PCP
PLANT & CELL PHYSIOLOGY

Regular Paper

Inhibition of NPR1 Leads to Shoot Growth Improvement under Low-Calcium Conditions in *Arabidopsis*

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(Received 24 May 2023; Accepted 30 August 2023)

Under low-Ca conditions, plants accumulate salicylic acid (SA) and induce SA-responsive genes. However, the relationship between SA and low-Ca tolerance remains unclear. Here, we demonstrated that the inhibition or suppression of nonexpressor of pathogenesis-related 1 (NPR1) activity, a major regulator of the SA signaling pathway in the defense response, improves shoot growth under low-Ca conditions. Furthermore, mutations in *phytoalexin-deficient 4* (*PAD4*) or *enhanced disease susceptibility 1* (*EDS1*), which are upstream regulators of NPR1, improved shoot growth under low-Ca conditions, suggesting that NPR1 suppressed growth under low-Ca conditions. In contrast, growth of *SA induction–deficient 2-2* (*sid2-2*), which is an SA-deficient mutant, was sensitive to low Ca levels, suggesting that SA accumulation by *SID2* was not related to growth inhibition under low-Ca conditions. Additionally, *npr1-1* showed low-Ca tolerance, and the application of tenoxicam—an inhibitor of the NPR1-mediated activation of gene expression—also improved shoot growth under low Ca conditions. The low-Ca tolerance of double mutants *pad4-1*, *npr1-1* and *eds1-22 npr1-1* was similar to that of the single mutants, suggesting that *PAD4* and *EDS1* are involved in the same genetic pathway in suppressing growth under low-Ca conditions as *NPR1*. Cell death and low-Ca tolerance did not correlate among the mutants, suggesting that growth improvement in the mutants was not due to cell death inhibition. In conclusion, we revealed that *NPR1* suppresses plant growth under low-Ca conditions and that the other SA-related genes influence plant growth and cell death.

Keywords: *Arabidopsis* • Calcium deficiency • Growth suppression • NPR1 • Salicylic acid

Introduction

Ca is an important nutrient for plant growth. Insufficient uptake of Ca causes Ca deficiency symptoms in plants, which are characterized by necrosis in the tips of tissues and inhibition of growth (de Freitas and Mitcham 2012). For example, blossom-end-rot, a disorder characterized by a blackened area in tomato fruit, is induced by low Ca levels (Taylor and Locascio 2004).

Several studies have identified the genes that affect the symptoms of Ca deficiency. CAX1 is an H^+/Ca^{2+} antiporter that transports Ca^{2+} from the cytosol to vacuoles (Hirschi et al. 1996). *cax1* mutants show increased low-Ca tolerance (Bradshaw 2005), whereas the overexpression of *CAX1* in crops results in severe Ca deficiency symptoms (Hirschi 1999, Zorrilla et al. 2019). Other genes involved in low-Ca tolerance are the callose synthase genes, *Glucan Synthase–Like* (*GSL*). Callose is synthesized and accumulated in the cell wall under low-Ca conditions, and *gsl10* exhibits growth defects and severe cell death under low-Ca conditions (Shikanai et al. 2020). GSL1 and GSL8 are also involved in low-Ca tolerance (Shikanai et al. 2022a, 2022b), suggesting that supporting the cell wall structure with callose is important for low-Ca tolerance.

A recent study revealed that defense response genes, including salicylic acid (SA)–related genes, are upregulated under low-Ca conditions (Shikanai et al. 2020). Furthermore, a positive correlation between susceptibility to low-Ca conditions and accumulation of SA has been observed in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*; Su et al. 2016). These findings suggest that SA may be involved in growth suppression and cell death development under low-Ca conditions.

Several genes are involved in SA biosynthesis and downstream signaling in defense responses, including *phytoalexin-deficient 4* (*PAD4*) and *enhanced disease susceptibility 1* (*EDS1*) (White 1979, Peng et al. 2021). PAD4 and EDS1 are major regulators of defense responses including resistance to *Peronospora parasitica*, an oomycete pathogen (Parker et al. 1996, Jirage et al. 1999, Lapin et al. 2020). PAD4 and EDS1 function as heterodimers that induce the expression of downstream genes including *SA induction–deficient 2/isochorismate synthase*

Plant Cell Physiol. 64(12): 1579–1589 (2023) doi:https://doi.org/10.1093/pcp/pcad096, Advance Access publication on 31 August 2023, available online at https://academic.oup.com/pcp
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1 (*SID2/ICS1*; Zhou et al. 1998, Feys et al. 2001, Wagner et al. 2013). In *Arabidopsis*, SID2 is responsible for biosynthesizing a majority of SA upon pathogen infection (Wildermuth et al. 2001). Biosynthesized SA binds to the nonexpressor of pathogenesis-related (PR) genes (NPRs), such as NPR1, to promote the expressions of *PR* genes (Moreau et al. 2012, Wang et al. 2020, Peng et al. 2021).

These SA-related genes contribute to the response to various environmental stresses. For example, SA-deficient mutant *nahG* and SA signaling mutant *npr1-1* exhibit salt tolerance (Hao et al. 2012). Mutation in *PAD4*, *EDS1* or *ICS1* leads to a sensitive phenotype to low-Fe stresses (Shen et al. 2016). However, the involvement of SA-related genes in response to low-Ca conditions remains unclear. In the present study, we showed that NPR1 inhibition improves shoot growth under low-Ca conditions. In addition, mutations in *PAD4* or *EDS1* improve growth and their growth-related effects are genetically on the same pathway as *NPR1*. Although *NPR1*, *PAD4* and *EDS1* are involved in SA signaling in pathogen-induced defense responses, SA accumulation by *SID2* does not seem to be the cause of growth suppression under low-Ca conditions. Furthermore, although *npr1-1*, *pad4-1* and *eds1-22* showed low-Ca tolerance, the appearance of symptoms of cell death varied among them, suggesting that growth improvement in the mutants was not due to the inhibition of cell death. Our study provides genetic evidence for the involvement of SA-related genes in growth suppression under low-Ca conditions.

