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RESEARCH PAPER

New evidence defining the evolutionary path of aquaporins regulating silicon uptake in land plants

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Abstract

Understanding the evolution events defining silicon (Si) uptake in plant species is important for the efficient exploration of Si-derived benefits. In the present study, Si accumulation was studied in 456 diverse plant species grown in uniform field conditions, and in a subset of 151 species grown under greenhouse conditions, allowing efficient comparison among the species. In addition, a systematic analysis of nodulin 26-like intrinsic proteins III (NIP-III), which form Si channels, was performed in >1000 species to trace their evolutionary path and link with Si accumulation. Significant variations in Si accumulation were observed among the plant species studied. For their part, species lacking NIP-IIIs systematically showed low Si accumulation. Interestingly, seven NIP-IIIs were identified in three moss species, namely *Physcomitrella patens*, *Andreaea rupestris,* and *Scouleria aquatica*, indicating that the evolution of NIP-IIIs dates back as early as 515 million years ago. These results were further supported from previous reports of Si deposition in moss fossils estimated to be from around the Ordovician era. The taxonomical distribution provided in the present study will be helpful for several other disciplines, such as palaeoecology and geology, that define the biogeochemical cycling of Si. In addition to the prediction of Si uptake potential of plant species based on sequence information and taxonomical positioning, the evolutionary path of the Si uptake mechanism described here will be helpful to understand the Si environment over the different eras of land plant evolution.

Keywords: Aquaporins, genomics, mosses, nodulin 26-like intrinsic proteins, plant evolution, silicon.

Introduction

Silicon (Si) plays an important role in plant biology by providing protection against many biotic and abiotic stresses in crop plants (Coskun *et al.*, 2019). While not considered a nutrient *per se* (Epstein, 2009), Si has recently been classified as a beneficial element by the International Plant Nutrition Institute (IPNI, www.ipni.net/nutrifacts-northamerican) on the basis of hundreds of reports describing its positive role. However, plant species do not respond similarly to Si because of their different ability to absorb the element, as a wide range of concentrations, from 0.1% to as high as 10% Si (DW basis), has been observed (Epstein, 1994). Initially, based on the concentrations measured, plants were categorized as low, intermediate, and high accumulators (Jones and Handreck, 1967). In general, there is a positive correlation between the ability of a species to absorb Si and the benefits it derives from the element, so it is important to properly assess the properties of a plant to take up Si. In this context, Hodson *et al.* (2005) conducted an exhaustive analysis of 735

plant species from 125 different studies and normalized the data based on measurements from at least two independent studies for each species, in order to classify plants according to their ability to accumulate Si. At the time when Si transporters had not yet been discovered, this dataset provided a valuable resource of phylogenetic distribution within the plant kingdom for Si accumulation. Even though the data normalization can sometimes be misleading considering the different conditions under which the experiments are conducted, the approach is convenient to determine variation due to genetic and environmental factors. More recently, Trembath-Reichert *et al.* (2015), using Hodson *et al.*'s dataset to normalize their own Si data from plant collections, drew conclusions from these renormalized data to establish a history of Si uptake in plants. As a result of this normalization, the study obtained overestimation or underestimation of Si in many species.

The discrepancy related to Si accumulation data in plants among different studies can be explained by many factors such as variable Si content in the growing medium, plant-available Si (PAS) in soil, age of the plant, Si quantification methods, etc. For instance, PAS in the growing medium or soil was rarely considered in the previous studies while comparing Si in plant tissues. The same plant species grown in different media or sampled at different ages can present large variations in Si content, such as a range of 0.2–3% observed in strawberry (Ouellette *et al.*, 2017). Therefore, to better categorize plant species in terms of their ability to accumulate Si, standard methods need to be implemented together with the integration of the molecular mechanisms involved in Si uptake by plants.

The seminal discoveries of Si transporters in rice by Ma *et al.* (2006, 2007) have laid the foundation to exploit the molecular mechanisms inherent to Si uptake to better understand how and what plants can accumulate the element. According to Ma *et al.* (2006, 2007), Si will enter the plant from the outside environment in the form of silicic acid through specific Si influx transporters (termed Lsi1), while efflux transporters (termed Lsi2) will translocate Si into the xylem which takes it through xylem flow to the aerial parts of the plants where it will deposit as amorphous Si (SiO_2). The Si influx proteins belong to the large family of aquaporins (AQPs) (Ma *et al.*, 2006). AQPs are a class of channel-forming proteins that facilitate the transport of water and many other small solutes across the cell membrane (Deshmukh *et al.*, 2016). They have a characteristic hourglass-like structure made up of six transmembrane (TM) domains, and two half TM helices protruding from opposite sides towards the center of the pore (Murata *et al.*, 2000). The two half TM helices form a constrict hosting two NPA motifs at the center of the pore. The pore forms another constrict, often referred to as a selectivity filter (SF), and is composed mostly of four amino acids. The amino acids at the SF are usually highly conserved and involved in the solute specificity of a given AQP. Interestingly, the phylogenetic distribution of Si influx transporters identified in different crop plants, including monocots and dicots, showed a specific clustering of all known Si influx transporters within the nodulin 26-like intrinsic protein III (NIP-III) group (Deshmukh and Bélanger, 2016). Moreover, all Si influx transporters in crop plants identified to date have an SF composed of a glycine–serine–glycine–arginine (GSGR) conserved sequence (Deshmukh and Bélanger, 2016). Therefore, plant AQPs belonging to the NIP-III group with a GSGR SF, six TM domains, and two NPA motifs can be categorized as candidate Si transporters.

While much is known about the structural and functional features of Lsi1 transporters and their influence on tissue Si content, very little is known about Lsi2 in comparison. No crystallographic structures have ever been resolved for Lsi2, which creates a bottleneck to understand how exactly Lsi2 actively transports Si (Ma *et al.*, 2006, 2007). To date only a few homologs have been reported in a limited number of plant species (Vatansever *et al.*, 2017). With new genomic data becoming available for an ever-expanding number of plant species, this should support future research to decipher with greater precision the properties of Si efflux transporters. Currently, while both influx and efflux Si transporters contribute to Si uptake from soil and subsequent transport to aerial tissues, it is clear that Lsi1s are essential by providing the primary entry of Si into the plant roots.

Confirming the presence of AQPs bearing all the molecular signatures of an Si influx transporter in a plant should theoretically lead to an accurate prediction of a plant's ability to take up Si. In this context, the rapidly increasing sequence resources for plants provide a perfect opportunity to mine putative Si transporters in many crop plants and model species and determine if their presence can be correlated with Si accumulation in plants. In an effort to provide a definitive and precise classification of Si-accumulating plant species, the objectives of this study were: (i) to define the effects of PAS in soil, plant age, plant organs, and plant genotypes on the phenotypes of Si accumulation; (ii) to identify molecular signatures associated with phenotypes; and (iii) to understand the evolution of NIP-IIIs in the plant lineage. On the basis of >1000 plant species' transcriptomes analyzed, our results show that only plant species carrying NIP-III AQPs with specific molecular signatures can accumulate significant Si. We conclude that plants can be classified directly as an Si accumulator or not, based on the acquisition/presence of these specific NIP-IIIs, a finding that is also useful to better understand the evolution of Si uptake in plant lineages.

Materials and methods

Plant material and growth conditions

Plant propagation material such as seeds, cuttings, and bulbs maintained at the Department of Horticulture, University Laval was used for growing plants on raised bed garden soil. The same genetic stock was used for the greenhouse experiment. For the evaluation of closely related species and intraspecies variation, seed material was obtained from the Germplasm Resources Information Network (GRIN), the United States Department of Agriculture (USDA).

In the greenhouse experiment, a total of 151 plant species were grown in 8 inch plastic pots with three replications. Potting mix was prepared from the Fafard AGRO Mix G10 (https://fafardpro.ca), garden topsoil, and washed fine sand at the ratio of 10:2:1 (v/v/v). Plants were irrigated with 1.7 mM Si provided in the form of potassium silicate (Kasil6, 23.6% SiO_2, National Silicates, Etobicoke, Toronto, Ontario, Canada). Growth conditions in the greenhouse

were maintained at ~22 °C day/18 °C night temperature, 80% humidity, and 16 h/8 h photoperiod cycle ensured with the artificial light source.

In the field experiment, raised beds prepared from uniform garden topsoil, farmyard manure (FYM), and compost were used to grow 456 plant species. The plants were irrigated through a precisely regulated drip irrigation system. Recommended seed treatment and agronomical practices were applied to raise healthy and disease-free plants in the greenhouse as well as in the field experiment.

Silicon quantification in plant and soil samples

Healthy and mature leaves were collected from 30-day-old plants grown in a greenhouse with Si supplementation and from plants grown on uniform raised beds in the field. Leaf tissues were directly collected in polypropylene tubes and dried in a hot-air incubator at 65 °C for 24 h. Dried leaves were powdered by using a bead homogenizer (Omni Bead Ruptor, Omni International). The dried leaf powder was then compressed into a 5 mm diameter size pellet using a manual compressor at a uniform pressure. The pellets were subjected to Si quantification using a portable X-ray fluorescence spectrometer (Niton XL3t900 GOLDD XRF analyzer; Thermo Scientific), according to the methods of Reidinger *et al.* (2012).

