ARTICLE ADDENDUM

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Silicon efflux transporters isolated from two pumpkin cultivars contrasting in Si uptake

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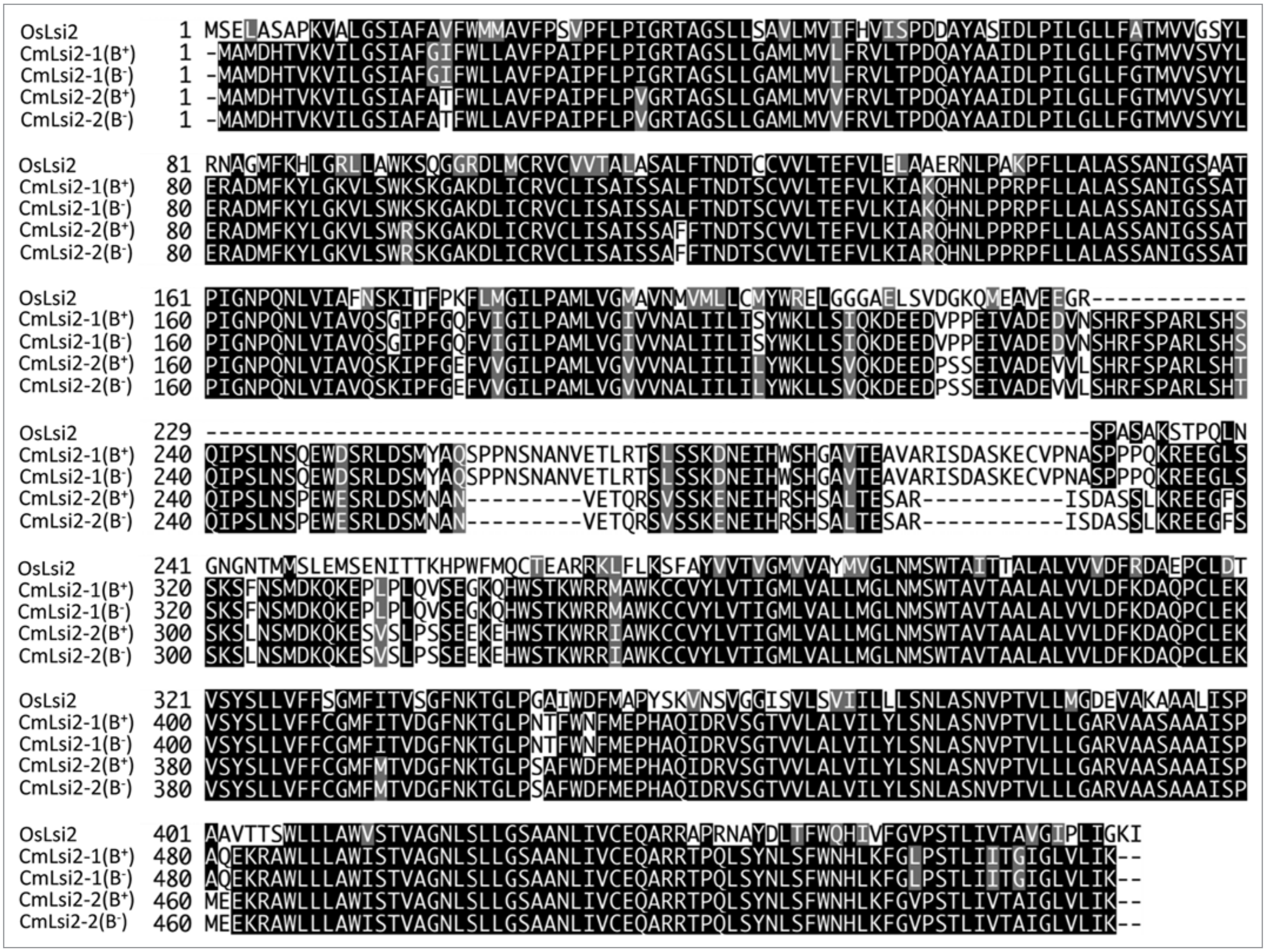
The accumulation of silicon (Si) differs greatly with plant species and cultivars due to different ability of the roots to take up Si. In Si accumulating plants such as rice, barley and maize, Si uptake is mediated by the influx (Lsi1) and efflux (Lsi2) transporters. Here we report isolation and functional analysis of two Si efflux transporters (CmLsi2-1 and CmLsi2-2) from two pumpkin (*Cucurbita moschata* Duch.) cultivars contrasting in Si uptake. These cultivars are used for rootstocks of bloom and bloomless cucumber, respectively. Different from mutations in the Si influx transporter CmLsi1, there was no difference in the sequence of either CmLsi2 between two cultivars. Both CmLsi2-1 and CmLsi2-2 showed an efflux transport activity for Si and they were expressed in both the roots and shoots. These results confirm our previous finding that mutation in CmLsi1, but not in CmLsi2-1 and CmLsi2-2 are responsible for bloomless phenotype resulting from low Si uptake.

