorescence probe in RotorGene cycler. Relative expression intensity was calculated using the Pfaffl-equation in comparison to the actin as internal control. Values are the means of four replicates ± SE.

3.3 Effect of silicon supply in nutrient solution on apoplastic pH in growing shoot tissues

To caliberate the system for measuring the apoplastic pH, different pH buffered dye (FITC-Dextran) standards were used. The images showed that absorbance clearly depend on pH, being lowest at pH 5.5 and highest at pH 7.0. While, the concentrations effect of the florescence dye on absorbance were eliminated by using the dual excitation technique (440/490 mm wavelengths).

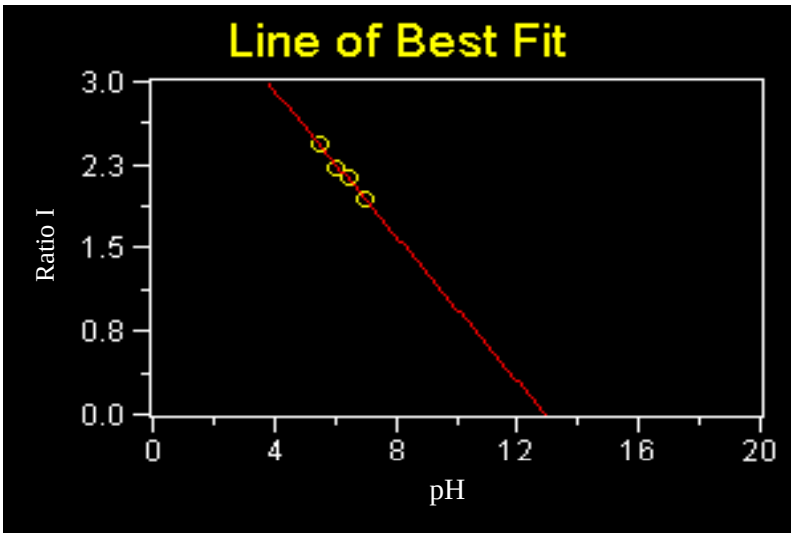

Figure 20: *In vivo*-calibration of maize leaves infiltrated with FITC-Dextran after excitation at 490 and 440 nm. The graph present calculated fluorescence ratio (440/490), which can be allocated to different pH values.

To assess the relevance of plasma-membrane H^+-ATPase pumping activity with the physiological pH, the apoplastic pH in growing tissue of maize shoots grown with and without 1 mM Si supply was measured. The leaf segments were infiltrated with FITC-dextran and apoplastic pH was measured. For the exact measurement of apoplastic pH it is very important that the fluorescence dye should not penetrate into the cytosol. The images confirmed that the dye accumulated selectively in cell-walls of maize leaves. The stomatal cavity showed higher pH and fluorescent intensities than epidermal cells (Figure 21) due to the infiltration process. The

ratio obtained for apoplastic pH of the two treatments did not show any difference and comparable values for the apoplastic pH were calculated. From these results it can be confirmed that *In vivo* supplied Si did not have any effect on plasma membrane H^+-ATPase activity and apoplastic pH (Figure 22).

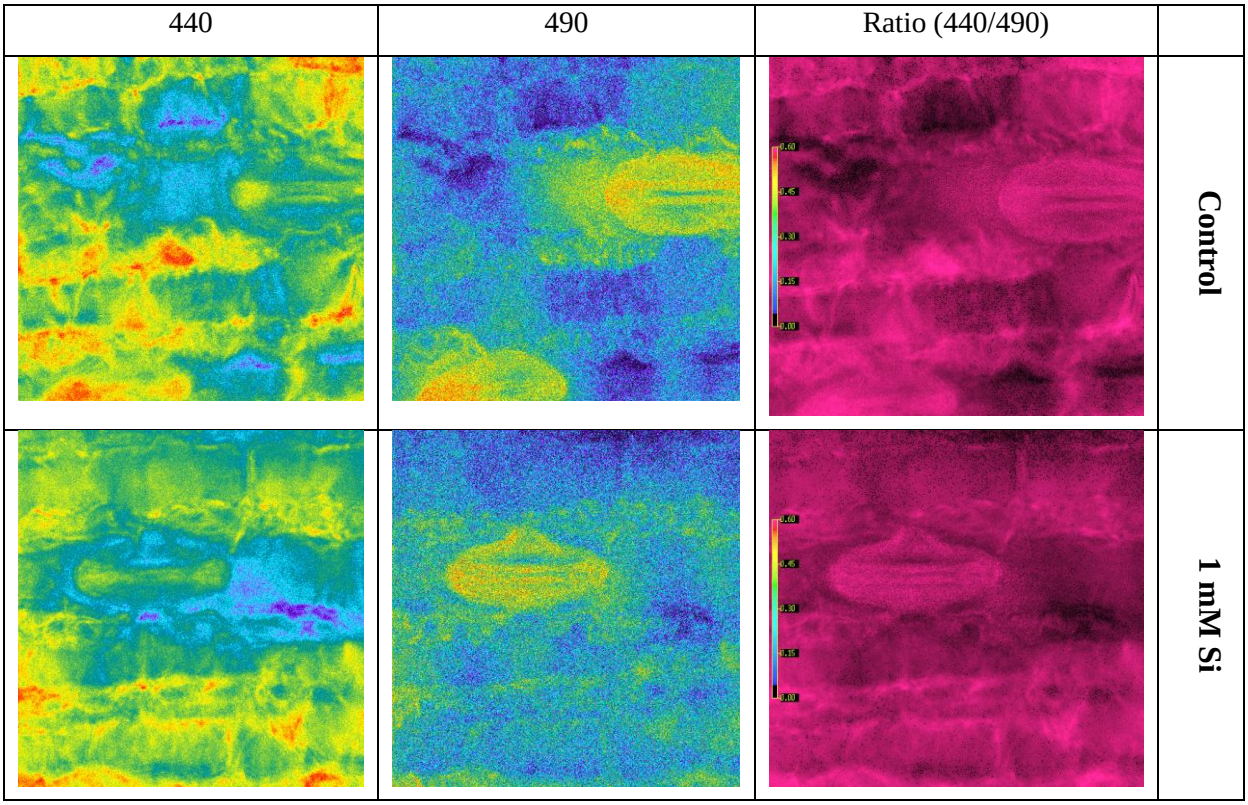

Figure 21: Fluorescent images of intact maize leaves after infiltration with FITC-Dextran. The images were taken with a 20× objective (Leica pH 1; 20×/0.40). The dye (FITC-Dextran, 20 mM) was infiltrated into the leaf apoplast by the vacuum-infiltration technique and the apoplastic pH was calculated by *In vivo*-calibration according to Mühling et al., (1995).

