Role of GSL8 in low calcium tolerance in \textit{Arabidopsis thaliana}

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Abstract Calcium (Ca) deficiency affects the yields and quality of agricultural products. Susceptibility to Ca deficiency varies among crops and cultivars; however, its genetic basis remains largely unknown. Genes required for low Ca tolerance in \textit{Arabidopsis thaliana} have been identified. In this study, we identified a novel gene required for low Ca tolerance in \textit{A. thaliana}. We isolated a mutant sensitive to low Ca concentrations and identified Glucan synthase-like (GSL) 8 as a gene responsible for low Ca tolerance. GSL8 is a paralog of the previously identified low Ca tolerance gene GSL10, which encodes β-1,3 glucan(callose) synthase. Under low Ca conditions, the shoot growth of \textit{gsl8} mutants were inhibited compared to wild-type plants. A grafting experiment indicated that the shoot, but not root, genotype was important for the shoot growth phenotype. The ectopic accumulation of callose under low Ca conditions was reduced in \textit{gsl8} mutants. We further investigated the interaction between \textit{GSL8} and \textit{GSL10} by testing the \textit{gsl8 gsl10} double mutant for sensitivity to low Ca concentrations. The double mutant exhibited a more severe phenotype than the single mutant under 0.3 mM Ca, indicating additive effects of \textit{GSL8} and \textit{GSL10} with respect to low Ca tolerance. These results establish that \textit{GSL} genes are required for low Ca tolerance in \textit{A. thaliana}.

Key words: \textit{Arabidopsis thaliana}, calcium deficiency, callose, necrosis, plant nutrition.

Introduction

Calcium (Ca) is one of the essential macronutrients in plants and has a variety of functions (Marschner 2011). Ca interacts with pectin in the cell wall and gelate the pectin and contributes to cell wall structure. Although extracellular Ca concentrations are high (several mM), intracellular concentrations are usually limited to about 1/10,000 of the extracellular concentration (White and Broadley 2003). A transient increase in intracellular Ca concentration allows it to function as a secondary messenger in signal transduction.

At the plant body level, Ca dynamics are closely related to transpiration flow (Gilliham et al. 2011). Ca is mainly transported by transpiration through the apoplastic pathway, with very little absorption and distribution through the symplastic pathway, and tends to be distributed to tissues exhibiting high transpiration (Gilliham et al. 2011). By contrast, young tissues with actively growing cell walls tend to require more Ca as a cell wall component, while their low transpiration results in less Ca distribution (Gilliham et al. 2011).

Thus, Ca deficiency symptoms often occur in young, rapidly enlarging tissues. One characteristic of Ca deficiency symptom is the development of necrosis. Well-known examples include blossom end rot in tomato and tip burn in Chinese cabbage (White and Broadley 2003). Because these tissues are commercially important parts in agriculture, preventing Ca deficiency may help improve agricultural productivity.

Ca fertilization is one option for controlling Ca deficiency symptoms. However, since the distribution of Ca in plants tends to be transpiration-dependent, Ca may not always be delivered to tissues with high Ca requirements. Ca fertilization alone may not be sufficient to completely suppress Ca deficiency.

Breeding cultivars with low Ca tolerance is an approach to control Ca deficiency. For efficient breeding, it is necessary to elucidate the Ca deficiency tolerance mechanisms of plants. In crops, Ca deficiency manifests

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as necrosis, such as blossom-end rot in tomato and tip burn in Chinese cabbage. Necrosis also occurs in *Arabidopsis thaliana* under low-Ca conditions (Shikanai et al. 2020). Resemblance of the symptoms suggests that identification of genes involved in low-Ca tolerance in *A. thaliana* will help to understand the mechanisms of Ca deficiency symptoms in crops. To date, several low Ca tolerance genes have been identified through forward genetic analysis of *A. thaliana* mutants sensitive to low Ca conditions (Li et al. 2017; Shikanai et al. 2015, 2020), including the callose (β-1,3 glucan) synthase gene GSL10 (Shikanai et al. 2020). In our previous analysis of GSL10, we showed that callose synthesis is essential for low Ca tolerance. However, the contribution of the other 11 GSL genes in the Arabidopsis genome to low Ca tolerance remains unknown.