The PAS was quantified using the calcium chloride extraction method (Liang *et al.*, 2015). Soil samples collected from seven different fields in Iowa, USA were air-dried and then digested with 0.01 M calcium chloride, centrifuged, and finally the dissolved amount of Si in the supernatant was quantified colorimetrically. At the same time, leaf samples from soybean genotype Majesta grown in the same fields were harvested in mid-July. Si concentration was then quantified in soybean leaves as described above.

Identification and characterization of aquaporins

Whole-genome sequence annotation information was retrieved from different databases (Supplementary Table S1 at *JXB* online). A local database of transcript and protein sequences of predicted gene models was created for each species using command-line BLAST utilities provided in the BioEdit software tool (version 7.0.9.0; Hall, 2011). To identify putative AQPs, a BLAST search was performed against the local database using query sequences of 342 known AQP genes (Supplementary Dataset S1). To claim a significant match, the cut-off of the e-value $<10^{-5}$ and bit-score >100 was used.

Protein tertiary structure modeling and pore characterization

Protein tertiary structure was developed using the I-TASSER (Iterative Threading ASSEmbly Refinement) server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). Subsequently, the MOLE online server was used to predict the pore and pore-lining residues (https://mole.upol.cz/).

Identification of NIP-IIIs in transcriptomic sequences

Transcriptome data available for 1000 diverse plant species (1KP) belonging to different orders was used to identify NIP-III homologs (Carpenter *et al.*, 2019; One Thousand Plant Transcriptomes Initiative, 2019). As described above, query sequences of known AQPs were used to perform BLAST search against the 1KP database (Supplementary Dataset S1). The NIP-IIIs in the AQP homologs were further identified based on the phylogenetic distribution.

Phylogenetic analysis

A phylogenetic tree was made by using the RAxML method provided in The CIPRES Science Gateway to identify genes specific to the NIP-III group. Similarly, the taxonomical tree was developed based on information retrieved from the NCBI taxonomy browser and visualized through the PhyloT (https://phylot.biobyte.de/).

Results

Plant age and tissue type affect Si accumulation

To evaluate the effects of plant age and tissue type on Si accumulation, soybean (dicot) and barley (monocot) plants were tested for Si uptake over a period of 5 weeks. Both species gradually accumulated Si over time, and the concentration in leaf tissues saturated after 4 weeks (Fig. 1a). The highest concentration of Si was observed in the leaves, whereas smaller amounts were detected in the stem and root tissues of both species (Fig. 1b).

Silicon accumulation depends on plant-available silicon in the soil

Soybean, previously described as a good Si-accumulating dicot plant species (Deshmukh *et al.*, 2016), was grown on different soils to evaluate the relationship between Si accumulation and PAS in soil. Si concentrations ranging from 0.4% to 1.5% were quantified in leaves of soybean genotype cv. Majesta grown in different fields across Iowa with PAS varying from 13 ppm to 139 ppm. The results showed a strong positive correlation between Si accumulation in soybean leaves and PAS in the soil despite possible variations in environmental conditions across the fields (Fig. 1c).

Silicon accumulation in 456 plant species grown in soil

A set of 456 diverse plant species (658 total genotypes) belonging to 73 families representing 32 different taxonomical orders were grown on raised beds prepared with uniform soil. Plant species belonging to orders Poales, Zingiberales, Boraginales, and Asterales showed the highest Si accumulation (Fig. 2). In contrast, members of the Solanaceae and Brassicaceae families showed very little Si accumulation (Supplementary Table S2). Overall, Si accumulation observed in the different species was lower than amounts previously reported in greenhouse studies (Deshmukh *et al.*, 2014), as a result of the low PAS (20 ppm) present in the soil. For instance, well-known Si accumulators such as maize and soybean contained as little as 0.5% Si (DW), while several Poaceae species recorded <1% Si (DW).

Silicon accumulation in plant species grown under optimal silicon feeding

A wide range of Si accumulation was observed in a set of 151 diverse plant species fed with 1.7 mM Si under greenhouse conditions over a 4 week period. Plant species belonging to Poaceae, Fabaceae, and Cucurbitaceae families accumulated in general well over 1% Si (Fig. 3; Supplementary Table S3). Surprisingly, several species found to be low Si accumulators under field conditions (Fig. 2) displayed a high affinity for the element under greenhouse conditions. Notable examples were soybean, maize, and Brachypodium, which contained >2% Si (DW) under a 1.7 mM treatment compared with ~0.5% Si (DW) in soil experiments (Supplementary Tables S2, S3).

Several families within the monocot clade, which are mostly considered as high Si accumulators, were found to contain

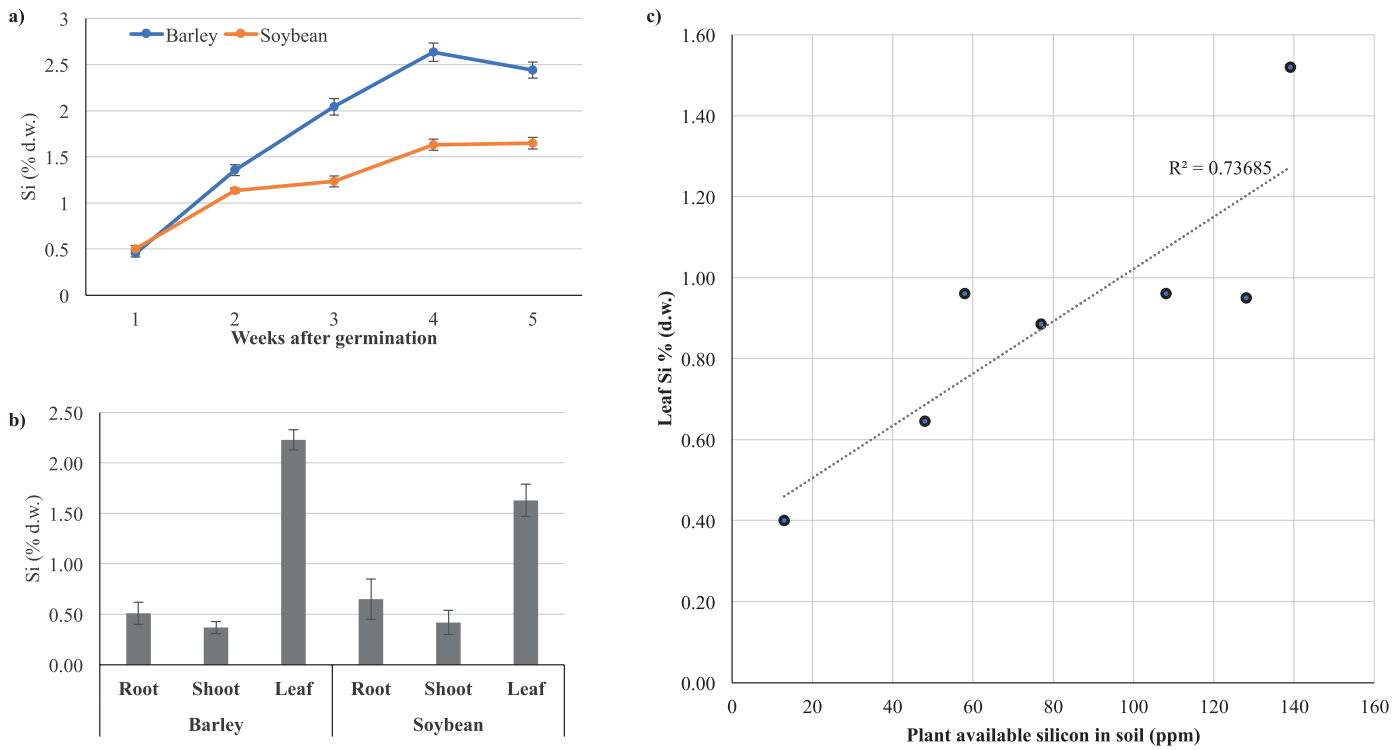

Fig. 1. Effect of plant age, tissue type, and plant-available silicon (PAS) in soil on silicon (Si) accumulation in plants. (a) Si accumulation in soybean and barley over a span of 5 weeks; (b) Si concentration in different tissues of soybean and barley; (c) univariate relationship between PAS and Si concentrations in soybean leaves.