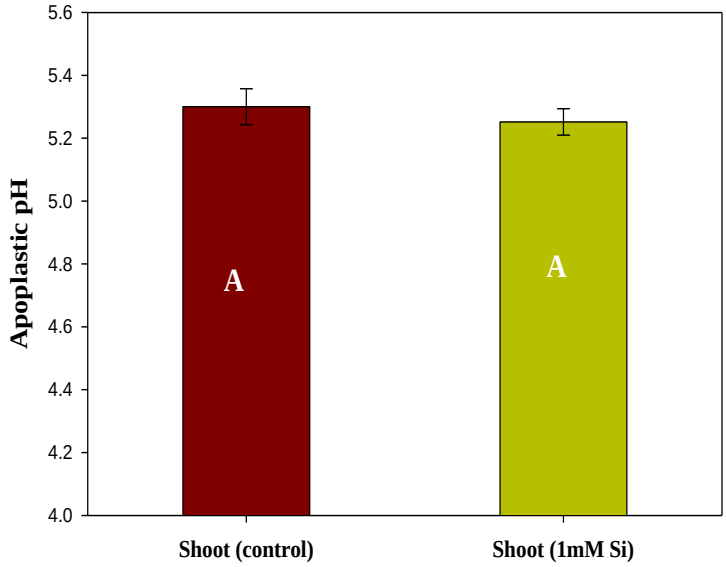

Figure 22: Effect of 1 mM Si supply in nutrient solution on the apoplastic pH of young expanding maize leaves. The leaf discs were infiltrated with the pH-sensitive FITC-Dextran and the images were taken with an inverse microscope (Leica DM IRB, Solms, Germany) connected to a high sensitive CCD-camera (CoolSNAP, Photometrics, Tucson, Arizona, USA). Images were processed and carried out with the Meta Fluor imaging system (Visitron, Puchheim, Germany) using the program Meta Series (Vers. 6.2). The values represent means ± S.E. (n = 12) of four independent experiments.

4 Discussion

4.1. Exogenously supplied silicon in nutrient solution improves maize growth

Silicon (Si) is the second most abundant element in earth crust and is considered as a non-essential beneficial element for growth of higher plants. Of all the non-essential elements taken up by plants, Si alone is consistently present at concentrations similar to those of some other macro-nutrients. However, there is very limited evidence available in literature that shows that Si may fulfill the criteria of essentiality (Arnon and Stout, 1939). It is rather established that most of the plants grown in solution culture without Si can complete their life cycle. However, this conclusion may not hold true because Si is ubiquitous in the environment as a contaminant and it is almost impossible to create conditions free of Si. Additionally, there is no such evidence that shows that Si is a part of some plant essential constituent or metabolite (Epstein, 1999). Among plant physiologists, there is no consensus about the direct metabolic role of Si. Recently, efforts have been made to identify the metabolic role of Si in plants at a molecular level. Fauteux et al. (2006) showed that Si supply to *Arabidopsis* changed the expression of only two out of more than 40,000 genes. From these results, the authors concluded that Si alone has apparently no effect on the metabolism of plants growing in a controlled environment. In contrast, Chain et al. (2009) and Brunings et al. (2009) reported that Si nutrition changed the expression of 47 and 221 genes in wheat and rice, respectively.

Generally, a direct metabolic role of Si in plant physiology is neglected since the ability to benefit from Si is not a universal characteristic of all plant species under different environmental and experimental conditions. Moreover, the beneficial effects of Si are more obvious under various biotic and abiotic stresses (Weise et al., 2005; Saqib et al., 2008). The non-consistent response of different plant species to additionally supplied Si may be due to inter and intra species differences in Si requirements. The direct beneficial effects of Si are observed in crop species that require more Si such as rice (Ma et al., 1989; Hossain et al., 2002), sugarcane (Datnoff et al., 2000), oat (Hossain et al., 2002), maize, and cucumber (Lewin and Reimann, 1969; Epstein, 1994; Shen et al., 2010).

As shown in this study, exogenously supplied Si in nutrient solution promoted plant growth at all concentrations. However, the maximum increase in growth attributes was observed at 0.8 - 1.2 mM Si (Figure 3). Silicon did not only increase plant biomass but also expansion growth characterized as plant height and leaf area of the young growing leaves was positively influenced. In line with these results, a number of studies showed that Si is a beneficial element and can increase the plant biomass of various plant species such as maize (Vaculík et al., 2009), rice (Alvarez and Datnoff, 2001), barley (Liang et al., 1997), cucumber (Zhu et al., 2004), cow pea (Mali and Aery 2009) and alfalfa (Guo et al., 2006). The beneficial effects of Si were more pronounced in young growing plant parts such as young expanding leaves. These results are consistent with the finding that the exogenously supplied Si promoted the growth of only young growing plant tissues such as the basal leaf part and apical and sub-apical zones in roots (Hossain et al., 2002; Hattori et al., 2003; Hossain at al., 2007). The possible reasons for the specific beneficial effects of Si on young growing tissues may be due to the depletion of endogenous Si of seeds, and Si present in nutrient solution as a contaminant may not be enough to maintain growth. As Si is immobile in plant tissues so a lack of movement from old to young tissues may be another possibility for the specific effect of exogenous Si on young tissues.

Various authors proposed different theories how Si can contribute to plant growth processes. Some studies suggested that additionally supplied Si increases plant growth by changing the morphological and biochemical characteristics of plants (Epstein, 1994). In this context, Si changed the morphology of leaves in terms of leaf area and leaf thickness (Epstein, 1999; Isa et al., 2010). Silicon increased the growth and yield of cucumber by increasing the rigidity of mature leaves which have a rough and tough texture and are held horizontally to intercept more light (Miyake and Takahashi, 1983; Adatia and Besford, 1986). Moreover, silicification of cell-walls requires only a fraction of energy in comparison to organic carbohydrate or lignin incorporation (Raven, 1983). In plants, the deposition of Si in cell-walls requires only 3.7 and 6.7% of the metabolic energy required for lignin and carbohydrate synthesis and incorporation, respectively (Raven, 1983; Epstein, 1994). Additionally, deposition of Si in leaves and Si-mediated increase in phenol and anthocyanin concentration act as a solar

screen and may protect the leaf tissues from damage by ultraviolet radiations (Goto et al., 2003; Shen et al., 2010).