Results

Mutation in *PAD4* or *EDS1* improved shoot growth under low-Ca conditions

In a previous study, mRNA accumulation of SA-related defense response genes, such as *PAD4*, *EDS1* and *SAG101*, was observed under low-Ca conditions using RNA sequencing (Shikanai et al. 2020). We confirmed the induction of these genes by qRT-PCR in Col-0 (**Fig. 1A**). As these are the major regulators of defense responses (Lapin et al. 2020), we focused on the contribution of these three genes to growth under low-Ca conditions using *pad4-1*, *eds1-22* and *sag101-3* mutants. Of these, *pad4-1* is an ethyl methanesulfonate (EMS) mutant (W359stop; Jirage et al. 1999), whereas *eds1-22* and *sag101-3* contain T-DNA insertions in exons *EDS1* and *SAG101* (Chen et al. 2015), respectively.

The effects of mutations on SA-related genes were detected by measuring the mRNA accumulation levels of *PR1*, a marker gene for SA-related defense responses (Linthorst and Van Loon 1991, Peng et al. 2021), in these mutants. The induction of *PR1* under low-Ca conditions has been reported previously (Shikanai et al. 2020). *PR1* expression under low-Ca conditions was lower in all mutants than in Col-0 plants (**Fig. 1B**), suggesting that the defense response was partly impaired in these mutants under low-Ca conditions.

Thereafter, the involvement of *PAD4*, *EDS1* and *SAG101* in growth under low-Ca conditions was evaluated by observing the growth of these mutants under normal- and low-Ca conditions. We found that *pad4-1* and *eds1-22* grew better than Col-0 under low-Ca (0.2 mM and/or 0.3 mM) conditions (**Fig. 1C**, D), whereas growth under the normal conditions was indistinguishable, indicating that *PAD4* and *EDS1* suppress shoot growth only under the low-Ca conditions.

In addition, cell death in newly emerging tissues was investigated as it is one of the symptoms of Ca deficiency disorders, together with growth suppression (de Freitas et al. 2016). Plants were grown in normal-Ca medium for 7 d. Thereafter, the tissues were transferred to low-Ca (0.2 mM) conditions and further incubated for 96 h. New true leaves emerged during the incubation period. We quantified cell death in the leaves using trypan blue staining and found that *pad4-1* and *eds1-22* showed severe cell death in true leaves, while *sag101-3* did not (**Fig. 1E**, **F**). These results suggest that the improved growth of *pad4-1* and *eds1-22* was not due to the alleviation of cell death.

Next, we determined Ca concentrations in the shoots to observe the possible involvement of these genes in Ca distribution. The Ca concentration in *pad4-1* was higher than that in Col-0 under both normal- and low-Ca conditions, whereas the Ca concentrations in *eds1-22* and *sag101-3* were not significantly different (Fig. S1A). Accordingly, there was no clear effect of mutations in SA-related genes on Ca distribution.

In conclusion, *pad4-1* and *eds1-22* mutants showed improved growth under low-Ca conditions, but it is unlikely that this was due to the alleviation of cell death or an increase in the Ca concentration. It is possible that PAD4 and EDS1 play roles in growth suppression under low-Ca conditions.

SA was induced by *SID2* under low-Ca conditions, but *SID2* mutants did not show low-Ca tolerance

PAD4 and EDS1 promote SA-dependent defense responses (Wiermer et al. 2005). Because SA can promote or inhibit plant growth in a dose-dependent manner (Kováčik et al. 2009, Nazar et al. 2015), we investigated how SA accumulation changes under low-Ca conditions. Under low-Ca conditions, SA accumulation in Col-0 plants was approximately 10-fold higher than that under normal conditions (**Fig. 2A**). This result is consistent with a previous report on Chinese cabbage in the viewpoint that SA is elevated under low-Ca conditions (Su et al. 2016).

To evaluate the effect of SA on growth under low-Ca conditions, sodium salicylate (NaSA) was added to the medium and Col-0 plants were grown for 18 d. Under both normal- and low-Ca conditions, the growth of Col-0 was inhibited in an NaSA-dependent manner (**Fig. 2B**, **C**), indicating that high concentrations of exogenous SA can suppress shoot growth, irrespective of Ca conditions.

Next, we observed the growth phenotype of the low-SA mutant *sid2-2* under low-Ca conditions. *SID2* is responsible for the majority of SA biosynthesis in *Arabidopsis* (Wildermuth et al. 2001, Peng et al. 2021), and its mRNA expression was induced under low-Ca conditions (**Fig. 2D**). *sid2-2* accumulated less than 10% of free and total SA under low-Ca conditions compared with Col-0 (**Fig. 2E**), indicating that *SID2* contributes to SA accumulation under low-Ca conditions. However, *sid2-2* was