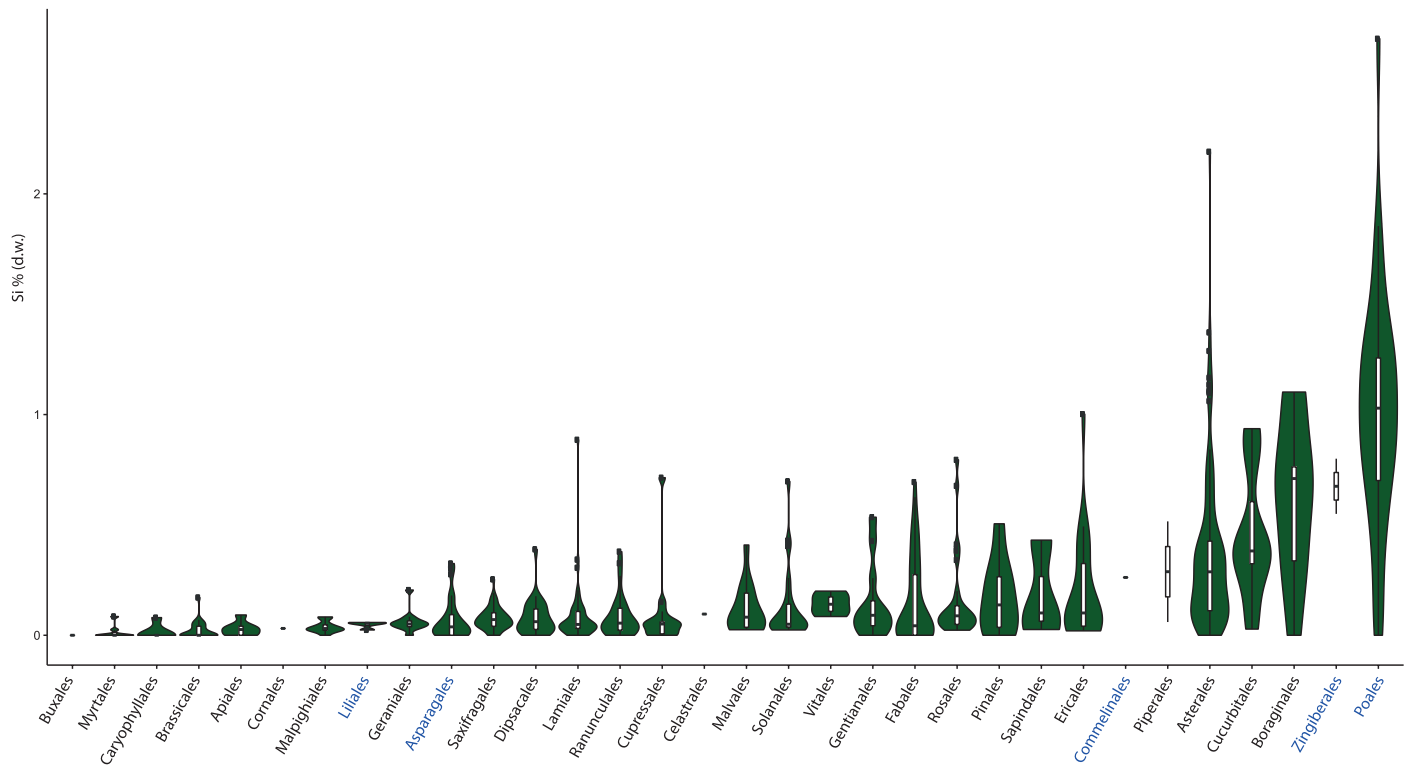

Fig. 2. Phylogenetic distribution of silicon (Si) accumulation in species belonging to diverse taxonomical plant orders. The entire set of genotypes representing 456 diverse plant species grown on soil supplemented with 20 ppm plant-available Si was evaluated for Si accumulation. The plant orders from the monocot clade are shown in blue text.

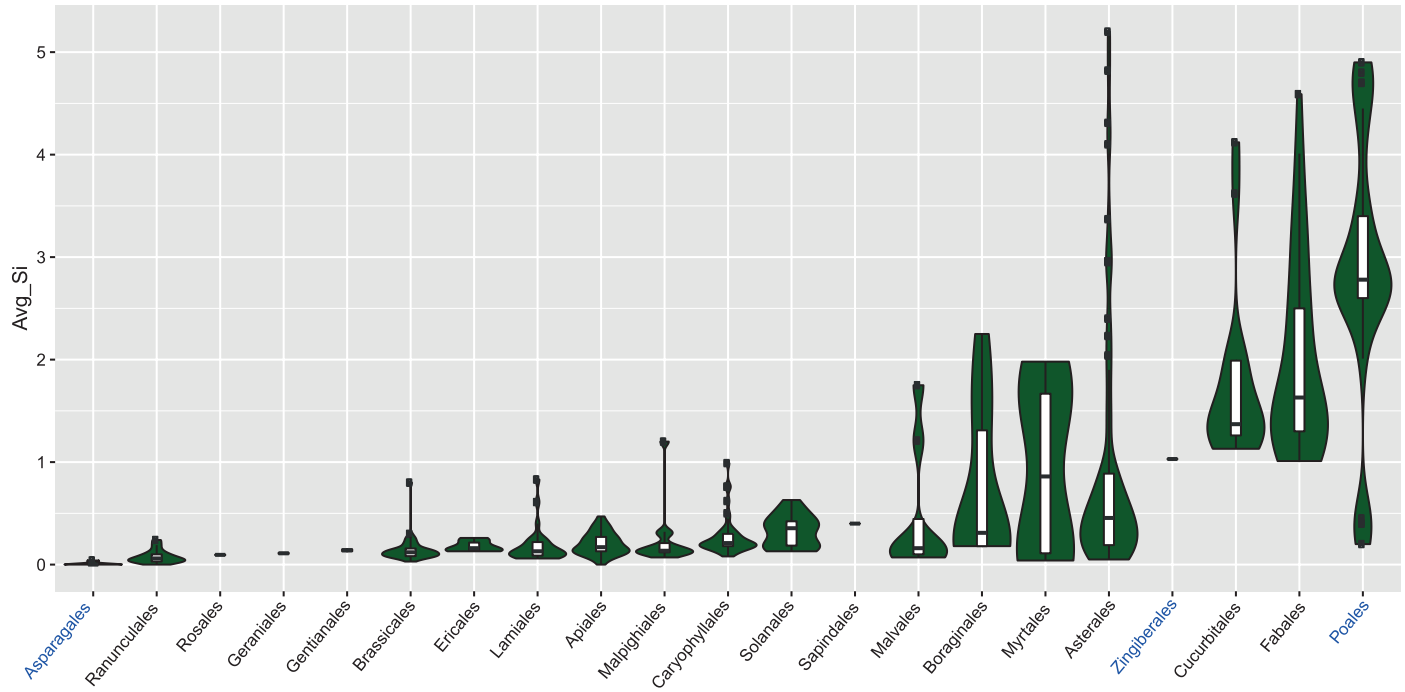

Fig. 3. Phylogenetic distribution of silicon (Si) accumulation in species belonging to diverse taxonomical plant orders grown under greenhouse conditions. The entire set of genotypes representing 151 diverse plant species grown with supplementation of 1.7 mM Si. The plant orders from the monocot clade are shown in blue text.

concentrations as low as 0.01%. For instance, very low Si concentrations were recorded for the plant members of the Asparagales and the Cannaceae families (Supplementary Tables S2, S3). Low accumulation in the latter family is more surprising since it belongs to the order Zingiberales which is taxonomically close to Poales.

Intraspecies variation for silicon accumulation

Si content among high and low accumulator species was analyzed on different genotypes of eight species to determine intraspecies variation. As shown in Fig. 4, regardless of the predisposition of a given species to accumulate Si, all genotypes within a species were remarkably similar in Si content. All genotypes of high-accumulating species had Si concentrations well above 1%, while those of low accumulating species were significantly below 0.5% when grown under greenhouse conditions.

Phylogenetic distribution and evolution of silicon influx transporter in plant lineages

A total of 4170 AQPs were identified in the set of 116 plant genomes through a BLAST search performed by using previously reported AQPs as query sequences (Supplementary Dataset S1). Subsequently, the identification of conserved motifs, TM domains, and the tertiary structure was evaluated to confirm the AQPs (Fig. 5a). Among those, a total of 280 AQPs with either both or one NPA motif missing were excluded from further analysis. The removed 280 AQPs only had partial sequences and were very short in length. A total of 140 NIP-IIIs (Si influx transporter) representing 81 plant genomes were identified based on the information including amino acid constitution, top hit against previously reported AQP genes, and clustering in the phylogenetic tree of 3890 AQPs (Supplementary Table S4). Similarly, a total of 16 875 AQPs initially identified using transcriptome data were filtered to 11 551 AQPs based on the presence of two NPA motifs. Finally, phylogenetic analysis of 1544 NIPs showed three distinct groups, namely NIP-I, NIP-II, and NIP-III. A total of 349 proteins belonging to 236 plant species were identified as NIP-III with the transcriptome data (Supplementary Table S5; Supplementary Fig. S1).