Silicon (Si) is the second most abundant elements in earth's crust.[1] Therefore, all plants rooting in soils contain Si in their tissues. However Si accumulation in the shoot differs greatly among plant species, ranging for 0.1 to 10% of dry weight.[1-3] In higher plants, only Poaceae, Equisetaceae and Cyperaceae show a high Si accumulation.[2,3] Si accumulation also differs with cultivars within a species.[4,5] These differences in Si accumulation have been attributed to the ability of the roots to take up Si.[6,7]

Genotypic difference in Si accumulation has been used to produce bloomless cucumber (*Cucumis sativus* L.).[8] Bloom (white and fine powders) on the surface of cucumber fruits is primarily composed of silica (SiO_2).[9] However, nowadays, cucumber without bloom (bloomless cucumber) is more popular in Japan due to its more attractive and distinctly shiny appearance. Bloomless cucumber is produced by grafting cucumber on some specific pumpkin (*Cucurbita moschata* Duch.) cultivars. These pumpkin cultivars used for bloomless cucumber rootstocks have lower silicon accumulation compared with the rootstocks used for producing bloom cucumber.[9]

Our study showed that the difference in Si accumulation between bloom and bloomless root stocks of pumpkin cultivars results from different Si uptake by the roots.[10] Si uptake has been demonstrated to be mediated by two different types of transporters (Lsi1 and Lsi2) in rice, barley and maize.[11-15] Lsi1 is an influx transporter of Si, belonging to a NIP subfamily of aquaporin family.[10,11,13,14] This transporter is responsible for transport of Si from external solution to the root cells.[11] On the other hand, Lsi2 is an efflux transporter of Si, belonging to putative anion transporter.[12] Lsi2 releases Si from the root cells towards the xylem. Both Lsi1 and Lsi2 are required for Si uptake by the roots.[11,12] To understand the mechanism underlying genotypic difference in Si uptake, we have isolated and functionally characterized an influx Si transporter CmLsi1 from two pumpkin cultivars used for rootstocks of bloomless



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OsLsi2        1 MSELASAPKVALGSIAFAVFWMMAVFPSVPFLPIGRTAGSLLSAVLMVIFHVISPDDAYASIDLPILGLLFATMVVGSYL
CmLsi2-1(B+)  1 -MAMDHTVKVILGSIAFGIFWLLAVFPAIPFLPIGRTAGSLLGAMLMVLFRVLTPDQAYAAIDLPILGLLFGTMVVSVYL
CmLsi2-1(B-)  1 -MAMDHTVKVILGSIAFGIFWLLAVFPAIPFLPIGRTAGSLLGAMLMVLFRVLTPDQAYAAIDLPILGLLFGTMVVSVYL
CmLsi2-2(B+)  1 -MAMDHTVKVILGSIAFATFWLLAVFPAIPFLPVGRTAGSLLGAMLMVVFRVLTPDQAYAAIDLPILGLLFGTMVVSVYL
CmLsi2-2(B-)  1 -MAMDHTVKVILGSIAFATFWLLAVFPAIPFLPVGRTAGSLLGAMLMVVFRVLTPDQAYAAIDLPILGLLFGTMVVSVYL