Therefore, in this study, we report the identification and characterization of GSL8 as a low Ca tolerance gene from the same screening system used for identification of GSL10. The results of this study support the importance of GSL genes for plant growth under low Ca conditions and emphasize the need to consider GSL genes in Ca deficiency tolerance breeding.

**Materials and methods**

**Plant materials and growth conditions**

*Arabidopsis thaliana* Col-0 and gsl8-11(lcs6) seeds were obtained from our laboratory stocks. The lcs6 line was isolated as previously described (Shikanai et al. 2015). For growth testing, surface sterilized seeds were sown on media containing nutrients (Yamagami medium; Shikanai et al. 2015) with 1% (w/v) sucrose (84097-250G; Sigma-Aldrich Corp., St. Louis, MO, USA) and 1.5% (w/v) or 0.7% (w/v) purified agar (01056-15; Nacalai Tesque, Kyoto, Japan). The seeds were vernalized at 4°C for 2–7 days and the plates were then positioned vertically with a 16-h light/8-h dark photoperiod at 22°C.

**Plant genotyping**

The primers used in this study are listed in Supplementary Table S1. To determine the gsl8-11(lcs6) and gsl8-12 (CS87712) mutations, we used a derived cleaved amplified polymorphic sequence marker (CAPS) and a cleaved CAPS (dCAPS) were used together with the corresponding restriction enzymes (Supplementary Table S1, Nos. 4–7).

**Grafting experiments**

Plants were grown on MGRL medium (Fujiwara et al. 1992) for 4 days (Col-0) or 5 days (lcs6) prior to grafting to ensure similar growth between lines. At this stage, Col-0 and lcs6 plants used for grafting typically had expanding 1st and 2nd true leaves with tiny 3rd true leaf. After grafting, the plants were incubated on ½× Murashige and Skoog medium for 7 days. Successfully grafted seedlings were transferred to Yamagami medium supplemented with various Ca concentrations as indicated for each experiment for 10 days.

**Aniline blue staining and callose quantification**

Shoots from 5-day-old *A. thaliana* seedlings were excised and incubated overnight at 4°C in a 24-well plate (15–20 shoots per well) with approximately 200 µl of fixative solution (methanol:water:acetic acid=5:4:1; Truernit et al. 2008). The fixative solution was removed, and 200–400 µl of 80% ethanol was then added to each well. The plates were incubated on a heating block at 80°C for 5–10 min (until the samples were colorless). Next, the ethanol was removed from each well and approximately 200 µl of chloral hydrate solution (chloral hydrate:distilled water=5:2 [w/w]) was added. The samples were incubated at 27°C overnight. The chloral hydrate solution was then removed, and the samples were incubated with 1 M glycine (pH 9.5) at 4°C for at least 3 h. Samples were then incubated with 0.1 mg ml⁻¹ aniline blue (016-21302; Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 1 M glycine (pH 9.5) for 2 h. Finally, the samples were mounted on glass slides with a 1:1 mixture of glycerol and 1 M glycine (pH 9.5), and then photographed using a Fluoview 1000 confocal laser scanning microscope (Olympus, Tokyo, Japan). Square images were acquired; each image was centered on the target cotyledon at an optical depth that showed the vascular bundles. The excitation wavelength was 405 nm and emission wavelengths were monitored from 480 to 530 nm. Each image was quantified using Fiji software (Schindelin et al. 2012; https://fiji.sc/); the number of particles above the threshold in the center of each image (i.e., the center square of a 3×3 grid) was counted using the “analyze particles” function. The threshold was set to ignore autofluorescence from dead cells, which was assessed using samples that were treated without aniline blue. The number of particles (equivalent to the number of callose spots) per 1 mm² leaf area was quantified for subsequent analyses.

**Statistical analyses**

Statistical analyses were performed using R software v. 4.0 (R Development Core Team, 2020). The fresh weight data were analyzed using Dunnett’s test or Tukey’s honest significant difference (HSD) test was used. The callose staining data were analyzed using the nonparametric Steel test.