Presence of NIP-IIIs and associated silicon uptake in angiosperms

Among the sequences of 715 angiosperm species analyzed in the present study, only 264 species harbored NIP-IIIs (Supplementary Tables S4, S5). Most of them displayed characteristic G-S-G-R Ar/R SF, with a few exceptions. For instance, an S-S-G-R SF was observed in two monocots, *Phalaenopsis equestris* and *Dendrobium catenatum*, and five dicots, *Schlegelia parasitica*, *Cota tinctoria*, *Matricaria matricarioides*, *Solidago canadensis*, and *Bituminaria bituminosa*. Another notable exception was an A-S-G-R amino acid motif present in a few species of the dicot families such as the Fabaceae, Chenopodiaceae, and Papaveraceae. Surprisingly, the Fabaceae species *Phaseolus vulgaris*, *Vigna angularis*, and *Vigna radiata*, which accumulated Si well above 1% (Supplementary Table S3), harbored NIP-IIIs with an A-S-G-R SF (Supplementary Tables S4, S5). Another exception was specifically observed in nine species (*Philoxerus vermicularis*, *Alternanthera* spp., *Alternanthera brasiliana*, *Alternanthera sessilis*, *Aerva persica*, *Alternanthera sessilis*,

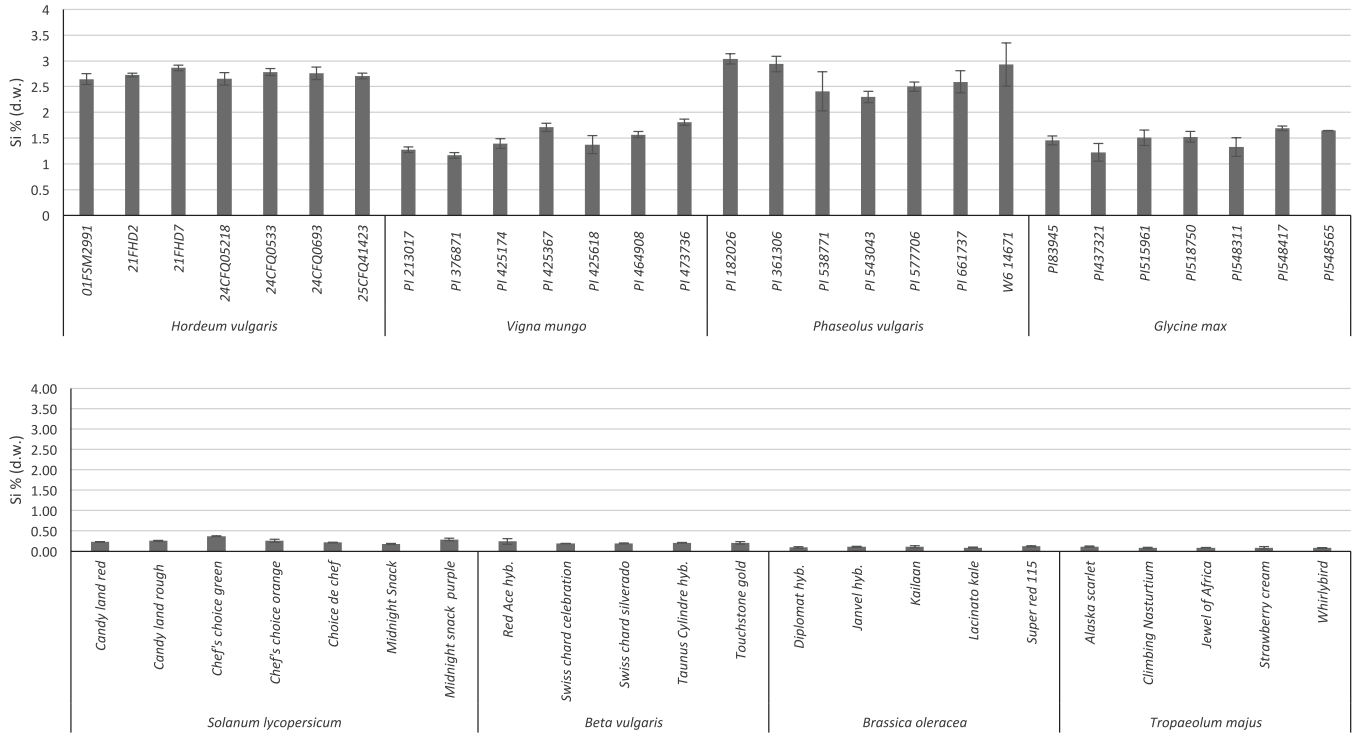

Fig. 4. Intraspecies variation in leaf silicon (Si) content in different genotypes of four species reported to be high Si accumulators (a), and four species reported to be low accumulators (b). The coefficient of variation for Si accumulation in these species are: *Hordeum vulgaris* 0.02, *Vigna mungo* 0.15, *Phaseolus vulgaris* 0.10, *Glycine max* 0.11, *Solanum lycopersicum* 0.23, *Beta vulgaris* 0.09, *Brassica oleracea* 0.11, and *Tropaeolum majus* 0.15.

Alternanthera caracasana, *Amaranthus retroflexus*, and *Amaranthus cruentus*) of the Amaranthaceae family that carried a V-S-[A/G]-R SF. Among them, *A. brasiliana* only accumulated 0.69% Si under greenhouse conditions. (Supplementary Table S3). Apart from *A. brasiliana*, four other members of the Amaranthaceae (*Amaranthus caudatus*, *Amaranthus paniculatus*, *Amaranthus tricolor*, and *Gomphrena globosa*) were also found to be low Si accumulators. Based on the premise of a conserved G-S-G-R SF, the first position, G, located in helix 2 (H2) was found to be the least conserved (Supplementary Table S4; Fig. 5a). Similarly, the fifth position of the Froger's residue (Froger *et al.*, 1998) present in transmembrane helix 6 (H6) was found to be the most variable across the NIP-IIIs from different species, but no association was observed with Si accumulation (Supplementary Fig. S2). Mitani's residue (Mitani *et al.*, 2011), earlier found to be associated with the genotypic variation for Si uptake in cucumber, was found to be the most conserved feature in NIP-IIIs. In the case of NPA domains, NPV sometimes replaced NPA in NIP-IIIs. Interestingly, Cucurbitaceae species such as *Cucumis melo* and *Cucumis sativus* carried NPV in loop E but were nevertheless considered high accumulators (Supplementary Table S4; Fig. 5a).

Analysis of the NPA motifs also revealed that a spacing of 108 amino acids between two NPA motifs was a conserved feature of Si-permeable NIP-IIIs. Indeed, among 20 species that were common between the greenhouse experiment and the sequencing dataset, all were found to carry an NPA spacing of 108 amino acids (Supplementary Tables S4, S5). In contrast, species having NIP-IIIs with an NPA spacing different from 108 showed consistently low Si accumulation. For instance, *Tragopogon porrifolius*, a species from the Asteraceae family, carrying two NIP-IIIs with G-S-G-R and G-I-G-R SFs, and NPA spacings of 105 and 136 amino acids, respectively, had a low Si content (Supplementary Tables S3, S5). Similarly, three species of the Solanaceae family, *Ipomoea purpurea*, *Solanum lycopersicum*, and *S. tuberosum* with NPA spacing of 110, 109, and 109 amino acids, respectively, were all low Si accumulators (Supplementary Tables S3, S4). Another low accumulator, *A. brasiliana* from the Amaranthaceae family, was found to have a V-S-A-R SF and an NPA spacing >108 amino acids.

Absence of NIP-IIIs in angiosperm families

The absence of NIP-IIIs from a plant genome is challenging to confirm with transcriptomic data because it represents only a portion of the total genes that are expressed. Therefore, only well-annotated genomic data were used to investigate the absence of NIP-IIIs within the angiosperm species. Out of 116 whole-genome sequenced plants, 35 plant species did not show the presence of NIP-IIIs (Fig. 6). Interestingly, the nine species within the Brassicaceae family showed complete absence of NIP-IIIs. Accordingly, other Brassicaceae species such as *Arabidopsis thaliana*, *Brassica napus*, *Brassica oleracea*, *Brassica rapa*, and *Camelina sativa*, tested for Si uptake, contained Si concentrations <0.2% (Supplementary Table S3). In the same manner, two species of the Solanaceae family, *Nicotiana tomentosiformis* and *Capsicum annuum*, lacked NIP-IIIs and were found to be low Si accumulators (Supplementary Table S3). Within the Solanaceae family, some species were found to carry NIP-IIIs, but all had an NPA–NPA spacing >108 amino acids and tested to be low Si accumulators (Supplementary Table S3).