Kaufman et al. (1979) proposed a "window hypothesis" for Si by suggesting that Si in form of silica bodies deposited in leaf epidermal cells could act as a "window" that enhances the light-use efficiency by facilitating the transmission of light to the photosynthetic active mesophyll tissues. Silicon supply to the nutrient solution also increased the net CO_2 fixation capacity in rice plants (Ma et al., 2002). Similarly, Adatia and Besford (1986) showed that cucumber plants supplied with Si showed 31 and 50% higher Rubisco activity on fresh weight and per unit area basis, respectively compared to the control treatment. Moreover, Si treatment increased the chlorophyll concentrations and also delayed leaf senescence that further contributed to CO_2 fixation. There are also studies that contradict the role of Si in enhancing photosynthetic activity. Silicon treatment to healthy unstressed tomato and maize plants induced minimal effect on photosynthesis or chlorophyll fluorescence (Al-aghabary et al., 2005; Hattori et al., 2005). The inconsistency concerning the beneficial effects of Si on photosynthesis may be due to differences in experimental conditions, plant species or genotype, and presence of Si in nutrient medium as a contaminant.

However, various authors proposed that Si plays some role in plant biology by maintaining the internal balance of the other mineral nutrients (Marschner et al., 1990). Silicon nutrition can affect the carbon and nitrogen metabolism by changing the amino acid composition and amount (Watanabe et al., 2001). Ma and Takahashi (1990) reported that Si application increased plant growth by increasing phosphorus metabolism within the plant. Plants deficient in Si have higher inorganic P compared to the control treatments. They also suggested that Si changes the precipitation of P together with Fe and Mn in plant tissues. Okuda and Takahashi (1962) reported that Si promoted the oxidation power of roots and thus decrease the availability and uptake of Fe and Mn. Results from the present study revealed that Si supply in root medium decreased Mn and Fe concentrations in plant tissues (Figure 8B and Figure 9B). Silicon supply in the root medium can affect the cation uptake in two ways: (1) a change in the solution chemistry and (2) in-planta mechanisms (Cocker et al., 1998b). Complexation of metal cation directly with

Si (Ma et al., 1997) in nutrient solution and Si-mediated release of phenolics contributed to the decreased availability of Al to plants (Kidd et al., 2001). The in-planta mechanisms such as co-precipitation of Si with metal cations in roots (Neumann and Nieden, 2001), reduced translocation from root to shoot (Shi et al., 2005) and compartmentation within the plant cells (Liang et al., 2007), can reduce the physiological availability of the metal cations. Lux et al. (2002) showed by using X-ray microanalysis coupled with environmental scanning electron microscopy that Si was mainly deposited at the endodermis of the roots. Moreover, Shi et al. (2005) found by using fluorescent tracers that Si was mainly deposited in the cell-walls of the endodermis but not of the epidermis in roots. The endodermis blocks the apoplastic movement of the mineral nutrients and facilitates their selective uptake by plant roots. Silicon deposition in the endodermis may contribute to the reduction of apoplastic transport of mineral cations and to a reduced uptake of various cations such as Ca and Na. It has been suggested that Si-induced decrease in Ca and Na concentration may be due to the reduced apoplastic transpirational bypass flow (Ma and Takahashi, 1993; Liang 1999; Yeo et al., 1999; Gong et al., 2006). Calcium is an important constituent of the cell-wall where it stabilizes the wall through the formation of ionic bonds with pectin (Hepler, 2005). Therefore, it was suggested that a Si-mediated decrease in Ca concentration may have contributed to increased shoot growth of rice (Ma and Takahashi, 1993). Furthermore, Si supply to the nutrient solution also prevents the phosphorus-induced leaf chlorosis by increasing the physiological availability of Zn under limited Zn supply (Marschner et al., 1990). Taken together, all these results suggest that Si plays an active role in plant physiology and takes part in a number of physiological processes that improve plant growth.

Silicon taken up by the root is readily transported to the stem and leaf tissues. The Si concentration in different plant species varies (Hodson et al., 2005) depending on the uptake ability of the roots (Tami and Ma, 2003). There is co-existence of Si passive and active components of transport in plants. It is a general characteristic of graminaceous plants that when supplied with Si they tend to accumulate Si in their shoot tissues (Yamaji et al., 2008). Recently, Si transporters were identified in rice, barley, maize and pumpkin that control the uptake and distribution of Si in plants (Ma et al., 2006; 2007; Mitani et al., 2009; 2011). Silicon influx transporters (ZmLsi1 and ZmLsi6) belonging to the aquaporin family have been characterized in

both maize roots and shoots (Mitani et al., 2009a; 2009b). On the basis of localization and expression patterns of these transporters, it was concluded that ZmLsi1 is responsible for the uptake of Si from external solution to the root cells, while ZmLsi6 is mainly involved in xylem unloading of Si into leaf sheaths and leaf blades (Mitani et al., 2009a). In contrast to Si influx transporters, an efflux transporter (ZmLsi2) is a putative anion transporter (Mitani et al., 2009b) localized only at the endodermis of maize roots. It is assumed that ZmLsi2 works together with *ZmLSi1* and transports Si from external solution to the root stele.

Once Si is transported to the leaf sheath and leaf blade, it is unloaded from xylem sap by Lsi6 and Si concentration starts to increase due to transpirational losses of water. In aqueous solutions, solubility of silicic acid at 25°C is 2 mM, and when the concentration exceeds 2 mM, it starts to polymerize into colloidal form and subsequently into silica gels. It has been observed that more than 90% of the total silica in plants is present as silica gel, while the colloidal and monomeric silica were in the range of 0.3 - 0.5 mg SiO_2 g^{-1} fresh weight of rice and cucumber shoots (Ma et al., 2002). There is some evidence about the chemical interaction between Si and cell-wall constituents in land plants. It was suggested that Si may be combined with phenolics (Inanaga and Okasaka, 1995), complex carbohydrates, and proteins (Ishii and Matsunaga, 2008) in the cell-wall of rice leaves.