OsLsi2       81 RNAGMFKHLGRLLAWKSQGGRDLMCRVCVVTALASALFTNDTCCVVLTEFVLELAAERNLPAKPFLLALASSANIGSAAT
CmLsi2-1(B+) 80 ERADMFKYLGKVLSWKSKGAKDLICRVCLISAISSALFTNDTSCVVLTEFVLKIAKQHNLPPRPFLLALASSANIGSSAT
CmLsi2-1(B-) 80 ERADMFKYLGKVLSWKSKGAKDLICRVCLISAISSALFTNDTSCVVLTEFVLKIAKQHNLPPRPFLLALASSANIGSSAT
CmLsi2-2(B+) 80 ERADMFKYLGKVLSWRSKGAKDLICRVCLISAISSAFFTNDTSCVVLTEFVLKIARQHNLPPRPFLLALASSANIGSSAT
CmLsi2-2(B-) 80 ERADMFKYLGKVLSWRSKGAKDLICRVCLISAISSAFFTNDTSCVVLTEFVLKIARQHNLPPRPFLLALASSANIGSSAT

OsLsi2       161 PIGNPQNLVIAFNSKITFPKFLMGILPAMLVGMAVNMVMLLCMYWRELGGGAELSVDGKQMEAVEEGR------------
CmLsi2-1(B+) 160 PIGNPQNLVIAVQSGIPFGQFVIGILPAMLVGIVVNALIILISYWKLLSIQKDEEDVPPEIVADEDVNSHRFSPARLSHS
CmLsi2-1(B-) 160 PIGNPQNLVIAVQSGIPFGQFVIGILPAMLVGIVVNALIILISYWKLLSIQKDEEDVPPEIVADEDVNSHRFSPARLSHS
CmLsi2-2(B+) 160 PIGNPQNLVIAVQSKIPFGEFVVGILPAMLVGVVVNALIILILYWKLLSVQKDEEDPSSEIVADEVVLSHRFSPARLSHT
CmLsi2-2(B-) 160 PIGNPQNLVIAVQSKIPFGEFVVGILPAMLVGVVVNALIILILYWKLLSVQKDEEDPSSEIVADEVVLSHRFSPARLSHT

OsLsi2       229 --------------------------------------------------------------------SPASAKSTPQLN
CmLsi2-1(B+) 240 QIPSLNSQEWDSRLDSMYAQSPPNSNANVETLRTSLSSKDNEIHWSHGAVTEAVARISDASKECVPNASPPPQKREEGLS
CmLsi2-1(B-) 240 QIPSLNSQEWDSRLDSMYAQSPPNSNANVETLRTSLSSKDNEIHWSHGAVTEAVARISDASKECVPNASPPPQKREEGLS
CmLsi2-2(B+) 240 QIPSLNSPEWESRLDSMNAN---------VETQRSVSSKENEIHRSHSALTESAR-----------ISDASSLKREEGFS
CmLsi2-2(B-) 240 QIPSLNSPEWESRLDSMNAN---------VETQRSVSSKENEIHRSHSALTESAR-----------ISDASSLKREEGFS