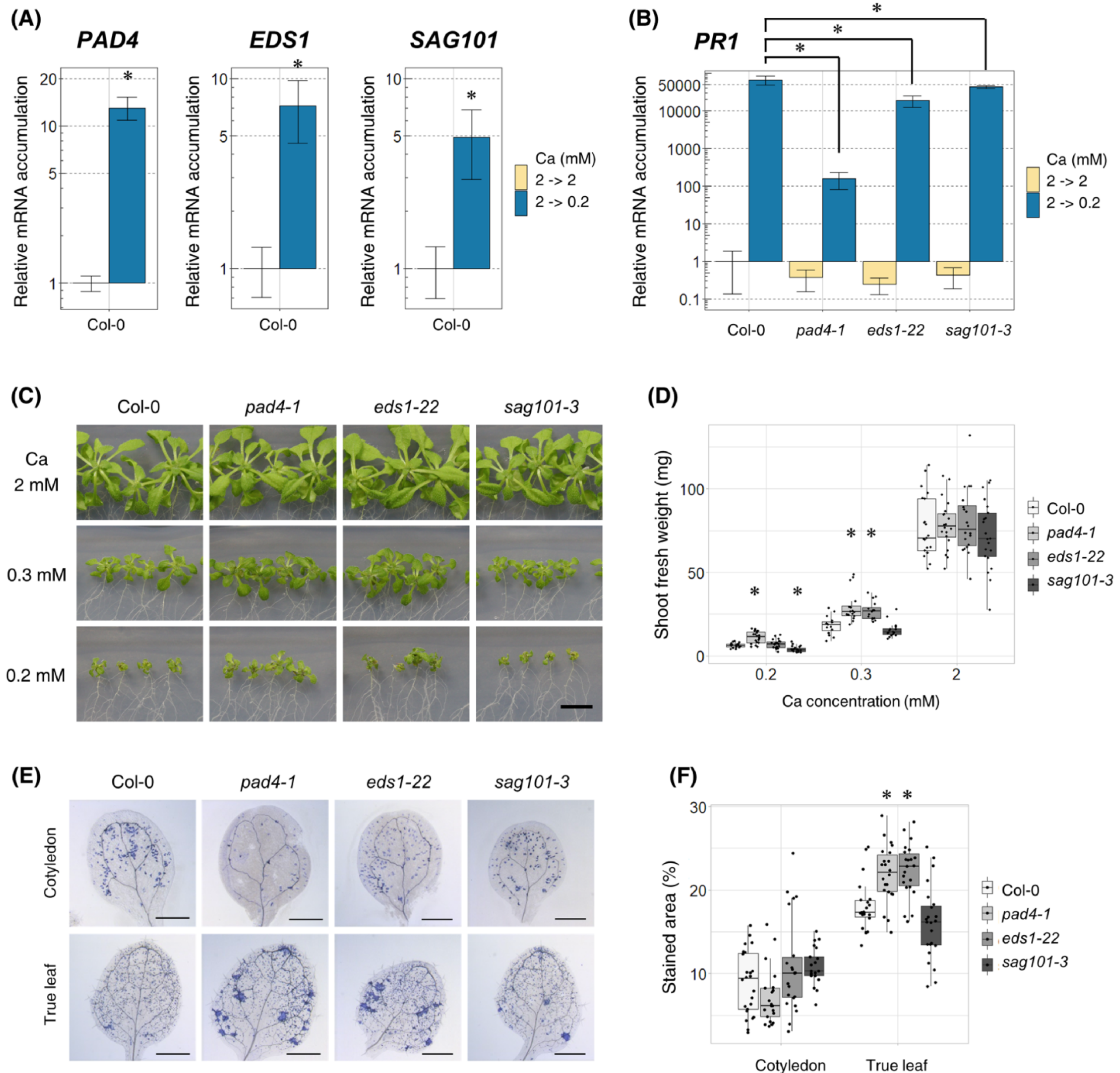

Fig. 1 Mutation in *PAD4* or *EDS1* improved shoot growth under low-Ca conditions. (A) The accumulation of mRNA of *PAD4*, *EDS1* and *SAG101* in Col-0. Plants were grown on 2 mM Ca medium for 7 d, transferred to 2 mM Ca or 0.2 mM Ca medium and grown for 96 h. Relative mRNA accumulation was determined by setting the accumulation under 2 mM Ca to 1. Mean ± SD; $n = 3–4$; Welch's *t*-test; *, $P < 0.05$. (B) Accumulation of *PR1* mRNA in *pad4-1*, *eds1-22* and *sag101-3*. The experiments were performed as described in (A). Mean ± SD; $n = 3–4$; Dunnett's test; *, $P < 0.05$. (C and D) Shoot growth and fresh weight of *pad4-1*, *eds1-22* and *sag101-3* under normal- (2 mM) and low-Ca (0.3 and 0.2 mM) conditions. Plants were grown under the indicated Ca conditions for 18 d. Images of shoots (C) and shoot fresh weight (D). $n = 16–21$; Dunnett's test compared with Col-0 in each condition; * $P < 0.05$; Bar = 1 cm. (E and F) The severity of cell death in *pad4-1*, *eds1-22* and *sag101-3*. Plants were grown on 2 mM Ca medium for 7 d, transferred to 0.2 mM Ca medium and then grown for 96 h. Whole shoots were stained with trypan blue and their cotyledons and true leaves were photographed (E). The stained regions of the leaves were quantified (F). The stained area (%) was calculated by dividing the cell death area by the total leaf area. $n = 21–24$; Dunnett's test compared Col-0 in each tissue; *, $P < 0.05$.

smaller than Col-0 under both normal- and low-Ca conditions, with increased cell death (Fig. 2F–I). These results suggest that the reduced accumulation of SA does not necessarily result in low-Ca tolerance.

Inhibition of NPR1 improved shoot growth under low-Ca conditions

Because *sid2-2* did not show low-Ca tolerance, we hypothesized that another gene in the SA pathway is involved in