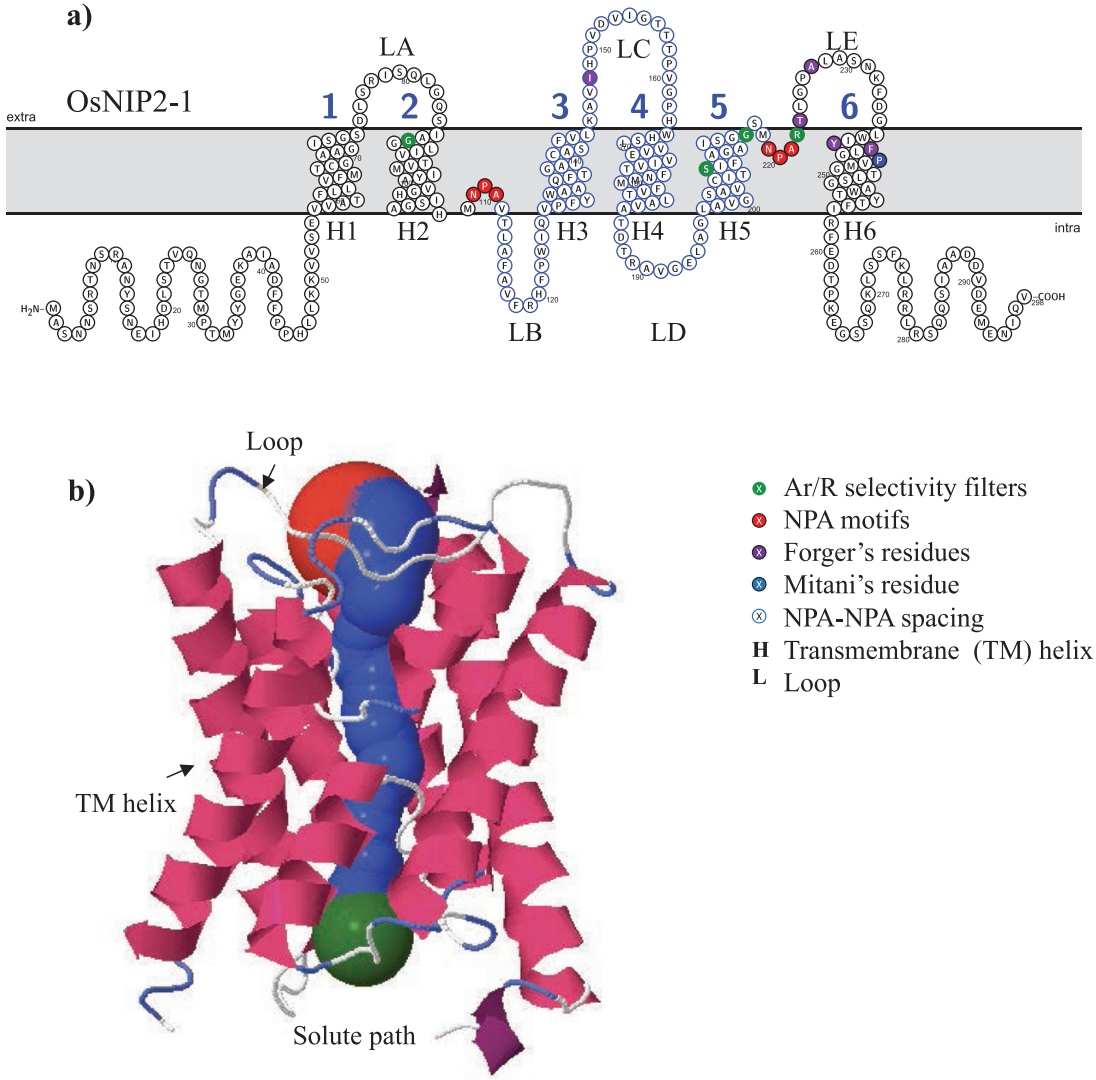

Fig. 5. Characterization and phylogenetic analysis of aquaporins. (a) Simplified 2-D structure of a typical NIP-III showing conserved amino acid residues and motifs known to have functional roles in silicon permeability. (b) The tertiary structure of rice NIP-III (OsNIP2-1) showing pore morphology and constricts formed by the Ar/R selectivity filter and NPA motifs.

Interspecies variation in Asteraceae

A surprisingly high level of interspecies variation was observed within the Asteraceae. For instance, sunflower contained the highest Si concentration among all dicot species analyzed in this study, whereas other Asteraceae species such as *Aster alpinus*, *Cladanthus arabicus*, *Erigeron speciosus*, *Hieracium villosum*, *Jacobaea maritima*, *Osteospermum ecklonis*, *Tragopogon porrifolius*, and *Cynara cardunculus* recorded <0.2% Si (Fig. 3; Supplementary Table S3). Among the 29 Asteraceae species evaluated in the greenhouse experiment for Si accumulation, only 11 species from the Heliantheae alliance showed Si accumulation >1% (Fig. 7). Further analysis of the very recently made available genome sequence of Asteraceae member *Cynara cardunculus* var. *scolymus* (http://www.artichokegenome.unito.it/) showed the absence of NIP-IIIs. In addition, *Tragopogon porrifolius* having a NIP-III with NPA spacing >108 amino acids is also present in the same clade of *C. cardunculus*. All the species taxonomically closer to *C. cardunculus* and *T. porrifolius* showed low Si content compared with the species from the Heliantheae alliance group (Fig. 7).

NIP-IIIs in gymnosperms

In gymnosperms, NIP-IIIs were observed in 16 out of 76 species (Supplementary Tables S4, S5). There was a clear delineation on the basis of order where NIP-IIIs were found in species within Cupressales and Pinales, and absent in species belonging to the Araucariales, Cycadales, Ginkgoales, Gnetales, and Welwitschiales. All NIP-IIIs in gymnosperms carried a very specific S-[S/A]-G-R SF, all with the specific 108 amino acid NPA spacing. Interestingly, as mentioned above, an S-S-G-R SF was also observed in seven angiosperms. Unlike angiosperms, where the second NPA motif in loop E sometimes showed an NPV variation, a similar variation was also observed in gymnosperms, but only in the first NPA motif (NP[A/V]) located in loop B (Supplementary Table S4, Fig. 8).

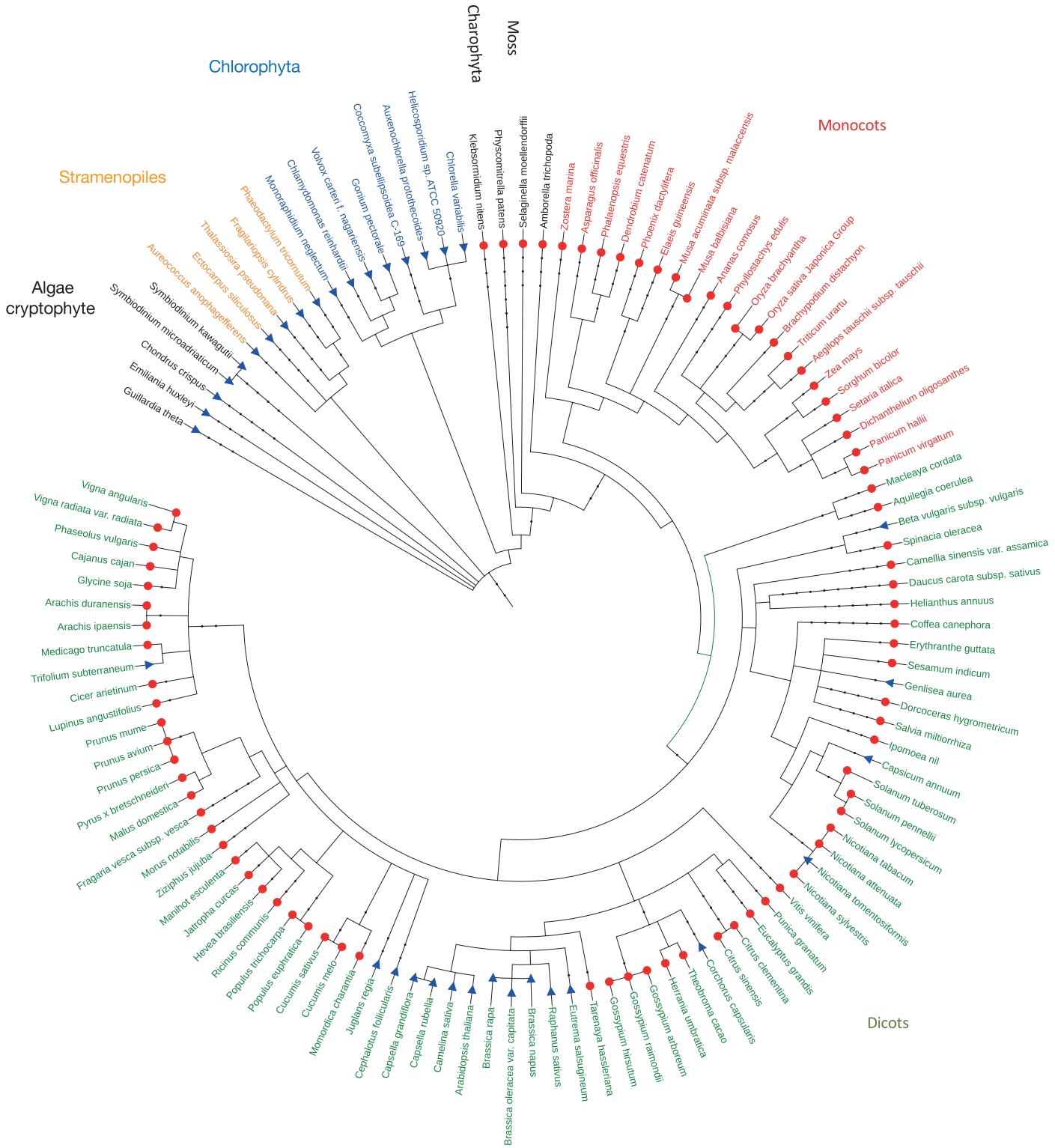

Fig. 6. Taxonomical phylogeny of 116 plant species based on whole-genome sequence data showing presence or absence of nodulin 26-like intrinsic protein-IIIs (NIP-IIIs). Presence of NIP-IIIs is denoted with a red circle, whereas a blue triangle represents the absence of NIP-IIIs.