OsLsi2       241 GNGNTMMSLEMSENITTKHPWFMQCTEARRKLFLKSFAYVVTVGMVVAYMVGLNMSWTAITTALALVVVDFRDAEPCLDT
CmLsi2-1(B+) 320 SKSFNSMDKQKEPLPLQVSEGKQHWSTKWRRMAWKCCVYLVTIGMLVALLMGLNMSWTAVTAALALVVLDFKDAQPCLEK
CmLsi2-1(B-) 320 SKSFNSMDKQKEPLPLQVSEGKQHWSTKWRRMAWKCCVYLVTIGMLVALLMGLNMSWTAVTAALALVVLDFKDAQPCLEK
CmLsi2-2(B+) 300 SKSLNSMDKQKESVSLPSSEEKEHWSTKWRRIAWKCCVYLVTIGMLVALLMGLNMSWTAVTAALALVVLDFKDAQPCLEK
CmLsi2-2(B-) 300 SKSLNSMDKQKESVSLPSSEEKEHWSTKWRRIAWKCCVYLVTIGMLVALLMGLNMSWTAVTAALALVVLDFKDAQPCLEK

OsLsi2       321 VSYSLLVFFSGMFITVSGFNKTGLPGAIWDFMAPYSKVNSVGGISVLSVIILLLSNLASNVPTVLLMGDEVAKAAALISP
CmLsi2-1(B+) 400 VSYSLLVFFCGMFITVDGFNKTGLPNTFWNFMEPHAQIDRVSGTVVLALVILYLSNLASNVPTVLLLGARVAASAAAISP
CmLsi2-1(B-) 400 VSYSLLVFFCGMFITVDGFNKTGLPNTFWNFMEPHAQIDRVSGTVVLALVILYLSNLASNVPTVLLLGARVAASAAAISP
CmLsi2-2(B+) 380 VSYSLLVFFCGMFMTVDGFNKTGLPSAFWDFMEPHAQIDRVSGTVVLALVILYLSNLASNVPTVLLLGARVAASAAAISP
CmLsi2-2(B-) 380 VSYSLLVFFCGMFMTVDGFNKTGLPSAFWDFMEPHAQIDRVSGTVVLALVILYLSNLASNVPTVLLLGARVAASAAAISP

OsLsi2       401 AAVTTSWLLLAWVSTVAGNLSLLGSAANLIVCEQARRAPRNAYDLTFWQHIVFGVPSTLIVTAVGIPLIGKI
CmLsi2-1(B+) 480 AQEKRAWLLLAWISTVAGNLSLLGSAANLIVCEQARRTPQLSYNLSFWNHLKFGLPSTLIITGIGLVLIK--
CmLsi2-1(B-) 480 AQEKRAWLLLAWISTVAGNLSLLGSAANLIVCEQARRTPQLSYNLSFWNHLKFGLPSTLIITGIGLVLIK--
CmLsi2-2(B+) 460 MEEKRAWLLLAWISTVAGNLSLLGSAANLIVCEQARRTPQLSYNLSFWNHLKFGVPSTLIVTAIGLVLIK--
CmLsi2-2(B-) 460 MEEKRAWLLLAWISTVAGNLSLLGSAANLIVCEQARRTPQLSYNLSFWNHLKFGVPSTLIVTAIGLVLIK--
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Figure 1. Alignment of amino acid sequence of Lsi2-like Si efflux transporters in rice (OsLsi2) and pumpkin. Two copies of Lsi2-like gene encoding CmLsi2-1 and CmLsi2-2 were obtained from pumpkin cultivars; Shintosa (B^+) and Super-unryu (B^-).

and bloom cucumber.[10] Sequence analysis showed only two amino acids difference of CmLsi1 between two pumpkin cultivars. However, CmLsi1 from bloom rootstock [CmLsi1(B^+)] showed transport activity for Si, whereas that from bloomless rootstock [CmLsi1(B^-)] did not.[10] Furthermore, we found that loss of Si transport activity was caused by one amino acid mutation at the position of 242 (from proline to leucine).[10] This mutation resulted in failure to be localized at the plasma membrane, which is necessary for functioning as an influx transporter. The mutated protein was localized at the ER.[10] Here, we report isolation and expression analysis of Si efflux transporters from two pumpkin cultivars contrasting in Si uptake and accumulation to examine whether Si efflux transporter is also involved in the bloom and bloomless phenotypes.