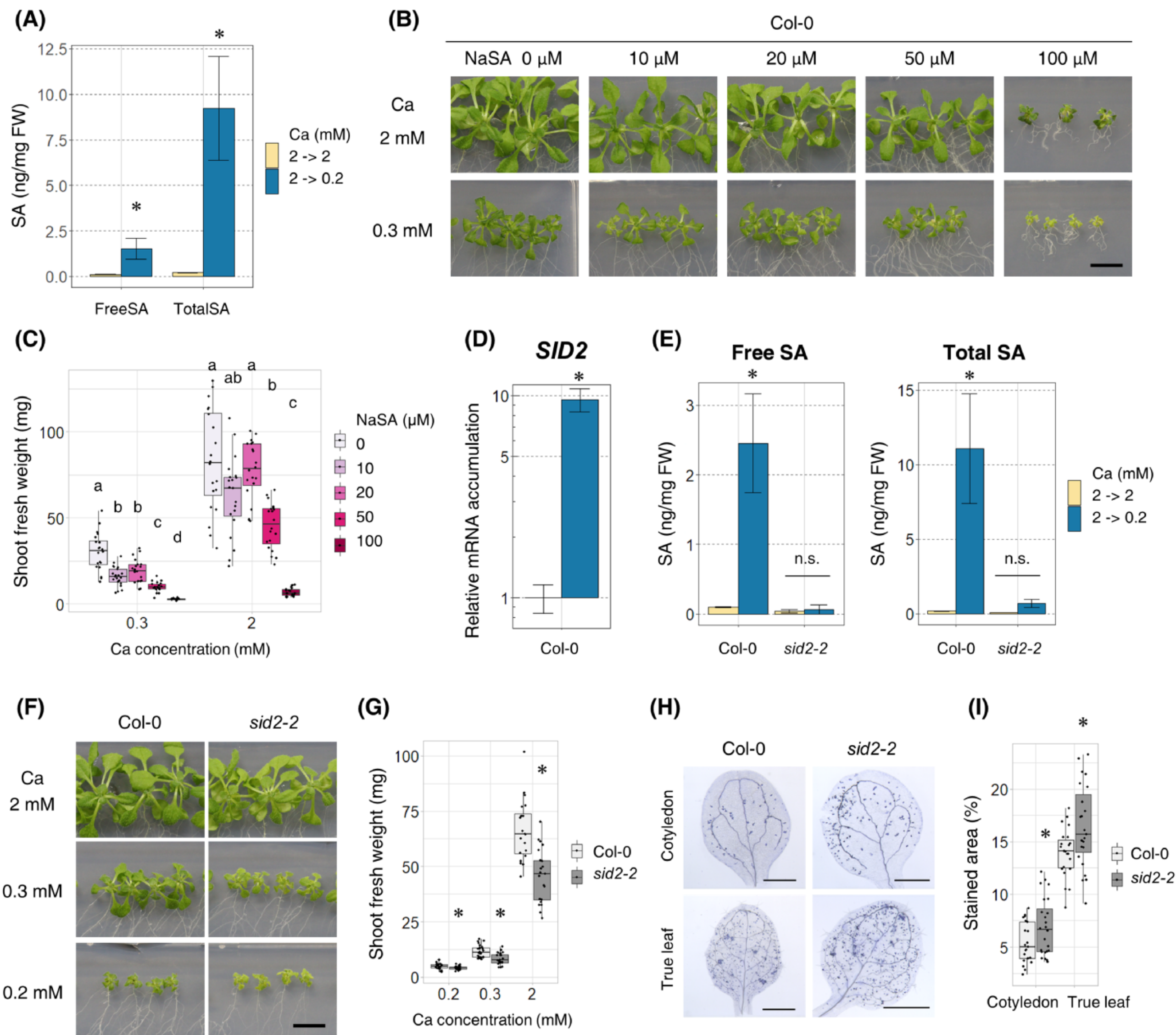

Fig. 2 SA accumulation required *SID2* under low-Ca conditions, but *sid2* mutant did not show low-Ca tolerance. (A) SA accumulation in Col-0. First, plants were grown in 2 mM Ca medium for 7 d, then transferred to 2 mM Ca or 0.2 mM Ca medium and allowed to grow for 96 h. Entire shoots were collected, and SA concentration was measured using HPLC. Mean ± SD; $n = 3$; Welch's *t*-test; *, $P < 0.05$. (B and C) Shoot growth and fresh weight of Col-0 under different concentrations of sodium salicylate (NaSA). Plants were grown with indicated Ca (0.3 and 2 mM) and NaSA (0, 10, 20, 50 and 100 µM) concentrations for 18 d. Shoots were photographed (B), and shoot fresh weight was measured (C). $n = 18–21$; Steel–Dwass test within samples of the same Ca concentration; $P < 0.05$. Different letters indicate significant differences. Bar = 1 cm. (D) The accumulation of *SID2* mRNA in Col-0. First, plants were grown in 2 mM Ca medium for 7 d, then transferred to 2 mM Ca or 0.2 mM Ca medium and allowed to grow for 96 h. The mRNA accumulation levels under the 2 mM Ca condition were set as 1. Mean ± SD; $n = 3–4$; Welch's *t*-test; *, $P < 0.05$. (E) SA accumulation in *sid2-2*. The experiments were performed as in (A). Mean ± SD; $n = 3$; Welch's *t*-test; *, $P < 0.05$. n.s., not significant. (F and G) Shoot growth and fresh weight of *sid2-2*. The experiments were performed as mentioned in (B and C). $n = 20–24$; Dunnett's test; *, $P < 0.05$; bar = 1 cm. (H and I) The severity of death in *sid2-2*. First, plants were grown in 2 mM Ca medium for 7 d, then transferred to 0.2 mM Ca medium and allowed to grow for 96 h. Entire shoot was stained with trypan blue, and true leaves and cotyledons were photographed (H). The stained regions in the leaves were quantified (I). The stained area (%) was calculated by dividing the cell death area by the whole leaf area for each leaf. $n = 21–24$; Welch's *t*-test; *, $P < 0.05$. Abbreviation: n.s., not significant.

growth inhibition independent of SA accumulation. One candidate gene is *NPR1*, which is involved in both SA-dependent and SA-independent defense responses (Lai et al. 2018, Yildiz et al. 2021). RNA sequence analysis has revealed mRNA accumulation of *NPR1* under low-Ca conditions in a previous study (Shikanai et al. 2020), suggesting the possibility of NPR1 involvement in low-Ca tolerance. To assess this, we first confirmed the RNA sequencing results using qRT-PCR, and a

1.5-fold *NPR1* induction was observed in Col-0 under low-Ca conditions (**Fig. 3A**). Next, to evaluate the effect of the NPR1 on low-Ca tolerance, we obtained an *NPR1* mutant (*npr1-1*) and a partial loss-of-function mutant (EMS mutation, H334Y, Cao et al. 1997, Ding et al. 2020). *npr1-1* showed improved growth under low-Ca conditions compared with Col-0 (**Fig. 3B, C**) and reduced cell death in true leaves (**Fig. 3D, E**). The Ca concentration in the shoots of *npr1-1* was not significantly different from that in Col-0 (Fig. S1C). These results suggest that *npr1-1* is low-Ca-tolerant and that this tolerance is not associated with an increase in Ca uptake.

To further confirm the effect of NPR1 on the shoot growth using a different approach, we observed the growth of Col-0 and *npr1-1* plants under different concentrations of tenoxicam (TNX). TNX is known as an anti-inflammatory drug in humans, and in *Arabidopsis*, TNX broadly suppresses SA-responsive genes by reducing NPR1 protein accumulation (Ishihama et al. 2021). In the presence of TNX, the shoot fresh weight of Col-0 under low-Ca conditions was 3–4 times higher than that under non-applied conditions (**Fig. 3F, G**), supporting the idea that inhibition of NPR1 activity can improve growth under low-Ca conditions. *npr1-1* also showed growth recovery at 10 or 20 μM TNX (**Fig. 3H, I**). Considering that the *npr1-1* mutation is not a null allele, the growth recovery of *npr1-1* following TNX application can be explained by the inhibition of NPR1, which partially functions in this mutant. We further tested the effects of TNX on NPR1 activity via *PR1* mRNA accumulation. *PR1* mRNA accumulation was lower under TNX-treated conditions than in non-treated conditions for both Col-0 and *npr1-1*, indicating the inhibition of NPR1 activity by TNX under low-Ca conditions (**Fig. 3J**). Based on these data, we concluded that NPR1 suppresses the shoot growth under low-Ca conditions.