**Results**

**Isolation and characterization of low calcium sensitive (lcs) 6**

We previously isolated and characterized several low Ca sensitive (lcs) mutants of *A. thaliana* (Li et al. 2017; Shikanai et al. 2015, 2020). In the present study, we characterized the lcs6 mutant, which showed slower growth under both normal and low Ca conditions than wild-type Col-0 plants (Figure 1A). Fresh weight of shoots was smaller in the mutant in both conditions (Figure 1B) and the reduction was much more severe.
under low Ca conditions (Figure 1C). On average, the shoot fresh weight of Col-0 (lcs6) plants under low Ca conditions was on average 47% (15%) of that under normal conditions. These results indicate that lcs6 is a poor-growth mutant and that this defect becomes more evident under low Ca conditions; thus, lcs6 is a low Ca sensitive mutant.

Identification of GSL8 as a causal gene for low Ca tolerance

To identify the gene responsible for the low Ca sensitivity of lcs6, we performed map-based cloning using simple sequence length polymorphism (SSLP) markers. We found that the candidate region for the causal gene was located downstream of 12.5 Mbp in chromosome 2 (Figure 2A). In this candidate region, we found Glucan synthase like (GSL) 8, AT2G36850; this is a paralogue of GSL10, which is a callose synthase gene that we had previously identified as a low Ca tolerance gene (Shikanai et al. 2020). Further genomic resequencing of lcs6 revealed that lcs6 contains a mutation that results in a non-synonymous substitution in GSL8 (S1044L) (Figure 2B). The mutation in GSL8 of lcs6 causes amino acid change from serine to leucine, which is highly conserved in homologous proteins of A. thaliana and yeast (Supplementary Figure S1). We also found that a TILLING line CS87712, which has a nonsynonymous mutation in GSL8 (S1088F) (Supplementary Figure S1), exhibited the low Ca sensitivity in a manner similar to lcs6 (Figure 2C, D, E), suggesting that GSL8 is the causal gene of the low Ca sensitivity of lcs6. To confirm this, we performed an allelism test by crossing lcs6 with the T-DNA insertion line, gsl8-9 mutant (SALK_098374) (De Storme et al. 2013). Because functional disruption of GSL8 causes seedling lethality (Guseman et al. 2010) and because we were unable to obtain a homozygous gsl8-9 mutant line, we crossed the gsl8-9 heterozygote with lcs6. We observed both the phenotype and genotype of the F1 crosses between gsl8-9/GSL8 heterozygous and lcs6, and found that the gsl8-9/lcs6 showed low Ca sensitivity, whereas the GSL8/lcs6 did not (Figure 2F). This result indicates that GSL8 is the causal gene of lcs6. Therefore, lcs6 and CS87712 are hereafter referred to as gsl8-11 and gsl8-12, respectively.

Shoot genotype is responsible for low Ca sensitivity

GSL8 is known to be expressed in both shoots and roots (De Storme et al. 2013). To determine whether shoot or root genotypes contribute to low Ca tolerance, we conducted grafting experiments (Figure 3). The results showed that gsl8-11 scion and Col-0 rootstock showed necrosis in newly emerging leaves of gsl8-11 scion and Col-0 rootstock, similar to tip burn, whereas Col-0 scion and gsl8-11 rootstock showed no necrosis. This result suggests that the GSL8 genotype in shoots, but not in roots is a determinant of low Ca sensitivity in shoots. Thus, GSL8 function in shoots is likely to be important for preventing necrosis under low Ca conditions.

Reduction of callose accumulation in gsl8-11 under low Ca

Previous studies have shown that GSL8 is involved in callose synthesis (Chen et al. 2009; Thiele et al. 2009). We previously observed ectopic accumulation of callose under low Ca conditions and that GSL10 contributed to this accumulation (Shikanai et al. 2020). These data made us to test if GSL8 is also involved in callose accumulation of under low Ca conditions.

We measured callose accumulation of in the gsl8-11 mutant under low Ca conditions by fluorescence observation using aniline blue staining. We found that

Figure 1. The lcs6 Arabidopsis thaliana mutant is sensitive to low calcium (Ca) conditions. (A) Shoot growth of wild-type Col-0 and lcs6 mutant plants. Plants were grown under the indicated Ca concentrations for 18 days. Scale bar = 1 cm. (B) Col-0 and lcs6 shoot fresh weight results. Plants were grown under the indicated Ca concentrations for 18 days. Data are means ± standard deviation (SD). Asterisks indicate significant differences compared to Col-0 (Student's t-test, *p < 0.05; n = 15–20). (C) Ratio of Col-0 and lcs6 mutant mean shoot fresh weight under 0.15 and 2 mM Ca conditions, calculated from the data shown in (B).
Calllose accumulation was significantly reduced in the *gsl8-11* compared with Col-0 under low Ca conditions (0.1 mM, Figure 4), as observed in *gsl10-5*. Under the normal Ca conditions (2 mM) no statistical difference was observed. We also observed decreased calllose accumulation in *gsl8-12* under low Ca conditions (Supplementary Figure S2). These results suggest that *GSL8* contributes to increased calllose accumulation under low Ca conditions, in a manner similar to *GSL10*.