Isolation of Si Efflux Transporters from Pumpkin

Based on the conserved sequence between *OsLsi2* and melon EST information, we designed the degenerated primers and performed PCR to obtain partial sequences of pumpkin *Lsi2* from two cultivars [Shintosa (B^+) and Super-unryu (B^-)] used for rootstocks of bloom and bloomless cucumber, respectively.[16] With the help of 5'- and 3'-RACE (Rapid Amplification of cDNA End), we were able to obtain two copies of the Si efflux transporter candidate genes, *CmLsi2-1* and *CmLsi2-2*, from either cultivar. There was no difference in the nucleotide sequences of either gene between two pumpkin cultivars (GenBank/EMBL accession numbers: AB551951 and AB551952; **Figs. 1 and 2** in amino acid sequence).

CmLsi2-1 and *CmLsi2-2* genes encode a peptide with 549 and 529 amino acids, respectively (**Figs. 1 and 2**). They shared 87% identity each other at the amino acid level. CmLsi2-1 and CmLsi2-2 share 80% identity with Os10g0547500, a homolog of OsLsi2 in rice (**Fig. 2**). The identity of CmLsi2-1 and CmLsi2-2 with OsLsi2 was 67% and 63%, respectively; at amino acid level (**Figs. 1 and 2**).

Efflux Activity of Si of CmLsi2-1 and CmLsi2-2

To investigate whether CmLsi2-1 and CmLsi2-2 have an efflux transport activity for silicic acid (Si) like OsLsi2, they were heterologously expressed in the *Xenopus laevis* oocytes. When 1 mM Si labeled with ^{68}Ge was preloaded to the oocytes by micro-injector, a significant higher release from the oocytes expressing either *CmLsi2-1* or *CmLsi2-2* was found than no expression control (water injected instead of cRNA) (**Fig. 3**). This result indicates that similar to OsLsi2, both CmLsi2-1 and CmLsi2-2 have an efflux transport activity for Si.

Expression Pattern of CmLsi2-1 and CmLsi2-2

Tissue-specific expression of both *CmLsi2-1* and *CmLsi2-2* was investigated by quantitative real-time RT-PCR in two pumpkin cultivars. Both *CmLsi2-1* and *CmLsi2-2* were expressed in the roots and shoots of either cultivar (**Fig. 4A and B**). This expression pattern is different from *OsLsi2*, *ZmLsi2* and *HvLsi2*, which are mainly expressed in the roots at young stage.[12,15] The expression level of either *CmLsi2-1* or *CmLsi2-2* in the shoots was similar between two cultivars, but that in the roots was higher in the bloom rootstock cultivar than bloomless rootstock cultivar (**Fig. 4A and B**). Although the efflux Si transporter Lsi2 is also important for Si uptake as demonstrated in rice,[12] the lower expression level of both *CmLsi2-1* and *CmLsi2-2* in the bloomless rootstock cultivar is not likely responsible for bloomless phenotype because the influx Si transporter *CmLsi1* in this cultivar is not able to transport Si from external solution to the root cells due to its subcellular mislocalization.[10]

CmLsi2-1 and CmLsi2-2 are the first Si efflux transporters identified in dicotyledonous plant. In monocots such as rice, barley and maize, one Si efflux transporter from each species has been functionally characterized. OsLsi2 from rice is localized at both exodermis and endodermis with polarity,[12] while ZmLsi2 and HvLsi2 are localized only at the endodermis without polarity.[15] In rice genome, there are four homologs of OsLsi2 (**Fig. 2**),[12] which has not been functionally characterized. CmLsi2-1 and CmLsi2-2 may be involved in Si uptake and/or cellular transport of Si. More detailed functional analysis on both CmLsi2-1 and CmLsi2-2 such as cellular localization and tissue specific expression will be required to understand the exact role of these two transporters in pumpkin.