NPR1 is on the same genetic pathway as *PAD4* and *EDS1* for growth suppression under low-Ca conditions

As we revealed that *PAD4*, *EDS1* and *NPR1* inhibited shoot growth under low-Ca conditions, we investigated whether these genes were involved in the same genetic pathway in the suppression of growth under low-Ca conditions. In the defense response, PAD4 and EDS1 function together and indirectly activate NPR1 (Cui et al. 2018; Peng et al. 2021). To test whether they are involved in the same genetic pathway in the suppression of growth under low-Ca conditions, double mutants, *pad4-1 npr1-1* and *eds1-22 npr1-1* were established. Under 0.2 mM Ca, the growth of *pad4-1 npr1-1* was not significantly different from that of *pad4-1* and *npr1-1* single mutants (**Fig. 4A, B**). Under 0.3 mM Ca, *pad4-1 npr1-1* showed similar growth to *pad4-1* but greater growth than *npr1-1*. *eds1-22 npr1-1* showed similar growth to *eds1-22* and *npr1-1* under 0.2 mM and 0.3 mM Ca conditions (**Fig. 4C, D**). These results showed that growth improvement was not an additive effect between *pad4-1* and *npr1-1* or between *eds1-22* and *npr1-1*, suggesting that *PAD4* and *NPR1* and *EDS1* and *NPR1* are in the same genetic pathway that suppresses growth under low-Ca conditions.

As shown in **Figs. 1A** and **3A**, we confirmed that *PAD4*, *EDS1* and *NPR1* are all induced under low-Ca conditions. The induction of these genes has been reported in response to other biotic and abiotic stresses (*Pst* infection, Bartsch et al. 2006, Zhang et al. 2012, low temperature, Chen et al. 2015, Olate et al. 2018), and SA-induced *NPR1* induction by *PAD4* or *EDS1* has been reported (Chen et al. 2021). To analyze whether the three genes were induced by each other under low-Ca conditions, we measured the mRNA accumulation of each gene in the other single mutants. *npr1-1* showed similar upregulated levels of *PAD4* and *EDS1* as Col-0 in response to low Ca, suggesting that their transcript-level regulation is independent of *NPR1* (**Fig. 4E**). In contrast, *pad4-1* did not accumulate *NPR1* under low-Ca conditions (**Fig. 4F**). These results suggest that *PAD4* is upstream and regulates mRNA accumulation of *NPR1*.

Discussion

Growth inhibition by NPR1 under low Ca is possibly regulated by PAD4 and EDS1 independent of SA

Under several abiotic stresses, such as low temperatures (Scott et al. 2004), heavy metals (Tao et al. 2013) and salt (Hao et al. 2012), growth was recovered when SA accumulation was suppressed, suggesting that SA accumulation inhibits growth under these conditions. Under low-Ca conditions, the accumulation of SA or mRNA induction of defense response genes has also been reported under low-Ca conditions (Su et al. 2016, Shikanai et al. 2020, **Fig. 2**). However, in our study, we demonstrated that SA accumulation in *sid2-2* was reduced, whereas growth inhibition under low-Ca conditions was maintained (**Fig. 2**), suggesting that SA accumulation is not likely responsible for growth inhibition under low-Ca conditions.

What could be the reason for the growth inhibition under low-Ca conditions? As shown in **Fig. 3**, a mutation in *NPR1* and TNX application improved growth under low-Ca conditions. In addition to *NPR1*, mutations in either *PAD4* or *EDS1* also improved growth under low-Ca conditions (**Fig. 1D**). The double mutants, *pad4-1 npr1-1* and *eds1-22 npr1-1* showed growth recovery similar to the single mutants (**Fig. 4**). The upregulation of *NPR1* was not observed in *pad4-1* (**Fig. 4F**). Taken together, we propose that PAD4 and EDS1 activate NPR1, leading to growth inhibition under low-Ca conditions in an SA-independent manner (**Fig. 5**).

NPR1 functions as a transcriptional co-activator (Lapin et al. 2020) and regulates gene mRNA accumulation in both SA-dependent and SA-independent manners (Lai et al. 2018, Yildiz et al. 2021). PAD4 and EDS1 form a heterodimer that functions as a major regulator of basal defense (Feys et al. 2001, Wagner et al. 2013). PAD4 and EDS1 induce the mRNA accumulation of genes such as AGD2-LIKE DEFENSE RESPONSE PROTEIN1, which activates NPR1 independent of SA (Cui et al. 2017, Sattely et al. 2018, Lapin et al. 2020, Yildiz et al. 2021). These previous studies support our hypothesis that NPR1 would be activated by PAD4 and EDS1 in an SA-independent manner under low-Ca conditions.