NIP-IIIs in ferns

In ferns, NIP-IIIs were identified in nine out of 60 species studies. Fern orders such as Equisetales, Ophioglossales, Osmundales, and Polypodiales have NIP-IIIs with a very distinct Ar/R SF. For instance, Equisetales, known as one of the highest Si-accumulating plants, has NIP-IIIs with T-N-A-R and F-A-A-R SFs, whereas Polypodiales and Osmundales have a G-I-G-R SF, all with conserved 108 amino acid NPA spacing.

Similar to previously reported Si influx transporters in *Equisetum arvense* (Grégoire *et al.*, 2012), NIP-IIs comprising S-T-A-R Ar/R SFs with characteristic 108 NPA spacing were identified in *Equisetum hyemale* and *E. diffusum*, and also

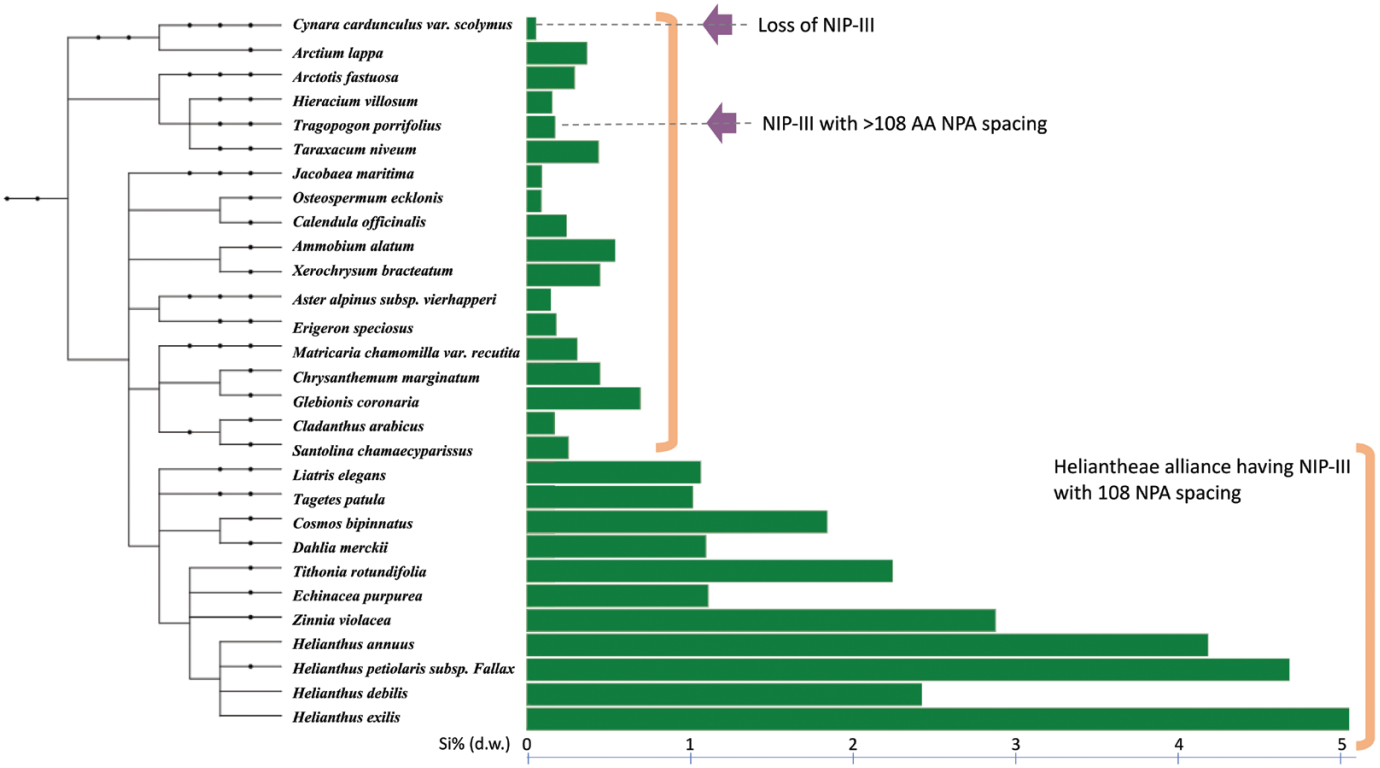

Fig. 7. Phylogeny based on the taxonomical distribution of species from the Asteraceae family showing silicon (Si) determined in the leaf tissue. Plants were analyzed for Si uptake under optimal Si supplementation in the greenhouse.

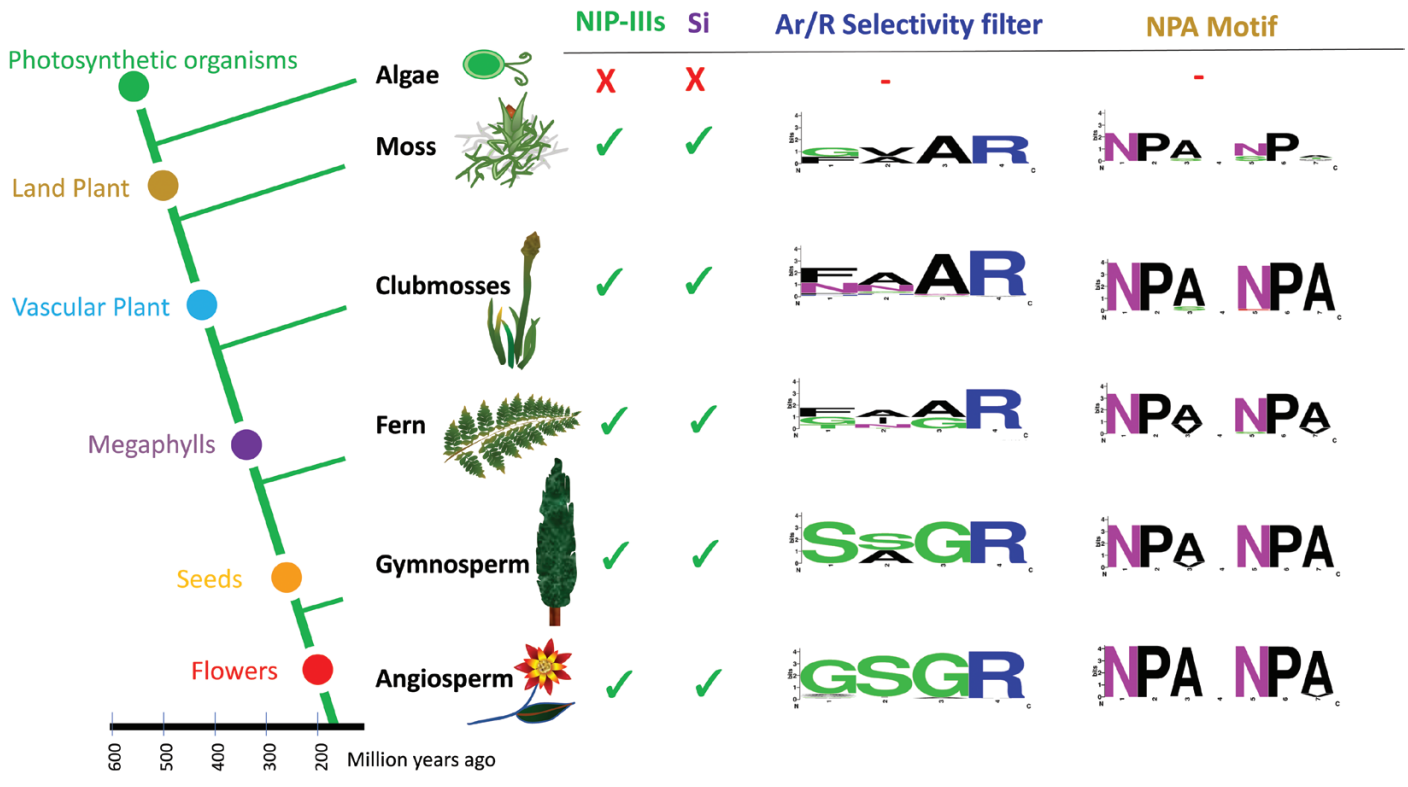

Fig. 8. Evolution of the Ar/R selectivity filter (SF) and NPA motifs in NIP-IIIs identified in plant lineages.

in a fern species *Mapania palustris.* Surprisingly, NIP-IIs with a S-T-A-R SF were also observed in two monocot species, *Dipteris conjugate* and *Lepidosperma gibsonii* from the Cyperaceae (Supplementary Table S6).