Unfortunately, there is limited information available about the critical concentrations of Si in crop plants. Only for rice an optimum concentration of Si is suggested in a range of 50-80 mg g^{-1} dry weight (Dobermann and Fairhurst, 2000). Korndorfer et al. (2001) established that Si gives an economic response when its concentration in rice exceeds 34 mg g^{-1} grown in field conditions. In the present study, maize plants showed a significant increase in plant growth and growth attributes when the concentrations of Si was in the range of 4 - 8 mg g^{-1} dry matter in maize shoots (Figure 10).

A number of methods have been proposed for the determination of Si in plant tissues, (Elliot and Snyder, 1991; Haysom and Ostatek-Boczynski, 2006; Masson et al., 2007; Guntzer et al., 2010). The most common and widely used method is the autoclave-induced digestion of

plant material and subsequent determination of Si by the molybdenum blue method (Elliott and Snyder, 1991). This autoclave-induced digestion is relatively rapid and cost-effective as it does not need any specialized instrumentation. However, results obtained by this method are highly variable and inconsistent (Taber et al., 2002; Haysom and Ostatek-Boczynski, 2006). Generally, the method used for the digestion of Si-containing plant material produces excessive foaming that could cause the variability. To overcome this problem, a microwave-assisted system with closed Teflon® vessels under high temperature and pressure was used in order to get accurate and reliable results (Haysom and Ostatek-Boczynski, 2006; Ostatek-Boczynski and Haysom, 1993). Since 90% of the Si in plant shoots are in the form of silica gels or amorphous Si, the solubilization of the amorphous Si was done by a two-step digestion. The determination of Si with molybdenum blue may not be suitable due to its unspecificity. Here it was shown that atomic absorption spectrometry can also be used as a cheap and reliable alternative to ICP for Si determination.

4.2. Effect of silicon on plasma membrane ATPase in maize shoots and roots

The experiments were performed to investigate the effect of Si supply to the nutrient solution on plasma membrane H^+-ATPase activity in maize shoots and roots. The objective of the experiments was to investigate the mechanisms lying behind the Si-mediated enhanced maize growth. Increased plant growth is a result of cell division and irreversible cell elongation (Hager et al., 1971). The plastic growth of a plant cell is the result of increased turgor pressure, loosening of the existing cell-wall, and synthesis and incorporation of new cell-wall components into the existing cell-wall matrix (Cosgrove, 2005). A number of studies showed that Si accumulation had no effect on leaf water potential (Agarie et al., 1998; Hattori et al., 2007; Pei et al., 2010; Chen et al., 2011). This is due to the fact that most of the Si in leaf tissue is in polymerized form as a silica gel that does not take part in turgor build-up. Cell-wall extensibility is another important factor contributing to expansion growth. Hossain et al. (2002) showed that Si supply to the nutrient solution increased the leaf expansion in rice, wheat and oat. Moreover, they also showed that Si-mediated increase in leaf length was not due to an increase in the numbers of cells but an increase of cell size. On the basis of these observations it was suggested

that increased shoot growth was due to a change in cell-wall extensibility. According to the acid growth theory, auxin-induced acidification of the apoplast is a pre-requisite for cell-wall extensibility (Rayle and Cleland, 1970; 1992; Cleland, 1977). Plasma membrane H^+-ATPase is one of the targets in auxin signaling cascade. Unfortunately, there is very little evidence that correlates the Si-induced increase of plant growth with the plasma membrane H^+-ATPase. Liang et al. (2006) showed that Si treatment had no effect on the hydrolytic activity of plasma membrane H^+-ATPase isolated from barley shoots. However, they determined only hydrolytic activity while H^+-pumping activity as a main contributory parameter of the enzyme in cell-wall acidification was not determined. It was hypothesized that the Si-mediated increase in maize growth was caused by an increase of the plasma membrane H^+-ATPase activity. To test this hypothesis the plasma membrane ATPase in vesicles isolated from maize shoots grown with or without Si supply in nutrient solution were characterized.

4.2.1 Effect of silicon supply in nutrient solution on the purity of plasma isolated from maize shoots and roots

To study the plasma membrane H^+-ATPase characteristics, isolated membrane vesicles are a useful tool. For the isolation of plasma membrane vesicles, two-phase partitioning (Larsson et al., 1985; Yan et al., 1998; 2002) has proved to be a good method as it gives plasma membrane vesicles virtually free of contaminating other membranes. In this study, the results (Table 3) show that Si supply in nutrient solution did not affect the purity of membrane vesicles. The membrane fractions isolated from Si-treated or non-treated maize shoots showed a similar sensitivity to the specific P-type ATPase inhibitor vanadate (O'Neill and Spanwick, 1984). The membrane vesicles showed only 9-13% activity in the presence of 100 µM vanadate and showed almost no sensitivity to nitrate and azide. Furthermore, it was observed that the presence of molybdate in the assay medium inhibited 5-11% of the ATPase hydrolytic activity. The molybdate-sensitive activity is due to the presence of non-specific acid phosphatases in plasma membrane fractions (Yan et al., 2002; Briskin and Poole, 1983). These soluble acid phosphatases are usually present in cytosol and vacuole (Butcher et al., 1977). During homogenization some of the phosphatases may be adsorbed to membrane surfaces or trapped within the membrane vesicles (Wang et al., 1995). Inclusion of 250 mM potassium iodide (KI) in the homogenization

buffer is suggested to remove these peripheral proteins (Briskin and Poole, 1983). Although KI was added to homogenization buffer, its effect remained unclear as contamination of non-specific acid phosphates was still detectable. In conclusion, it is clear from the results that the isolated membrane fractions were highly enriched with plasma membrane vesicles. The membrane fractions were free of mitochondrial, chloroplastic, and vacuolar membranes with little contamination of acid phosphatases.