**Severe low Ca sensitivity in the *gsl8* *gsl10* double mutant**

The phylogenetic tree of GSL amino acid sequences shows that GSL8 belongs to the same clade as GSL10 (Hong et al. 2001). The results of the present study suggest that GSL8 contributes to low Ca tolerance via a similar mechanism to that of GSL10. To investigate the relationship between GSL8 and GSL10 in the context of low Ca tolerance, we generated a *gsl8-11 gsl10-5* double mutant and evaluated its low Ca sensitivity.

The growth of aerial plant portions was reduced in *gsl8, gsl10*, and the *gsl8 gsl10* double mutants compared...
with that of Col-0 (Figure 5A). At 0.3 mM Ca, the growth reduction of the double mutant was more severe than that of the single mutants (Figure 5A). These observations were confirmed by our shoot fresh weight results; at <0.3 mM Ca, the shoot fresh weight of the gsl8 gsl10 double mutant showed enhanced low Ca sensitivity compared with the single mutant (Figure 5A, B). Thus, GSL8 and GSL10 appear to contribute independently to low Ca tolerance independently at least partially.

Discussion

In this study, we demonstrated that GSL8 is an essential gene for low Ca tolerance in A. thaliana. The gsl8 mutants exhibited a phenotype similar to that of the gsl10 mutant, and the gsl8 gsl10 double mutant showed more severe low Ca sensitivity than the single mutants, suggesting that GSL8 and GSL10 confer low Ca tolerance via similar mechanisms.

Possible involvement of GSL8 phosphorylation for its activity and low Ca tolerance

Non-synonymous substitutions in the gsl8-11 (S1044L) and gsl8-12 (S1088F) mutants were serine residues that are conserved in the GSL family (Supplementary Figure S1). Previous studies have reported evidence of β-1,3 glucan synthase phosphorylation in yeast and A. thaliana (Ellinger and Voigt 2014; Qadota et al. 1996). The S1044 and S1088 residues of GSL8 are located in the large cytoplasmic loop which is annotated as Glycosyl transferase family 48, β-1,3 glucan synthesis catalytic

Figure 4. Ectopic callose accumulation was reduced in gsl8-11 mutant plants under low Ca conditions. (A) Aniline blue callose staining in Col-0 and gsl8-11 cotyledons. Plants were grown under the indicated Ca conditions for 5 days, and then fixed and stained with aniline blue. Scale bar=200 µm. (B) Quantification of callose accumulation in cotyledons. Numbers of particles above the threshold per unit area are shown. Asterisks indicate significant differences compared with Col-0 (Steel test, p<0.05). ns, no significant difference. DIC, differential interference contrast.

Figure 5. Low Ca sensitivity in gsl8 gsl10 double mutant plants. (A) Plants were grown under the indicated Ca conditions for 13 days. Scale bar=1 cm. (B) Shoot fresh weights of the Col-0, gsl8-11, gsl10-5, and gsl8 gsl10 seedlings. Plants were grown under the indicated Ca conditions for 13 days. Different letters indicate significant differences among Ca conditions (Tukey’s honest significant difference test, p<0.05; n=19–20).
domain (Blum et al. 2021; Saatian et al. 2018). Based on NetPhos 3.1 server, these residues are potential phosphorylation sites (Blom et al. 1999). Although no studies have examined whether these residues in gsl8-11 and gsl8-12 are phosphorylation sites, it is possible that the activation of GSL8 through the phosphorylation of these residues is required for callose synthesis and low Ca tolerance.