Clubmosses, mosses, and liverwort

We identified NIP-IIIs in a total of 22 clubmosses. The majority of them had F-A-A-R and N-N-A-R Ar/R SFs. Furthermore, a NIP-III with a G-S-G-R SF along with 108 amino acid NPA spacing and a conserved Mitani's residue, as observed in flowering plants, was also present in *Phylloglossum drummondii*, a species from the clubmoss order Lycopodiales. Since this finding was unusual, the NIP-III sequence from *P. drummondii* was further confirmed by a BLAST search performed against the 1KP database (Supplementary Table S5). As expected, the sequence matched perfectly with the sequence from *P. drummondii* (self-match) but, surprisingly, the second top hit was the NIP-III sequence from *Cana* sp., a member of the flowering plants. There was no sequence similarity with other clubmoss sequences. Therefore, a BLAST search was also performed by using the NIP-III with the F-A-A-R SF from *P. drummondii* as a query sequence, and the top hit after the self-match was, as expected, a NIP-III sequence from *Huperzia selago*, a member of the Lycopodiaceae family.

A total of seven NIP-IIIs were identified in three moss species (*Physcomitrella patens*, *Andreaea rupestris*, and *Scouleria aquatic*). They carried F-A-A-R and G-V-A-R SFs with NPA spacing of 108 and 107/106, respectively. In liverwort, two NIP-IIIs from *Pellia* cf. *Epiphylla* with a F-A-A-R/P SF and with NPA spacing of 116 and 107 amino acids were observed (Supplementary Table S5). Algae were devoid of NIP-IIIs. In addition, the NIPs identified in algae can be considered as the closest possible homolog of NIP-IIIs and have an NPA spacing >108 amino acids.

Discussion

Si is now widely accepted as a beneficial element for plant growth because of its protective role observed under stress conditions (Coskun *et al.*, 2019). In general, high Si accumulator plant species are found to attain more benefits compared with low accumulating speicies (Coskun *et al.*, 2019). However, ascertaining the ability of a given species to accumulate Si has led to conflicting reports (Hodson *et al.*, 2005; da Silva Lobato *et al.*, 2013; Ouellette *et al.*, 2017). Earlier efforts to classify the Si-accumulating properties of plants have painstakingly compiled data from over a hundred studies, and did not have access to genomic resources (Hodson and Sangster, 2002; Hodson *et al.*, 2005). In the present study, Si uptake in >450 diverse plant species was evaluated under uniform growth conditions, extensive sequence analysis was performed in >1000 plant species to identify NIP-IIIs, and the information was used to corroborate with Si uptake data. As a result, we have identified conserved features of NIP-IIIs explaining the evolutionary history associated with the functionality of NIP-IIIs with respect to Si permeability, and factors affecting inter- and intraspecies variations related to Si uptake at the genetic and phenotypic levels.

Classifying the ability of a plant to accumulate Si has always been challenging because of the inconsistencies associated with PAS in growing media, plant age, plant tissue, and plant physiology (Rosen and Weiner, 1994; Hodson *et al.*, 2005; da Silva Lobato *et al.*, 2013; McLarnon *et al.*, 2017; Ouellette *et al.*, 2017). To draw the correct inference from the comparison of Si uptake in different species, controlled experiments with uniform growing conditions is a prerequisite. In the present study, we have shown that plant age, tissue type, and PAS can all affect Si concentrations. In terms of plant age, a 4 week growth period was necessary to reach saturation in both barley and soybean (Fig. 1a), although other species such as strawberry and white spruce have shown continued accumulation well beyond that period, probably because of simultaneous accumulation in the multiple branch crowns and needles, respectively (Hodson and Sangster, 1998; Ouellette *et al.*, 2017). Regarding Si accumulation in different plant tissues, the comparisons made in the present study clearly showed the highest level of Si in the leaves (Fig. 1b), a result consistent with the absorption transport model described by Ma *et al.* (2006). As expected, PAS had a significant effect on Si uptake (Fig. 1c). Under low soil Si concentrations (<20 ppm), soybean plants accumulated <0.4% Si which would classify them as low accumulators, while concentrations above 60 ppm yielded nearly 1% Si. These results explain why soybean has been considered a low accumulator in some work (Van der Vorm, 1980; Arsenault-Labrecque *et al.*, 2012) and a moderate to high accumulator in other works (Deshmukh *et al.*, 2013). While large variations in Si accumulation exist among species, it was quite noteworthy to observe more uniform Si accumulation among genotypes within a species (Fig. 4). This indicates that the phenotype is under strong genetic control and that it is highly conserved within a species.

As a general rule, monocots are considered high Si accumulators and dicots low accumulators (Hodson *et al.*, 2005; da Silva Lobato *et al.*, 2013; Ouellette *et al.*, 2017). In the present study, several dicot families, including the Cucurbitaceae, Fabaceae, and Asteraceae, showed high Si accumulation, while only monocot species within the Poaceae family did the same. Therefore, the concept that monocots and dicots belong in different classes of Si accumulators appears inappropriate. For instance, the dicot species sunflower accumulated as much Si as rice, a monocot species often referred to as the highest accumulator of commercial crops.

Evolution of NIP-IIIs in plant lineages

Understanding the evolution of NIP-IIIs is important to provide insights into why some plant species evolved with this trait, whereas others lost or lacked it. In this regard, the seminal discovery of Si influx transporters belonging to NIP-IIIs provided the basis to track the evolution of the gene (Ma *et al.*, 2006). At the same time, recent advances in sequencing technologies helped to develop useful resources for that purpose (Matasci *et al.*, 2014). In this study, by exploiting >1000 transcriptomic data available for diverse plant species, we were able to make phylogenetic inferences to analyze the evolution

of the NIP-IIIs. Along with the transcriptomic data, we also used whole-genome sequencing information available for >00 plant species representing all the major clades of land plants (Fig. 6).

Identification and in-depth analysis of NIP-IIIs performed here highlighted several interesting facts. For instance, unlike the earlier assumption that the NIP-III Ar/R SFs are exclusively composed of G-S-G-R SF (Trembath-Reichert *et al.*, 2015), our results suggested a more diversified pattern. In angiosperms, the first position appears to be the least conserved, suggesting a minimal role in functionality. In legumes such as *Phaseolus vulgaris*, *Vigna angularis*, and *Vigna mungo*, Si accumulation was recorded at >2% (DW) in spite of an A-S-G-R SF (Supplementary Table S3). Interestingly, results of Mitani-Ueno *et al.* (2011) supported our findings by showing that substitution of the first amino acid of the SF in the rice NIP-III (OsLsi1) did not affect Si permeability. Nevertheless, the predominance of G at the first position may indicate specificity for other solutes or be attributable to confounding effects of surrounding convergent amino acid sequences constituting specific structural features. Similarly, the change of NPA to NPV does not seem to have an impact on the functionality of the NIP-IIIs. Our results showed high Si uptake in Cucurbitaceae species in spite of having NPV instead of NPA (Supplementary Tables S3, S4). While most AQPs contain NPA motifs, several variations, including NPV, NPS, NPL, NPC, and NPT, were reported in different plant species (Sonah *et al.*, 2017). The NPA motif is also common to AQPs in fungi, insects, and mammals with few exceptions, as observed in plants (Ikeda *et al.*, 2011; Xu *et al.*, 2013; Lu *et al.*, 2017). In the mammalian AQP11, Ikeda *et al.* (2011) found that the change in a wild-type NPC to NPA had no effect on the subcellular localization but affected its oligomerization. In addition, the change from NPC to NPA was shown to reduce the water permeability of AQP11 (Ikeda *et al.*, 2011). Similar mutagenesis experiments are required to understand the effect of sequence variation at the NPA motif in plant AQPs.