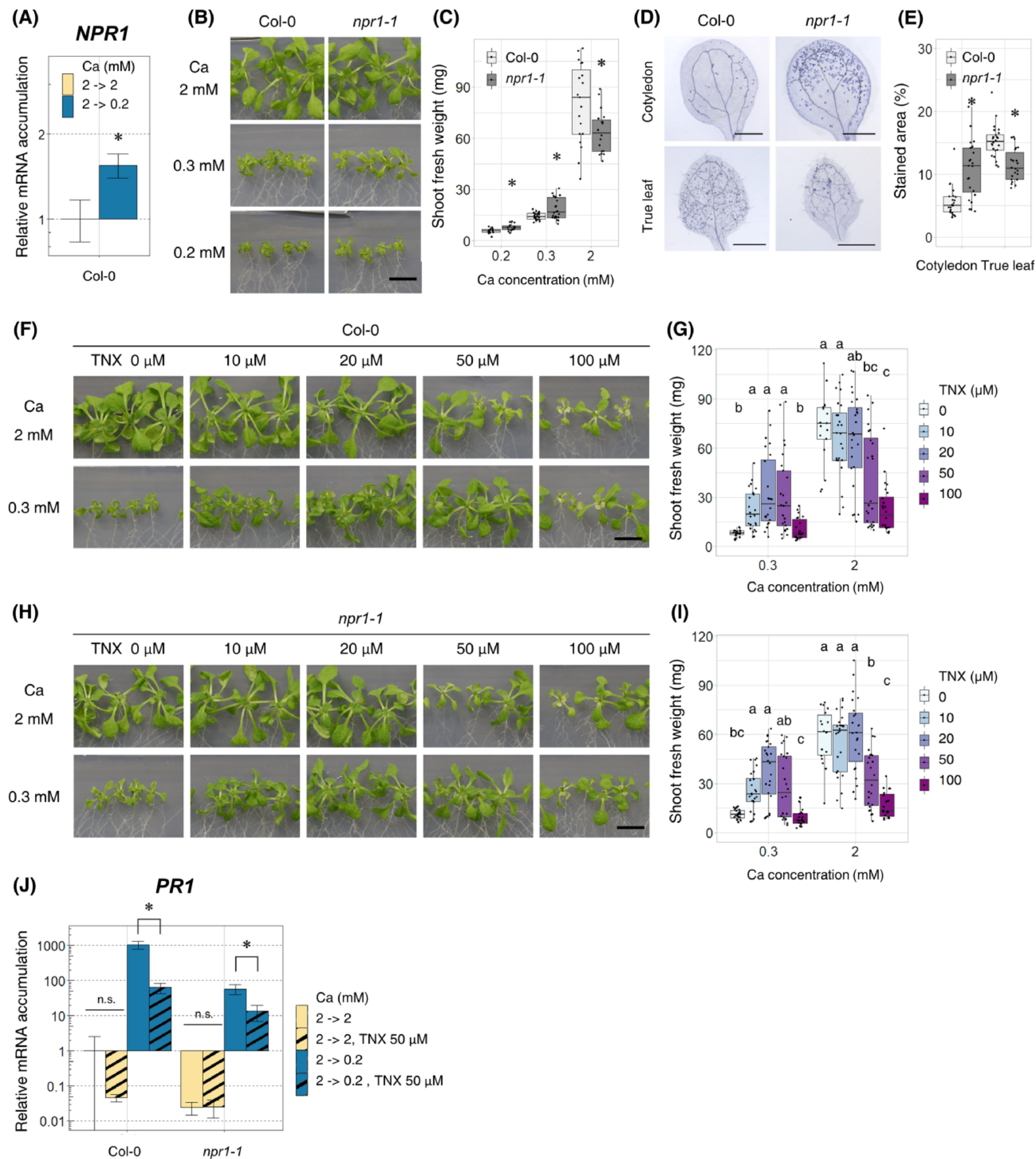

Fig. 3 Inhibition of NPR1 improved the shoot growth under low-Ca conditions. (A) Accumulation of *NPR1* mRNA in Col-0. First, plants were grown in 2 mM Ca medium for 7 d, then transferred to 2 mM Ca or 0.2 mM Ca medium and allowed to grow for 96 h. mRNA accumulation under 2 mM Ca conditions was set as 1. Mean ± SD; $n = 3–4$; Welch's *t*-test; *, $P < 0.05$. (B and C) Shoot growth and fresh weight of *npr1-1* under normal- (2 mM) and low-Ca (0.3 and 0.2 mM) conditions. Plants were grown with indicated Ca conditions for 18 d. Image of shoot was photographed (B), and shoot fresh weight was measured (C). $n = 16–24$; Welch *t*-test; *, $P < 0.05$; bar = 1 cm. (D and E) The severity of cell death of *npr1-1*. First, plants were grown in 2 mM Ca medium for 7 d, then transferred to 0.2 mM Ca medium and allowed to grow for 96 h. Entire shoot was stained with trypan blue, and true leaves and cotyledons were photographed (D). The stained regions in the leaves were quantified (E). The stained area (%) was calculated by division of the cell death area by the whole leaf area for each leaf. $n = 22–24$; Welch's *t*-test; *, $P < 0.05$. (F and G) Shoot growth and fresh weight of Col-0 under different concentrations of TNX. Plants were grown with indicated Ca (0.3 and 2 mM) and TNX (0, 10, 20, 50 and 100 μM) concentrations for 18 d. The image of shoot was photographed (F), and the shoot fresh weight was measured (G). $n = 15–25$; Steel–Dwass test within samples of the same Ca concentration; $P < 0.05$. Different letters indicate significant differences. Bar = 1 cm. (H and I) Shoot growth and fresh weight of *npr1-1* under different concentrations of TNX. The experiments were performed as in (F) and (G). $n = 18–24$; Steel–Dwass test within samples of the same Ca concentration; $P < 0.05$. Different letters indicate significant differences. Bar = 1 cm. (J) Accumulation of *PR1* mRNA in the presence of TNX. Plants were first grown in 2 mM Ca, 0 μM TNX medium for 7 d and then transferred to 2 mM or 0.2 mM Ca and 0 μM or 50 μM TNX medium for 96 h. Average mRNA accumulation levels under 2 mM Ca with the 0 μM TNX condition was set as 1. Mean ± SD; $n = 3–4$; Welch's *t*-test; *, $P < 0.05$. Abbreviation: n.s., not significant.