**Role of callose in low Ca tolerance**

GSL8 encodes a callose synthase; callose is a cell wall polysaccharide that is essential for growth. Callose accumulates transiently during cell plate formation; however, this process is delayed in gsl8-disrupted mutants, resulting in seedling lethal due to insufficient cell plate formation (Thiele et al. 2009). The growth of gsl8-11 and gsl8-12 were worse than that of Col-0 not only under low Ca conditions but also under normal conditions (Figure 2C, D, E); poor growth under normal conditions is possibly caused by the reduction of callose synthesis activity required for normal development.

In addition to its developmental roles, callose synthesis is strongly affected by environmental stimuli such as the disease response. In response to bacterial or fungal attack, ectopic callose accumulates at the site of infection, contributing to the prevention of mycelial penetration and to suppression of symplastic diffusion through plasmodesmata (Ellinger et al. 2013; Gao et al. 2020; Guseman et al. 2010). In both our previous (Shikanai et al. 2008) and present study, we demonstrated that the ectopic callose accumulation under low Ca is reduced in the gsl mutants. These results support the idea that ectopic callose accumulation under low Ca conditions is necessary to suppress necrosis under low Ca. Indeed, the diffusion of both micro- and macromolecules is enhanced in gsl8 leaves (Gao et al. 2020; Guseman et al. 2010). Ectopic callose accumulation may limit the area of necrosis by inhibiting the symplastic diffusion of reactive oxygen species, cytoplasmic Ca, and cell death related proteins via the plasmodesmata.

**Relationship between GSL8 and GSL10 in low Ca tolerance**

GSL8 belongs to the same clade as GSL10, which we previously identified as a low-Ca resistant gene. In the present study, gsl8 and gsl10 mutants showed similar low Ca responses to each other. This result is consistent with those of a previous study of RNAi lines of GSL8 and GSL10 in the Töller et al. (2008). In addition, we analyzed the combination of defects in GSL8 and GSL10 and showed much severer low Ca sensitivity of gsl8 gsl10 double mutant. There are at least two possible reasons why the double mutant shows a stronger phenotype than the single mutants.

One possibility is that GSL8 and GSL10 physically interact with each other, and that the presence of multiple mutations in the GSL8-GSL10 complex destabilizes its callose synthesis. Based on the yeast experimental system, Saatian et al. (2018) proposed that GSL8 and GSL10 may interact with each other. The overlap of weak alleles in gsl8 and gsl10 may have an additive effect on the function of GSL8-GSL10 complex, resulting in enhanced low Ca sensitivity. However, if GSL8 and GSL10 always form a complex and are totally dependent on this complex for its synthesis activity, then GSL8 disruption should also result in male gametophyte lethal, i.e., they should produce no seeds, rather than exhibiting seedling lethal. Our observation indicate that this is not the case, suggesting that the GSL8-GSL10 complex may be formed, but GSL8 and GSL10 function at least in part independently to each other.

The second possibility is that GSL8 and GSL10 synthesize callose independently to each other without forming GSL8-GSL10 complex, and that callose syntheses by both GSL8 and GSL10 are required for normal growth under low Ca conditions. To our knowledge, there is no report that yeast homologous proteins Fks1p and Fks2p form homo- or hetero- dimer (or multimer) for its activity. In a publicly available gene expression database and co-expression database (eFP browser; Winter et al. 2007, ATTED-II; Obayashi et al. 2022), the spatiotemporal expression patterns of GSL8 and 10 are similar to each other, including their response to environmental stimuli, suggesting that GSL8 and 10 contribute to the low-Ca tolerance at similar spatiotemporal locations independently. Based on this assumption, higher sensitivity of the gsl8 gsl10 double mutant than the gsl8 or gsl10 single mutant at 0.3 mM Ca (Figure 5) can be explained as follows: Ca deficiency symptom appears in a callose-amount dependent manner in a certain location in the tissue/cell. In A. thaliana, both GSL8 and GSL10 independently contribute to callose deposition, and this callose-deposition is likely to be more reduced in the double mutant than in the single mutant.

In conclusion, the results of this study confirm the importance of GSL genes for low Ca tolerance breeding. Thus, GSL gene polymorphism in each crop species should be considered when planning breeding varieties resistant to Ca deficiency.

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Author contribution
YS, MA, YE, MY, TK, TF designed research, YS, MA, TS, YE, MY, KY, SS, TK conducted research, YS, MA, YE, MY, TK, TF analyzed the data, YS, TK, TF prepared the manuscript.

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Conflict of interest
The authors declare no conflict of interest.

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