4.2.2 Effect of silicon on plasma membrane ATPases in maize shoots

Plasma membrane ATPases isolated from maize shoots were characterized on the basis of ATP-hydrolyzing power and H^+ transport across the membrane vesicles. Changes in the enzyme characteristics were studied by determining the changes in properties of the enzymes such as substrate affinity, turnover number, ability to pump H^+ and maintenance of the pH gradient across the membrane vesicles, transcription of different isoforms and changes in the enzyme concentrations. The results show that Si supply in the root medium had no effect on the V_{max}, K_m (Table 5), initial rate of H^+ pumping, maximum pH gradient (Table 4), and hydrolytic activity (Figure 11). However, the membrane fractions isolated from Si-supplied shoots showed 77% more enzyme protein. Increased concentration of the enzyme protein in membrane fractions of the Si-treated plants may by be due to higher transcription of plasma membrane H^+-ATPase and increased synthesis of membrane protein, or may be due to lower degradation rate of the enzyme. It has been reported that auxin treatment increases the H^+-ATPase protein in plasma membrane by increasing the membrane flow from endoplasmic reticulum to plasma membranes (Hager et al., 1991) and induces the higher H^+-ATPase mRNA transcription (Frias et al., 1996). Nevertheless, caution should be taken in interpreting the results of H^+-ATPase protein concentrations. Although the antibody used in this study was raised against the most conserved central part of the plasma membrane H^+-ATPase there might be some proteinogenic alterations that circumvent detection of some isoforms. It has been shown in some earlier studies that plasma-membrane vesicles from the same preparation showed different reactivity to two different antibodies raised against plant plasma membrane H^+-ATPases (Arend et al., 2004).

The discrepancy between the concentration of plasma membrane H^+-ATPase and its activity in isolated membrane vesicles can be explained in two different ways: A) *In vitro*-measured activities of the enzyme may not give a true picture of the cytosolic environment, B) expression of different plasma membrane H^+-ATPase isoforms may have contributed to a decrease in enzyme activity. As plasma membrane ATPases are the major plasma membrane proteins and high ATP consumers they need a tight regulation to fulfill physiological functioning of the plant cells. It has been shown that phosphorylation of plasma membrane H^+-ATPase may activate or may deactivate the enzyme (Trofimova et al., 1997; Fuglsang et al 2007). On the other hand, dephosphorylation may also activate the enzyme (Desbrosses et al., 1998). It is therefore suggested that Si nutrition may have brought some changes in the enzyme conformations which affects the H^+-ATPase activity.

It is worth to describe the structure of plasma membrane H^+-ATPase before describing the role of Si in its activation. The plasma membrane H^+-ATPase contains ten trans-membrane helices and a large cytoplasmic domain. Functionally the cytoplasmic domain can be further sub-divided into four domains: (1) nucleotide-binding domain (that phosphorylates the aspartyl residues), (2) phosphorylation domain, (3) actuator domain (that dephosphorylates the aspartyl residue), and (4) regulatory domain (Fuglsang et al., 2011; Palmgren and Niessen, 2011). The regulatory domain consists of a carboxy-terminal (Palmgren et al., 1991; Portillo et al., 1989) and some part of NH_2-terminal (Ekberg et al., 2010) of the enzyme. There is a tight self-regulatory mechanism in H^+-ATPase activation, which is controlled by the auto-inhibitory function of the regulatory domain. The plasma membrane H^+-ATPase is activated by various stimuli that suppress the auto-inhibition by the regulatory domain. Phosphorylation of the penultimate threonine residue at the C-terminus of the plasma membrane H^+-ATPase and subsequent binding of the 14-3-3 protein detach the regulatory domain and change the enzyme from low activity to high activity state (Fugalsang et al., 1999; Maudoux et al., 2000). The auto-inhibitory function of the regulatory domain is further supported by observations that the proteolytic removal or truncation of some part of the C-terminal and/or N-terminal lead to an increased plasma membrane H^+-ATPase hydrolytic and pumping activity (Portillo et al., 1989; Palmgren et al., 1999; Ekberg et al., 2010). Therefore, it was hypothesized that Si plays a direct

role in enzyme activation by interacting and changing the confirmation of the enzyme. To test this hypothesis, we determined the activity of plasma membrane H^+-ATPase in the presence of Si in the assay medium.

In order to measure *in vitro* effects of Si on hydrolytic and pumping activities, silicic acid was added in the reaction medium. The results show that the presence of 1mM Si in the assay medium decreased the hydrolytic activity significantly by 14% (Figure 15) and the initial rate of H^+ pumping insignificantly by 24% (Table 6). Moreover, Si in the assay medium had an only effect on the plasma membrane vesicles isolated from Si-supplied shoots. The inhibitory effect of Si on plasma membrane ATPase activity can be explained by a possible binding of Si with the enzyme. The binding site in question may be usually necessary for the phosphorylation/dephosphorylation and subsequent activation of the plasma membrane ATPase. Numerous studies showed that there is more than one phosphorylation site at the C-terminal domain of the H^+-ATPase (Nuhse et al., 2003; 2007; Whiteman et al., 2008) that can affect the enzyme activity. For example, the protein kinase *PKS5* inhibits the plasma membrane ATPase activity by inducing the phosphorylation of the Ser931 residue of the *Arabidopsis* H^+-ATPase (AHA_2). Phosphorylation of Ser931 prevents the binding of the 14-3-3 proteins (Fugalsang et al., 2007; Duby et al., 2009) and kept the enzyme at low activity state. From these results it is suggested that Si (silicate) may interact with some of the binding sites specific for the phosphorylation in the regulatory as well as in the phosphorylation domain of the plasma membrane H^+-ATPase. Furthermore, it was found that Si in assay medium not only decreased the enzyme activity but also changed the coupling ratio of ATP hydrolysis and H^+ pumping.

To get deeper insight in Si-mediated decrease in hydrolytic activity after addition to the assay medium, kinetic characteristics of the enzyme were determined. The results show that silicic acid presence in the assay medium did not have any effect on K_m but significantly decreased the V_{max} at 25°C (Table 7). From these results it can be suggested that Si in assay medium did not have any effect on kinase function of the ATPase while the phosphatase function of the enzyme was somehow inhibited. Umemura et al. (1961) reported that the addition of Si to the reaction mixture containing acid phosphatases isolated from rice leaves and potato tubers

reduced the enzyme activities. On the other hand, they also showed that Si did not have any effect on the activity of the yeast hexokinase. A decrease in reaction velocity (V_{max}) can be due to the expression of some of the H^+-ATPase isoforms that may have some specific binding sites for Si that may decrease the dephosphorylation.

The differential responses the plasma membrane H^+-ATPases isolated from Si-treated and control shoot such as raise the possibility of expression of different plasma membrane H^+-ATPase isoforms. The expressed isoforms vary from cell to cell and tissue to tissue depending on type, age, as well as environmental condition. Several reports showed that isoforms differ in their enzymatic characteristics such as affinity towards substrate, turnover rate, optimum pH, and sensitivity to vanadate (Palmgren and Christensen 1993; Luo et al., 1999). Comparison of three *Arabidopsis* isoforms namely *AHA1*, *AHA2*, and *AHA3* revealed that *AHA3* had a ten fold higher K_m value for ATP hydrolysis and three fold higher sensitivity to vanadate (Palmgren and Christensen, 1993). Simultaneous expression of more than one isoforms belonging to different subfamilies helps the plants to adapt to the environment.