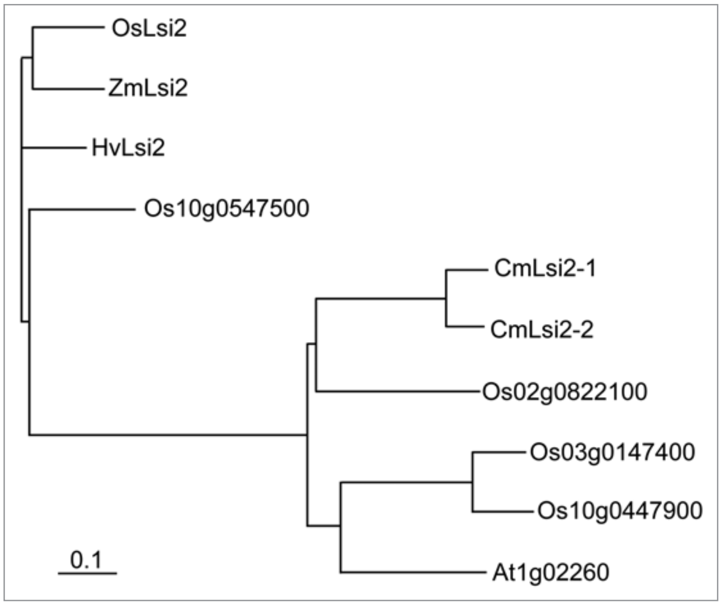

Figure 2. Phylogenetic tree of Si efflux transporter gene homologs in pumpkin (Cm-), rice (Os-), maize (Zm-), barley (Hv-) and Arabidopsis (At-). The 0.1 scale shows substitution distance.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AB551951 and AB551952 for *CmLsi2-1* and *CmLsi2-2*, respectively.

Acknowledgments

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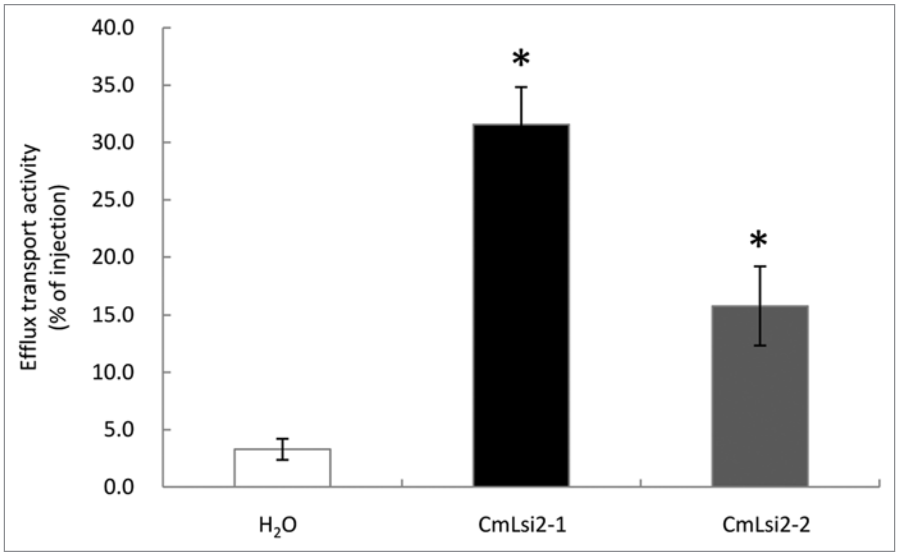

Figure 3. Efflux transport activity for Si in Xenopus oocyte. Oocytes expressing *CmLsi2-1*, *CmLsi2-2* or no expression control (water injected) were injected with Si labeled with ^{68}Ge. The radioactivity released from the oocytes was determined. Data are means ± SD of three biological replicates. * means significant difference at $p < 0.05$ (by student's t test).