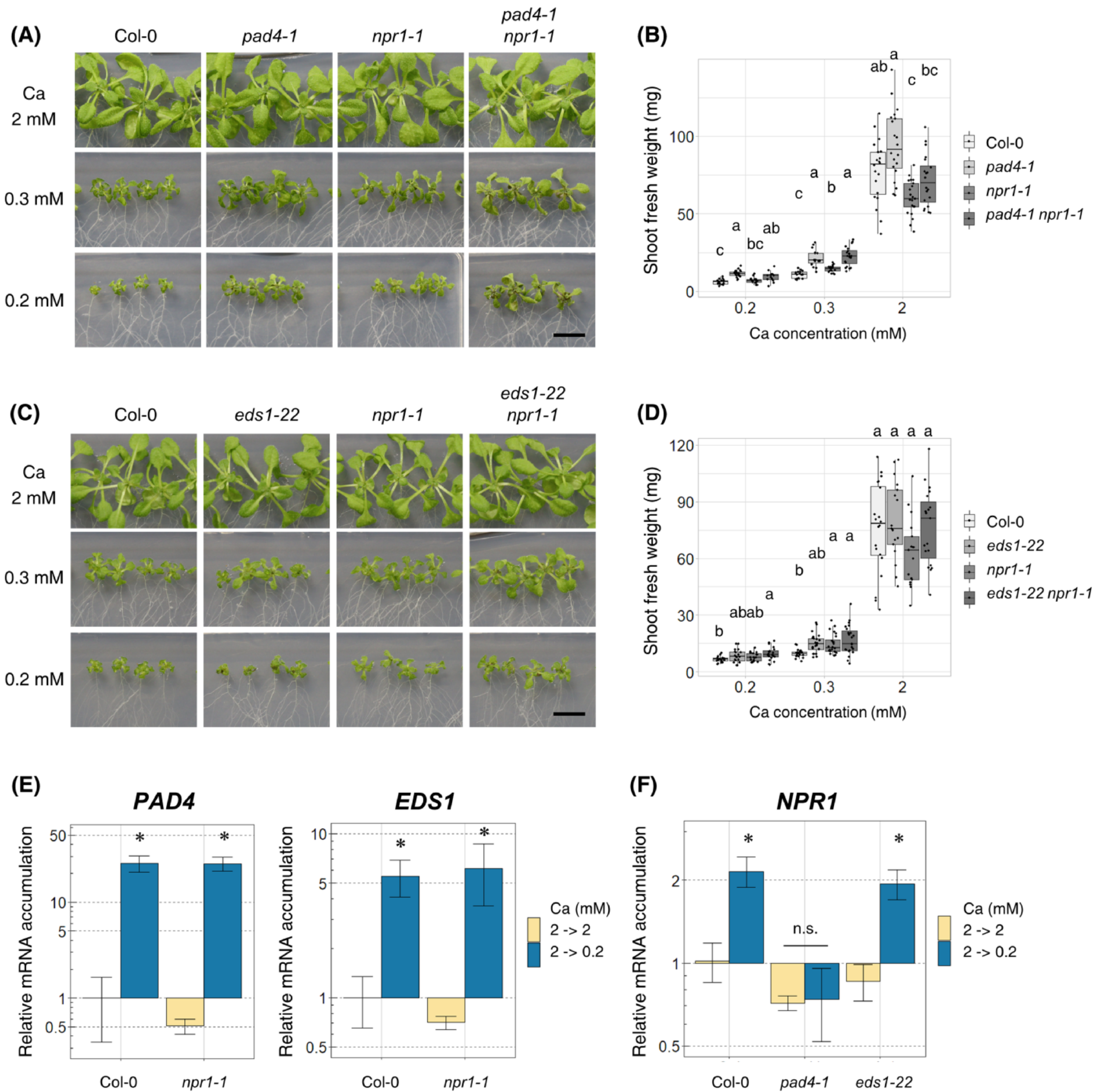

Fig. 4 *NPR1* is included in the same genetic pathway for growth regulation under low-Ca conditions as *PAD4* or *EDS1*. (A and B) Shoot growth and fresh weight of *pad4-1 npr1-1* under normal- (2 mM) and low-Ca (0.3 and 0.2 mM) conditions. Plants were grown with indicated Ca conditions for 18 d. Shoot was photographed (A), and the shoot fresh weight was measured (B). $n = 14–21$; Steel–Dwass test within samples of the same Ca concentration; $P < 0.05$. Different letters indicate significant differences. Bar = 1 cm. (C and D) Shoot growth and fresh weight of *eds1-22 npr1-1*. The experiments were performed as in (A) and (B). $n = 17–24$; Steel–Dwass test within samples of the same Ca concentration; $P < 0.05$. Different letters indicate significant differences. Bar = 1 cm. (E) The mRNA accumulation of *PAD4* and *EDS1* in *npr1-1*. First, plants were grown in 2 mM Ca medium for 7 d, then transferred to 2 mM Ca or 0.2 mM Ca medium and allowed to grow for 96 h. Average mRNA accumulation under 2 mM Ca conditions was set as 1. Mean ± SD. $n = 3–4$; Welch's *t*-test; *, $P < 0.05$. (F) *NPR1* mRNA accumulation in *pad4-1* and *eds1-22*. The experiments were performed as in (E). Mean ± SD. $n = 3-4$. Welch's *t*-test; *, $P < 0.05$. Abbreviation: n.s., not significant.

NPR1 mRNA accumulation was not observed in *pad4-1* mutants under low-Ca conditions (**Fig. 4F**), whereas *NPR1* mRNA was induced in the *eds1-22* mutant, similar to that in Col-0 (**Fig. 4F**), suggesting that PAD4 could be responsible for *NPR1* mRNA accumulation. A previous study has shown that EDS1 forms a heterodimer with PAD4 (Feys et al. 2001, Wagner et al. 2013), suggesting that PAD4 and EDS1 function together. These results suggest that PAD4 may contribute to both *NPR1*

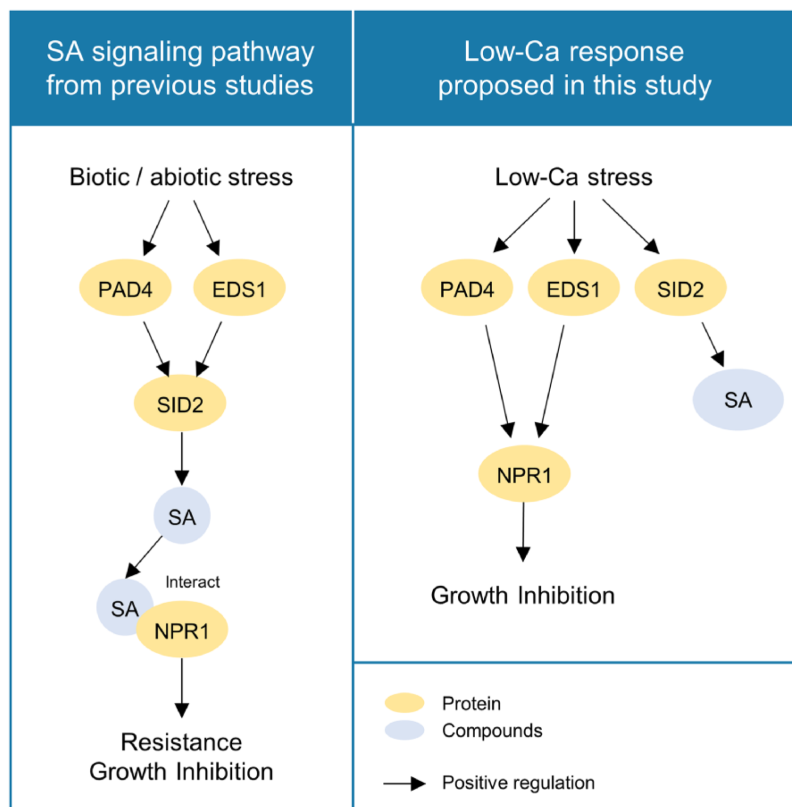

Fig. 5 The proposed model for growth regulation under low-Ca conditions. Left: Reported SA signaling pathway. PAD4 and EDS1 induce mRNA accumulation of *SID2*, which is followed by SA biosynthesis (Zhou et al. 1998). The SA produced binds to NPR1, which induces gene expression, leading to stress resistance and growth inhibition. Right: Proposed SA signaling pathway under low-Ca conditions: mRNA accumulation of *PAD4*, *EDS1* and *SID2* was induced. SA is synthesized by *SID2* induction but is not responsible for growth inhibition. PAD4 alone induces *NPR1*, and also, PAD4 and EDS1 together activates NPR1, leading to growth inhibition.

mRNA accumulation and its protein activity, while EDS1 may contribute to NPR1 activity alone.