Plant plasma membrane H^+-ATPases belong to a multigene family and are subdivided into five subfamilies on the basis of their amino acid sequence (Oufattole et al., 2000; Gaxiola et al., 2007). The cDNA library of tobacco, *Arabidopsis*, and rice showed at least 9-11 different H^+-ATPase isoforms (Moriau et al., 1999; Axelsen and Palmgren, 2001; Spernadio et al., 2010). In plant systems transcriptional regulation of plasma membrane H^+-ATPase is reported under NO_3/NH_4^+ nutrition (Zhu et al., 2009), aluminum toxicity (Shen et al., 2005), salt stress (Zörb et al., 2005); iron deficiency (Santi et al., 2005), and metabolizable sugar supply (Mito et al., 1996). Unlike in other plant species, in maize only four H^+-ATPase isoforms have been identified so far (Jin and Bennetzen, 1994; Frias et al., 1996; Santi et al., 2003). Among the isoforms identified, *MHA1* belongs to subfamily I while *MHA2*, *MHA3*, *MHA4* belong to subfamily II (Santi et al., 2003).

The transcriptional regulation of plasma membrane H^+-ATPases in young growing shoots tissues by Si treatment was determined by using isoform-specific primers (Santi et al., 2003;

Zörb et al., 2005). The results show that the relative transcription of *MHA3* was decreased in Si-supplied plants while *MHA1* remained unchanged. *MHA2* was not detectable while *MHA4* primer gave two peaks in qRT-PCR melt curves. Moreover, relative transcription of the H^+-ATPase family showed a similar trend as *MHA3*. These results are in line with the findings of Shahzad (personal comm.) and Fatima (personal comm.) since *MHA3* is a major contributor in expression of the *MHAfam* gene. Furthermore, the transcription to translation ratio showed a lower mRNA/MHA_{fam} protein in Si-treated shoots. The difference may be due to changes in enzyme stability caused by Si treatment. It is interesting that Si-treated shoot plasma-membrane vesicles having a higher enzyme concentration had the same hydrolytic and pumping activity compared to the vesicles isolated from plants grown without Si supply. Moreover, Si presence in the assay medium decreased hydrolytic activity in plasma membrane vesicles isolated from Si-supplied maize plants. These results suggest that *MHA3* can be classified as an efficient isoform (Shahzad, 2011; personal commun.). Some earlier studies showed that a change in the expression of one isoform can be compensated by down or up-regulation of other isoforms (Duby and Bounty, 2009). Therefore, it can be suggested that Si application in nutrient solution down-regulates an efficient H^+-ATPase isoform which is compensated by an increased expression of other unknown inefficient isoforms.

4.2.3 Effect of silicon supply in nutrient solution on plasma membrane ATPases in maize roots

The second objective of the experiments was to study the effect of Si supply in nutrient solution on root plasma membrane ATPase. The electrochemical H^+ gradient generated by plasma membrane H^+-ATPase is responsible for the uptake and transport of nutrients (Jahn et. al, 1998). Hundreds of proteins are involved in the uptake of cations, anions and neutral solutes, which are energized with the proton motive force (Palmgren, 2001). To date, many studies showed that the H^+-ATPase plays a key role in uptake and transport of nutrients such as potassium (Hoth et al., 1997; Kochian and Lucas, 1985), phosphorus (Yan et al., 2002; Shen et al., 2006), nitrogen (Santi et al., 1995; 2003), chlorine (Yamashita et al., 1996), iron (Dell'Orto et al., 2000; Santi et al., 2005), and zinc (Pinton et al., 1993). But so far, there is no such evidence regarding the interaction of Si uptake and transport with the plasma membrane H^+-ATPase.

In this study, results show that Si supply had no effect on plasma membrane H^+-ATPase hydrolytic activity. These results are in agreement with the observations of Liang et al. (1999; 2006) who showed that Si supplied to barley roots did not affect ATP hydrolysis of plasma membrane H^+-ATPase. Furthermore, results from the present experiments showed that plasma-membrane vesicles isolated from roots supplied with or without Si showed similar values for the initial rate of H^+ pumping, maximum pH gradient, and passive transport of H^+ across the membrane vesicles.

In vitro added silicic acid resulted in a gradual decrease in hydrolytic activity (Figure 16). The effects of Si in the assay medium were more pronounced only in those membrane vesicles that were isolated from Si-treated roots. *In vitro* addition of 1 mM Si in the assay medium significantly decrease (23%) the hydrolytic activity (Figure 16) while the initial rate of H^+ pumping remain unchanged. From these results it is suggested that Si presence in the assay medium improved H^+/ATP coupling ratios. These results are in contrast to the shoots membrane vesicles that showed a decrease in H^+/ATP coupling ratios. Therefore, it can be assumed that Si differentially regulated the plasma membrane H^+-ATPase in maize shoots and roots. Therefore, it is suggested that Si supply to the nutrient medium induced some specific changes in the membranes of Si-supplied roots. These changes may involve the structure of the enzyme that may have binding sites for Si and may also involve the transcription of specific H^+-ATPase isoforms. Silicon supply in nutrient solution had no effect on the H^+-ATPase protein concentrations in roots. Therefore, it can be suggested that a Si-induced decrease in hydrolytic activity of the Si-treated maize roots can be as a result of expression of some specific isoforms. The results show that Si treatment did not change the expression of *MHA1* while there was a slight down-regulation of *MHAfam* and *MHA3* (Figure 19B). These results further support the conclusion that Si nutrition can change the plant metabolic processes by changing the transcription and translation of H^+-ATPase isoforms.

4.3 Effect of silicon supply in nutrient solution on apoplastic pH in maize leaves

In accordance with the acid-growth theory, auxin-induced H^+ extrusion into the apoplast is responsible for expansion growth (Hager et al., 1971; Rayle et al., 1973; Jahn et al., 1996). A

lower apoplastic pH activates cell-wall-loosening proteins for example expansins (McQueen-Mason et al., 1992) and disfavors the conditions for peroxidases that play a critical role in cross-linking of the specific components of the cell-wall. Additionally, H^+ efflux into the apoplast hyperpolarizes the plasma membranes which facilitates the solute uptake and increases turgor inside the cell, hence creating conditions for the cell to expand.