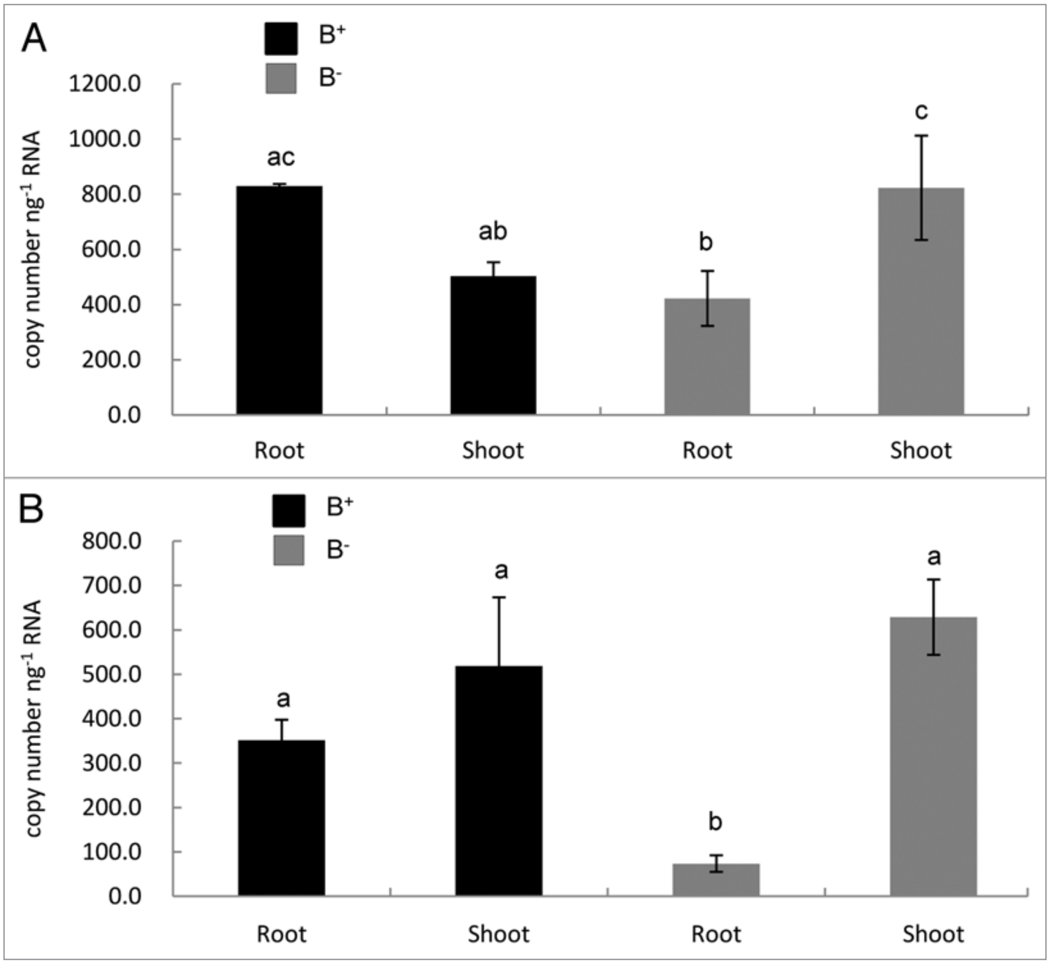

Figure 4. Tissue-specific expression of *CmLsi2-1* (A) and *CmLsi2-2* (B) in the roots and leaves of two pumpkin cultivars: Shintosa (B^+, bloom rootstock) and Super-unryu (B^-, bloomless rootstock). The expression level was determined by absolute quantitative RT-PCR. Data are means ± SD of three biological replicates. Different letters above the columns indicate statistically significant differences at $p < 0.05$ by Tukey's test.