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Identification and characterization of silicon efflux transporters in horsetail (*Equisetum arvense*)

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ABSTRACT

Silicon (Si) is a beneficial element to plants, and its absorption via transporters leads to protective effects against biotic and abiotic stresses. In higher plants, two groups of root transporters for Si have been identified: influx transporters (Lsi1) and efflux transporters (Lsi2). Lsi1 transporters belong to the NIPIII aquaporins, and functional Lsi1s have been found in many plants species. Much less is known about Lsi2s that have been characterized in only a few species. Horsetail (*Equisetum arvense*), known among the highest Si accumulators in the plant kingdom, is a valuable model to study Si absorption and deposition. In this study, we first analyzed discrete Si deposition patterns in horsetail shoots, where ubiquitous silicification differs markedly from that of higher plants. Then, using the sequenced horsetail root transcriptome, two putative Si efflux transporter genes, *EaLsi2-1* and *EaLsi2-2*, were identified. These genes share low sequence similarity with their homologues in higher plants. Further characterisation of *EaLsi2-1* in transient expression assay using *Nicotiana benthamiana* epidermal cells confirmed transmembrane localization. In order to determine their functionality, the *EaLsi2-1* was expressed in *Xenopus* oocytes, confirming that the translated protein was efficient for Si efflux. Both genes were equally expressed in roots and shoots, but interestingly, showed a much higher expression in the shoots than in the roots in contrast to *Lsi2s* found in other plants, a result consistent with the specific anatomy of horsetail and its rank as one of the highest Si accumulators among plant species.

1. Introduction

Silicon (Si) is ubiquitous in nature as the second most abundant element in the soil. Silicon is absorbed by plants in its soluble form, silicic acid, yielding remarkable beneficial effects (Epstein, 1994). The absorption and accumulation of Si protect plants against several biotic and abiotic stresses, including fungal diseases, insects, drought, lodging, salinity and nutrient imbalance (Epstein, 1999; Fauteux et al., 2005; Liang, 1999; Liang et al., 2015; Rémus-Borel et al. 2005). Despite its beneficial effects, Si is not considered as an essential element for plants, since it is dispensable for the completion of the life cycle of most species, with the significant exception of horsetail (Chen and Lewin, 1969). In plants, the final concentration of this element varies between 0.1 to 10% Si in top dry weight, with the strongest Si accumulators belonging to the orders Poales and Equisetales (Hodson et al., 2005). Most importantly, the plant species that accumulate the highest amounts of Si seem to benefit the most from its protective effects (Epstein, 1999; Arsenault-Labrecque et al., 2011). Plants take up Si from the soil solution as silicic acid [$Si(OH)_4$], through transporters recently discovered in rice (Ma et al., 2006). The Si transport from the soil solution to the shoots involves influx and efflux by two different families of proteins (Ma, 2010).

Firstly, Si influx transporters, also known as Lsi1, are passive channels belonging to the NIP subfamily (nodulin 26-like proteins)

Abbreviations: Si, silicon; PCR, polymerase chain reaction; NCBI, national center for biotechnology information; RT-PCR, reverse transcriptase PCR; CDS, coding DNA sequence; cDNA, complementary DNA; cRNA, complementary RNA; GFP, green fluorescent protein; DEPC, diethylpyrocarbonate; RACE, rapid amplification of cDNA ends.

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of aquaporins (Gomes et al., 2009; Deshmukh and Bélanger, 2015). These transporters allow the passive passage of silicic acid from the soil solution to the root cells. Lsi1 homologues have been identified originally in rice (*Oryza sativa*; Ma et al., 2006) and in many other plant species since (Chiba et al., 2009; Mitani et al., 2009a; Mitani et al., 2011; Deshmukh et al., 2013; Deshmukh et al., 2015). Functional Lsi1s have been reported to be essential for a plant species to accumulate Si (Deshmukh et al., 2015). Secondly, Si efflux transporters, also called Lsi2, are transmembrane proteins with 9–12 transmembrane domains, and belong to the less-studied family of putative anion transporters. Lsi2s are believed to be active transporters, driven by the proton gradient, based on experiments using protonophores (Ma, 2010). Unlike Lsi1s, only a few Si efflux transporters have been discovered so far in higher plants, namely in rice, barley, maize and pumpkin (Ma et al., 2007; Mitani et al., 2009b; Mitani-Ueno et al., 2011). Si transporters were also discovered in the salt-water unicellular diatoms, (Hildebrand et al., 1997) but have no sequence similarity with those from plants.

Equisetum is generally considered a living fossil and has great importance in ecological and evolutionary studies. The genus *Equisetum* is monophyletic, with around 30 species, and is a pteridophyte, a relative of living ferns (Des Marais et al., 2003). *Equisetum arvense* (horsetail) has been used as a model to study silicon mineralization in land plants because of its extremely high Si content (Bauer et al., 2011). But until recently Si transport mechanism in horsetails was totally unknown at the molecular level probably due to the lack of resources and a very large genome of 14 Gb (Bainard et al., 2011; Grégoire et al., 2012). We recently identified a multigene family of horsetail Si influx transporters that have distinct characteristics from those of higher plants (Grégoire et al., 2012). In this study, we report the discovery of two unique Si efflux transporters from horsetail. The properties and Si efflux activity of these proteins were investigated using plant and *Xenopus* oocytes heterologous expression systems. Our results support the concept that these Si efflux transporters, in conjunction with the numerous Si influx transporters, explain the outstanding Si accumulation of horsetail.