Notwithstanding the structure of NPA motifs, their spacing appears to be one of the most conserved and important features with respect to Si permeability of NIP-IIIs (Supplementary Tables S3, S4). In the greenhouse experiments, all species carrying a NIP-III with 108 amino acid NPA spacing recorded high Si accumulation (Supplementary Tables S3, S4). In contrast, species with an NPA spacing deviating from 108 amino acids showed lower Si accumulation. Some dicot families such as the Solanaceae and Amaranthaceae, in particular, appear to have evolved an NPA spacing different from 108, which correlates with their low Si accumulation (Supplementary Tables S3, S4). Earlier, Deshmukh *et al.* (2015) provided evidence that a 109 amino acid spacing in tomato NIP-III (SlNIP2-1) significantly reduced Si permeability compared with a 108 spacing. Interestingly, NIP-IIs cloned from *Equisetum arvense* with an NPA spacing of 108 amino acids were observed to have high Si transport activity in the oocyte assay in spite of having a S-T-A-R SF instead of the common G-S-G-R (Grégoire *et al.*, 2012). Sequence analysis performed here also showed NIP-IIs with an S-T-A-R SF in two more species from the Equisetales, suggesting its conservation across the order (Supplementary Table S6). The presence of similar NIP-IIs with an S-T-A-R SF in the monocot species *Lepidosperma gibsonii* and the fern species *Dipteris conjugata* is more surprising. It is not clear whether most of the angiosperm species lost the NIP-IIs with an S-T-A-R SF or if the feature has evolved specifically in these two species. In any event, the strong association of a 108 amino acid spacing between NPA motifs with high Si uptake in plants suggests the importance of this trait for the permeability of Si influx proteins.

By using whole-genome sequencing information available for >100 plant species, we uncovered new characteristics of NIP-III evolution. The ubiquitous presence of NIP-IIIs in the 21 monocot genomes analyzed combined with their absence in only a few specific dicot families suggests a recent loss of this specific AQP (Fig. 6). This is further supported by the failure of some monocots to efficiently take up Si, which is associated with the loss of functionality of the NIP-IIIs. This may be explained by several factors, including variation in conserved features, improper folding of the protein, improper subcellular localization, transcriptional activity, post-translational modifications, etc. Presently, we know that the constitution of the Ar/R SF, NPA motifs, and NPA spacing are linked with the ability for Si uptake in some species. However, limited information is available to explain how other factors affect protein functionality in NIP-IIIs. Recently, Coskun *et al.* (2019) identified a single-residue conformational change in tobacco NIP-III that affected Si permeability in spite of the presence of the other essential features. In addition, NIP-IIIs are known to be efficient transporters of many solutes such as Si, boric acid, arsenic, urea, and water (Mitani-Ueno *et al.*, 2011), and the variation observed in the conserved features may not necessarily be associated with the transport activity of other solutes.

Based on our phylogenetic analyses (Fig. 7; Supplementary Table S3), we can conclude that NIP-IIIs are not necessarily well conserved within families, as evidenced in the case of Asteraceae. The variations range from complete loss of NIP-IIIs to structural variations in the conserved attributes, suggesting that selection pressure for Si uptake does not extend to all members within a family. In contrast, our results revealed that Si uptake, and by association NIP-IIIs, showed very limited genotypic variation within a species (see Fig. 4).

In gymnosperms, only a few studies have suggested a potential role for Si (Hodson and Sangster, 1999; Prabagar *et al.*, 2011; Hogan *et al.*, 2018). In our study, none of the identified NIP-IIIs contained a G-S-G-R SF, which would suggest an inability to take up high concentrations of Si and explain the scarcity of reports linking Si with benefits in gymnosperms.

In ferns, different orders such as Equisetales, Ophioglossales, Osmundales, and Polypodiales have NIP-IIIs with distinct SFs. Earlier reports showed a wide range of Si concentrations from 0.1% to 3.9% among 27 fern species (Höhne and Richter, 1981). Within the Equisetales, known for their very high Si uptake, NIP-IIIs with very specific T-N-A-R SFs were observed, but is it unknown if such SFs allow Si permeability. In previous work, Grégoire *et al.* (2012) showed that Equisetales had evolved Si influx transporters belonging to NIP-IIs, a rare feat probably linked to their atypical plant anatomy dependent on Si. However, based on limited sequence availability at the time,

the authors did not identify NIP-IIIs, so it remains to be determined if the latter also contribute to Si uptake in Equisetales.

Liverwort and mosses depend greatly on transcellular AQP transport because of their undeveloped vascular system. This explains the larger number of AQP subfamilies observed in the mosses (Danielson and Johanson, 2008). In general, gymnosperms and angiosperms have five distinct AQP subfamilies, while seven subfamilies were reported in mosses (Danielson and Johanson, 2008). Our results identified for the first time the presence of NIP-IIIs in mosses and liverwort, which redefines the evolutionary origin of the NIP-IIIs. Ma *et al.* (2001) found >1% Si in several species belonging to the bryophytes, clubmosses, and Equisetopsida. Similarly, >3% Si was observed in *Ceratophyllum demersum* a hornwort species of the Ceratophyllales order (Schoelynck *et al.*, 2010). In an ultrastructural study of the liverwort (*Mizutania riccardioides*), Si deposition was noted on the cell wall lining (Pressel *et al.*, 2011). Based on the previous studies and our sequence analysis results, it can be inferred that Si uptake and specific patterns of Si distribution have evolved millions of years ago in non-vascular plants.

Land plants (Embryophytes) are estimated to have evolved during the middle Cambrian–early Ordovician era, dating back to 515 million years ago (Ma) to 470 Ma (Morris *et al.*, 2018). The occurrence of NIP-IIIs in today's Bryophyta (first land plants) is not sufficient alone to claim the existence of Si uptake mechanisms in Cambrian Bryophyta. For this reason, fossil records have enormous importance to connect today's information with historical records. Recently, Si deposition was observed in moss fossils estimated to date from the Ordovician era around 455–454 Ma (Cardona-Correa *et al.*, 2016). The moss fragment fossils described by Cardona-Correa *et al.* (2016) were claimed to be the oldest fossils presenting distinctive features that helped to link them with modern vascular plants. These findings also correlate well with molecular evidence that estimated the peat moss evolution dating back 607–460 Ma (Laenen *et al.*, 2014; Morris *et al.*, 2018).

We identified NIP-IIIs in 22 clubmosses, a seemingly logical finding since NIP-IIIs were also observed in mosses and liverwort. However, the NIP-III with a G-S-G-R SF identified in the clubmoss species *P. drummondii* is very surprising. To better understand this exception, sequence analysis in other species related to *P. drummondii* is required. Earlier, two clubmoss species were found to accumulate >3% Si (Ma and Takahashi, 2002). Although some species within the bryophytes and primitive vascular plants are found to be high Si accumulators and carry NIP-IIIs, their mode of Si uptake remains largely unknown.

The advent of conserved features such as the Ar/R SF and NPA motifs was shown to occur through stepwise changes over the course of plant evolution (Fig. 8). In the bryophytes, the first two positions of the Ar/R SF and the third position of both NPA motifs were less conserved. However, in clubmosses and ferns, only the first position of the Ar/R SF is less conserved while the second position became conserved. Similarly, both NPAs became more conserved. In general, there was a clear trend of selection for conserved sequences during each new era of plant evolution for the Ar/R SF and NPA motifs. As expected, bryophytes and primitive vascular plants displayed higher diversity at the Ar/R SF when compared with gymnosperms and angiosperms.

The present study exploited the most comprehensive genomic resources to date in an attempt to explain the origin and evolution of Si uptake ability in plants. Phenotypic evaluations further showed that factors such as plant age, tissue type, plant physiology, and PAS could all significantly affect Si accumulation in plants and their classification as accumulators or non-accumulators. Our results further showed that monocots and dicots are not as distinctly separated in terms of Si uptake as previously reported. The taxonomical distribution provided in the present study will be helpful for several other disciplines such as palaeoecology and geology that define the biogeochemical cycling of Si. In addition to the prediction of Si uptake potential of plant species based on sequence information and taxonomical positioning, the evolutionary path of the Si uptake mechanism described here will be helpful to understand the Si environment over the different eras of land plant evolution. Results presented here clearly indicate that the evolution of NIP-IIIs as primary Si influx transporters dates back as early as 515 Ma.

Supplementary data

The following Supplementary data are available at *JXB* online.

Table S1. List of 116 whole-genome sequenced plant species used for NIP-III identification.

Table S2. Silicon (Si) accumulation observed in leaves of diverse plant species from different orders and familiess.

Table S3. Silicon (Si) accumulation observed in the leaves of diverse plant species grown under greenhouse conditions with 1.7 mM Si supplementation.

Table S4. Details of 140 NIP-IIIs identified in 81 whole-genome sequenced plant species.

Table S5. Details of 349 NIP-IIIs identified using 1KP transcriptome data.

Table S6. Details of NIP-IIIs with S-T-A-R Ar/R selectivity filters identified in Equisetales, ferns, and angiosperms.

Fig. S1. Phylogenetic tree depicting three groups of nodulin 26-like intrinsic proteins identified by transcriptomic and genomic sequence data of 1133 plant species.

Fig. S2. Evolution of the Ar/R selectivity filter (SF), NPA motifs, Froger's residue, and other features in NIP-IIIs identified in plant lineages.

Dataset S1. Sequences of known aquaporins from diverse plant species used as query sequences for BLAST search.

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Author contributions

RD and HS designed the study, conducted the experiments, and prepared the first draft of the manuscript. RD, HS, and RB contributed to finalize the draft. RB provided critical inputs, materials, and financial requirements.

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