The apoplastic pH of young expanding maize leaf segments was measured to determine the role of Si in expansion growth. The leaf segments of the Si-treated and non-treated plants showed a comparable apoplastic pH. While, in an earlier experiment plasma membrane isolated from shoot tissues supplied with Si in nutrient solution showed 77% more H^+-ATPase protein with respect to control treatment (Figure 17) . There is evidence that show that transgenic tobacco plants with four fold higher enzyme concentration (*PMA4*) compared to non-transgenic plants had the same apoplastic pH (Gévaudant et al., 2007). The author proposed that transgenic plants have some *in vivo* down-regulatory mechanism that keeps the plasma membrane H^+-ATPase in a low activity state. It is evident from various studies that the native plasma membrane H^+-ATPase is partially in an uncoupled state (Kinoshita and Shimazaki, 1999). Post translational modifications change the enzyme from inactive to an active state that subsequently can affect the H^+ pumped/ATP hydrolyzed (Baunsgaard et al., 1996). Venema and Palmgren (1995) proposed that H^+/ATP coupling ratio is not always the same for the ATPase rather it is flexible depending on the regulatory state of the H^+-ATPase.

Taken together, it can be suggested that in Si-treated shoot plasma membrane H^+-ATPases have an unknown self-regulatory system that kept the enzyme in a low-activity state. It is concluded that a Si-mediated increase in shoot growth was due to some factors other than apoplast acidification. The acid growth hypothesis is still controversially discussed as decreased apoplastic pH does not always induce the expansion growth (Grebe, 2005; 2006; Kutschera, 2006). Similarly, Keller et al. (1998) concluded that to some extent apoplastic acidification is required for tobacco leaf elongation, but most of the auxin-induced cell elongation does not involve an increase in cell-wall acidification. Moreover, several studies showed that plant expansion growth had no correlation with apoplastic pH change. The plants with distinct leaf

length and leaf elongation rates showed no difference in apoplastic pH in salt stressed and control plants (Neves-Piestun and Bernstein 2001). These results are further supported by the observations of Staal et al. (2011) who found that the fusicoccin-induced decrease in apoplastic pH was unable to change the length of root epidermal cells (trichoblast). Therefore, it is assumed that some other wall loosening processes that are independent of cell-wall acidification may contribute to expansion growth. Fry et al. (1992) showed that xyloglucan endotransglucosylases may be involved in wall loosening. These enzymes take part in breakage and reformation of glycosidic linkages among the polymers of the walls. It has been known that these enzymes are mostly active at pH 6 and are not thought to be involved in acid-induced wall loosening (Purugganan et al., 1997). Similarly, Pitann et al. (2009) and Wakeel et al. (2010) showed that salt stress reduced the growth of maize (SR03) and sugar beet, but did not change the apoplastic pH. The authors proposed that salt stress induces wall-stiffening processes that were responsible for the reduced expansion growth.

Cell-wall composition is another possible factor that can contribute to cell-wall extensibility. It was shown that exogenously supplied Si increases the cell-wall extensibility by decreasing the cellulose, ferulic and diferulic acid contents per unit length of the basal part of oat leaves (Hossain et al., 2007). The synthesis and subsequent incorporation of phenolic compounds into the cell-wall matrix strengthens the cell-wall and decreases the wall extensibility. Silicon supply in the nutrient medium decreased the activity of phenylalanine ammonia lyase (Carver et al., 1998) and tyrosine ammonia lyase activity (Hossain et al., 2007) that take part in the synthesis of phenolic compounds. Goto et al. (2003) showed that Si fertilization of rice resulted in a lower immuno-reactive cinamyl alcohol dehydrogenase (CAD) amount. CAD is a key enzyme involved in the biosynthesis of monolignol before it is incorporated into the cell-wall (Ma, 2010). A decrease in activity and amount of the enzymes involved in the synthesis and production of cell-wall cross-linking phenols can also be a possible factor for Si-mediated enhanced maize shoot growth. From these results it can be concluded that apoplast acidification can be one of the possible factors for elongation growth but is not the sole factor for cell expansion.

5 Summary

Silicon is the second most abundant element in the earth crust and all plants grown in soil contain some amount of Si in their body. Despite of the ubiquitous nature of Si in soil-plant systems the essentiality of Si is not yet proven for higher plants. The beneficial effects of Si are more pronounced in plants grown under various biotic and abiotic stresses. Unfortunately, there is little evidence that Si application takes part in some of the physiological and biochemical processes in plants. Some earlier studies suggested that Si can increase the expansion growth in various plant species by changing the cell-wall extensibility in young growing shoot and root tissues. Moreover, some studies also suggested that Si uptake and translocation are energy requiring processes and may require a proton gradient. Plasma membrane H^+-ATPase is a master enzyme and it extrudes H^+ out of the cytosol and creates an electrochemical H^+ gradient. The plasma membrane H^+-ATPase generated H^+ gradient is responsible for cell wall extensibility and expansion growth. Moreover, the electrochemical H^+ gradient energizes various proteins involved in nutrient and solute uptake and translocation. Although few attempts have been made to elucidate the role of Si in plant growth and cell-wall extensibility, mechanisms lying behind are not fully understood. Therefore, it was assumed that Si-enhanced plant growth and uptake and translocation of Si from nutrient solution may require increased plasma membrane H^+-ATPase activity.

To determine the effect of Si nutrition on maize growth and its relationship with the plasma membrane H^+-ATPase regulation the following hypotheses were tested: (1) Exogenously supplied Si in nutrient solution improves maize (*Zea mays L* cv. Amadeo) growth under normal growth conditions (2) Changes in plasma membrane H^+-ATPase are responsible for the Si-induced maize growth (3) Silicon-nutrition increases maize shoot growth by inducing the apoplast acidification.

The results are summarized as:

1. Silicon application in nutrient solution increased maize growth at all concentrations. The maximum increase in plant growth attributes was observed at 0.8 - 1.2 mM Si in nutrient

solution. Furthermore, the results showed that Si nutrition had a balancing effect on other mineral nutrients in maize plants. Silicon application in nutrient solution changed the concentrations of different cations in maize roots and shoots tissues especially Ca, Zn, Mn, and Fe.