2. Materials and methods

2.1. Plant material

Horsetail material was collected from a natural colony of asexually reproducing plants growing at the Jardin Van den Hende botanical garden on the Université Laval campus, Québec, Canada. For RNA and genomic DNA extractions, roots were collected from a single plant in late June, during a period of active growth. For the Si uptake experiment (see below), horsetail, Arabidopsis and rice plants were grown in a greenhouse under a 16 h/8 h photoperiod, 22 °C day/18 °C night, 80% humidity, in 20 cm pots containing commercial potting mix (Connaisseur Premium Potting Soil, Fafard, http://www.fafard.com) and fertilized twice a week with a modified Hoagland solution containing 1.7 mM Si as potassium silicate (Kasil #6, National Silicates, http://www.silicates.com; Guével et al., 2007). Potassium levels in the Hoagland solution were adjusted in control conditions to compensate for the additional input of K from potassium silicate.

2.2. Scanning electron microscopy (SEM) and X-ray microanalysis mapping

Scanning electron microscopy and X-ray microanalysis mapping were used to locate Si deposition in soil-grown horsetail fed with Si for 30 days. Shoot samples were prepared as described by Guével et al. (2007). Briefly, shoots were lyophilized and coated with gold and palladium to provide conductivity to the samples. Samples were analyzed using a CAMECA SX-100 Universal EPMA microscope (Cameca instruments Inc, www.cameca.com) operating at a voltage of 15 kV and a current of 20 nA.

2.3. Nucleic acid extraction and cDNA synthesis

Horsetail total RNA was extracted from roots and shoots using TRIzol reagent (Invitrogen, http://www.invitrogen.com) according to the manufacturer's instructions, checked for integrity on a denaturing agarose gel, and stored at −80 °C until use. Arabidopsis total RNA was extracted from roots and shoots using an RNA purification kit (Qiagen, http://www.qiagen.com) and stored at −80 °C until use. Horsetail genomic DNA were extracted from leaves and stems using a DNeasy plant mini kit (Qiagen), and stored at −80 °C until use. For horsetail, Arabidopsis and rice, first-strand cDNAs were prepared from 1 μg total RNA treated with RQ1 RNase-free DNase (Promega, http://ww.promega.com), then reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen) and oligo$(dT)_{18}$ primers.

2.4. Identification and cloning of horsetail EaLsi2 genes

A horsetail transcriptome database was constructed as described in Grégoire et al. (2012). To identify possible homologues of Lsi2 in horsetail, the assembled contigs were searched by tBLASTn using known full-length Lsi2 sequences from rice (Ma et al., 2006) and pumpkin (Mitani-Ueno et al., 2011). The candidate *EaLsi2-1* and *EaLsi2-2* full-length coding sequences (CDS) were amplified using the high-fidelity Phusion DNA polymerase (New England Biolabs, http://www.neb.com) from horsetail root cDNA using specific primers listed in Supplementary Table 1. Full length CDS were amplified and subcloned in the pGEM-T-Easy vector (Promega, www.promega.com). Subsequent Sanger sequencing of cloned products on a 3730xl DNA Analyzer (Applied Biosystems, http://www.appliedbiosystems.com) revealed two distinct CDS. These constructions were named pGEM.EaLsi2-1 and pGEM.EaLsi2-2. For each CDS, eight clones were sequenced. To obtain full-length cDNA sequences, both 5′ and 3′ RACE (Frohman et al., 1988) were performed using HotMaster Taq DNA (5 PRIME, http://www.5prime.com). The amplification products were subcloned into pGEM-T Easy vector. For each construction, ten clones were sequenced. After sequencing, RNA-Seq reads were mapped back to the cloned cDNA sequences using CLC Genomic Workbench (http://www.clcbio.com/) to correct any sequencing or enzymatic errors. This also allowed a digital comparison of expression levels of both *Lsi2* genes in roots. To sequence the full-length *EaLsi2* genes, PCR was performed on genomic DNA using Phusion DNA polymerase and the same primers used for CDS cloning (Supplementary Table 1). A 7 kb PCR product was visualized on an agarose gel, and subcloned into pGEM-T Easy vector. For each gene, 12–20 clones were sequenced using internal primers that were designed as needed. To identify the intron–exon gene structure, full gene sequences were compared using Spidey (http://www.ncbi.nlm.nih.gov/spidey) for perfect sequence match with the corresponding cloned cDNA. The translated EaLsi2-1 protein sequence was used to perform a BLASTp search against the nr database at the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Reverse-transcription (RT-PCR) was performed on horsetail root and shoot cDNAs using primers EaLsi2 F/EaLsi2 R535 (Supplementary Table 1). The resulting PCR products were separated on a 2% agarose gel. Since these primers targeted both genes, the resulting product from shoots was digested with the restriction enzyme *Taq*I to distinguish between the two sequences.