2. Silicon nutrition changed the plasma membrane H^+-ATPase characteristics that were: (I) Plasma membrane vesicles isolated from Si-treated shoots had 77% more enzyme protein. (II) The plasma membrane H^+-ATPase isolated from Si-treated maize shoots showed inhibited hydrolytic and pumping activities by Si addition in the assay medium in comparison to the vesicles isolated from plants grown without Si. (III) Silicon nutrition induced differential transcription of plasma membrane H^+-ATPase isforms. The plants supplied with Si had reduced *MHA3* and *MHAfam* transcription. (IV) Similar to the shoots, Si nutrition brought some changes in the characteristics of plasma membrane H^+-ATPase in roots as well. The addition of 1 mM Si in the assay medium significantly reduced the hydrolytic activity of plasma membrane H^+-ATPase isolated from plants supplied with Si in nutrient solution.

3. Silicon-induced expansion growth in maize shoot showed no correlation with apoplastic pH. The plants grown with and without 1 mM Si supply in nutrient solution had a comparable value for their *in vivo*-measured apoplastic pH. Therefore, it is assumed that the Si-mediated increased shoot growth was due to unknown factors other than apoplastic pH.

6 Zusammenfassung

Silicium ist das zweithäufigste chemische Element der Erdkruste und somit ubiquitär im Boden vorhanden. Obwohl sich in allen im Boden wachsenden Pflanzen Si wiederfindet, konnte bisher nicht nachgewiesen werden, inwieweit Si als Nährstoff für die Pflanze essentiell ist. Vor allem unter biotischem und abiotischem Stress aber wird ein positiver Effekt einer Si-Ernährung auf das Pflanzenwachstum festgestellt. Leider gibt es aber bis heute kaum Belege dafür, dass Si an physiologischen und biochemischen Prozessen in der Pflanze beteiligt ist. Jedoch wurde in früheren Studien vorgeschlagen, dass Si das Streckungswachstum verschiedener Pflanzenarten verbessern kann, indem es die Zellwandextensibilität des jungen Spross- und Wurzelgewebes verändert. Darüber hinaus wird angenommen, dass sowohl die Aufnahme als auch die Translokation von Si Energie benötigt und durch einen Protonengradient angetrieben wird. Das Masterenzym, welches Protonen aus dem Cytosol der Zelle in die Zellwand pumpt und auf diese Weise einen elektrochemischen Protonengradient aufbaut, ist die Plasmalemma-H^+-ATPase. Der durch dieses Enzym generierte Protonengradient ist verantwortlich für die Zellwandextensibilität und das Streckungswachstum bei Pflanzen. Des Weiteren werden durch den Aufbau des elektrochemischen Protonengradienten verschiedene Proteine aktiviert, welche beispielsweise für die Aufnahme und Translokation von Pflanzennährstoffen notwendig sind. Obwohl über die Jahre bereits zahlreiche Versuche unternommen wurden, die Rolle von Si bei Pflanzenwachstum und Zellwandextensibilität zu beschreiben, sind die zugrunde liegenden Mechanismen bis heute nicht aufgeklärt. Vor diesem Hintergrund wurde in dieser Arbeit angenommen, dass das durch Si-verbesserte Pflanzenwachstum, sowie die Aufnahme und Translokation von Si aus dem Nährmedium in die Pflanze auf eine erhöhte Aktivität der Plasmalemma-H^+-ATPase zurückgeht.

Um den Einfluss von Si auf das Wachstum von Mais unter Berücksichtigung einer möglichen Regulation der Plasmalemma-H^+-ATPase zu untersuchen, wurden folgende Hypothesen aufgestellt: (1) Exogen appliziertes Si in der Nährlösung verbessert das Wachstum von Mais (*Zea mays* L.) unter normalen Wachstumsbedingungen. (2) Änderungen in der Aktivität der Plasmalemma-H^+-ATPase sind verantwortlich für das durch Si-induzierte

Wachstum von Mais. (3) Si-Ernährung verbessert das Sprosswachstum von Mais durch eine gesteigerte Zellwandansäuerung.

Die aus dieser Arbeit gewonnenen Ergebnisse lassen sich wie folgt zusammenfassen:

1. Die Applikation von Si zur Nährlösung verbessert das Wachstum von Mais unter allen angebotenen Si-Konzentrationen. Hierbei wurde der größte Wachstumszuwachs bei Si-Konzentrationen in der Nährlösung von 0,8 bis 1,2 mM festgestellt. Darüber hinaus zeigte sich, dass in Mais eine Si-Ernährung förderlich für das Gleichgewicht anderer Pflanzennährstoffe ist. So wurden durch die Zugabe von Si die Konzentrationen verschiedener Kationen wie Ca, Zn, Mn und Fe in Spross und Wurzel von Mais verändert.
2. Die Ernährung der Pflanze mit zusätzlichem Si veränderte auch die Eigenschaften der Plasmalemma-H^+-ATPase: (I) Aus dem Spross Si-ernährter Pflanzen isolierte Plasmamembran-Vesikel zeigten eine um 77% gesteigerte Proteinmenge. (II) Die Plasmalemma-H^+-ATPase Si-behandelter Pflanzen zeigte im Vergleich zu unbehandelten Pflanzen eine gehemmte hydrolytische und Pumpaktivität nach Zugabe von Si zum Analysemedium. (III) Die Zugabe von Si zum Nährmedium führte zu einer veränderten Transkription verschiedener Plasmalemma-H^+-ATPase-Isoformen. Insbesondere die Transkription der Isoformen *MHA3* und *MHAfam* war unter Si-Einfluss reduziert. (IV) Ähnlich wie im Spross, resultierte die Zugabe von 1 mM Si zum Analysemedium in einer signifikanten Abnahme der hydrolytischen Aktivität der Plasmalemma-H^+-ATPase in der Wurzel Si-behandelter Maispflanzen.
3. Das durch Si verbesserte Streckungswachstum des Maissprosses ist nicht auf eine verbesserte Zellwandansäuerung zurückzuführen. Sowohl Si-behandelte als auch unbehandelte Maispflanzen zeigten nach *in vivo*-Messung einen vergleichbaren apoplastischen pH. Hieraus kann abgeleitet werden, dass das durch Si verbesserte Sprosswachstum durch andere, noch unbekannte Faktoren bedingt wird.

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Hafiz Faiq Siddique Gul Bakhat

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