How does NPR1 activation inhibit the plant growth under low-Ca conditions? There is limited information available regarding the function of NPR1 in plant growth. Among the few studies conducted, Vanacker et al. (2001) showed that *npr1* has a reduced leaf cell number and increased DNA content. This study suggests that NPR1 promotes cell division and/or suppresses endoreduplication.

Interestingly, under low-Ca conditions, *npr1-1* showed better growth compared to wild type (**Fig. 3C**). This suggests that the activation of NPR1 could prevent growth under low Ca. This is in contradiction to the findings of Vanacker et al. (2001). Based on our study, NPR1 would inhibit growth under low Ca as further growth may exacerbate the Ca deficiency. Further study is needed to elucidate the function of NPR1 in plant growth and the molecular mechanism of NPR1 for growth inhibition under low-Ca conditions.

Possible function of SA in low-Ca tolerance

In this study, we quantified the severity of cell death in SA mutants, as cell death is a representative symptom of Ca deficiency disorder (de Freitas et al. 2016). The accumulation of SA causes a hypersensitive response (HR), a type of programmed cell death, in response to pathogen infection. The degree of HR severity varies depending on the mutant: disruption of *PAD4* or *EDS1* alleviates HR (Rustérucci et al. 2001, Wiermer et al. 2005), whereas *npr1* displays a stronger HR compared to the wild type (Rate and Greenberg 2001). In contrast, under low-Ca conditions, the SA mutants used in this study showed the opposite cell death phenotype: *pad4-1* and *eds1-22* exhibited severe cell death compared to Col-0, whereas *npr1-1* did not (**Fig. 1F**, **3E**). Accordingly, the cell death symptom under low-Ca conditions seems to occur by a different process from HR. *sid2-2*, a mutant defective in SA production, exhibited more severe cell death than Col-0 (**Fig. 2I**). *sid2-2*, a mutant defective in SA production, exhibited more severe cell death than Col-0 (**Fig. 2I**). The result suggested that SA accumulation inhibits cell death under low-Ca conditions. SA contributes to cell wall modification under biotic stress by enhancing lignin and callose production (Li et al. 2017, van Butselaar and Van den Ackerveken 2020). Recent studies have shown that callose plays an important role in alleviating cell death under low-Ca conditions (Shikanai et al. 2020, 2022a, 2022b). Therefore, SA

accumulation under low-Ca conditions may strengthen the cell wall structure, leading to the inhibition of cell death.

Conclusion

In this study, we found that SA-related genes including *NPR1* regulate growth under low-Ca conditions. Our data demonstrated that *PAD4*, *EDS1* and *NPR1* inhibited growth under low-Ca conditions, whereas SA accumulation by *SID2* was unlikely to be the cause of growth inhibition. Additionally, the growth improvement in the mutants are unlikely to be a result of the inhibition of cell death. These findings provide new insights into how plants regulate their growth in low-Ca environments. Our research is expected to be useful for understanding low-Ca responses in plants and their applications in agriculture.

Materials and Methods

Plant materials and growth conditions

Mutants *pad4-1* (CS3806; Jirage et al. 1999), *eds1-22* (SALK_071051; Chen et al. 2015), *sag101-3* (SALK_022911; Chen et al. 2015), *sid2-2* (CS16438; Wildermuth et al. 2001) and *npr1-1* (CS3726; Cao et al. 1997) were obtained. Double mutants *pad4-1*, *npr1-1* and *eds1-22 npr1-1* were generated by crossing, and homozygous seeds were selected by genotyping. The primers used for genotyping are listed in Supplementary Table 1.

The composition of the medium was based on a previous study (Yamagami medium; Shikanai et al. 2015) with some modifications: 1% (w/v) sucrose (Sigma-Aldrich, Burlington, Massachusetts, code 84097-250G) and 1% (w/v) agar (Nacalai Tesque, Kyoto, Japan, code 01056-15). For application of NaSA or TNX, the chemicals were added after autoclaving the remaining medium.

All the wild type (ecotype Col-0) and mutant seeds were surface-sterilized with commercial chlorine bleach and sown on medium plates. The plates were kept at 4°C for 2 d for vernalization and then transferred to a growth chamber. Growth chamber was kept at 22°C, relative humidity 50%, 16 h light and 8 h dark. To measure the shoot fresh weight and Ca concentration in the shoots, plants were grown under indicated Ca conditions for 18 d. For RNA extraction, SA measurement or trypan blue staining, plants were first grown under normal-Ca (2 mM) conditions for 7 d and then transferred to normal-Ca (2 mM) or low-Ca (0.2 mM) conditions for 96 h.

Determination of Ca concentrations

The shoots of 2–6 plants (with dry weight of 5–10 mg) were collected for each sample and dried at 60°C overnight. The dried samples were weighed and transferred to Teflon tubes for nitric acid digestion. After adding 1 ml of nitric acid, the tubes were heated at 100°C until nitric acid evaporated. This step was repeated with 400 µl of nitric acid, followed by the addition of 400 µl of hydrogen peroxide and heating until evaporation. The residues were dissolved in 1 ml of 0.08 N nitric acid and diluted 10 times with 0.08 N nitric acid containing 1 ppb beryllium as an internal standard. Elemental concentrations were measured using inductively coupled plasma mass spectrometry (Agilent 7800).

RNA extraction and qRT-PCR

Total RNA was extracted from frozen shoots harvested from 4–6 plants. Extraction was performed using the NucleoSpin RNA Plant (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. About 300 ng of total RNA was reverse transcribed using PrimeScript RT Master Mix (Perfect Real Time) (Takara Bio) and prepared as 50 µl of cDNA solution. A total of 2 µl of the cDNA solution was used for real-time PCR. Thermal Cycler Dice Real-Time System III (Takara Bio, Kusatsu, Japan) and SYBR Premix Ex Taq II (Tli RNase H Plus) (Takara Bio) were used for real-time PCR. The expressions of *ACT8* were used for normalizing mRNA accumulation of target genes. The primers used in this experiment are listed in Supplemental Table 2.

SA quantification

The shoot from 10–20 plants (80–160 mg) per sample was used for SA quantification. The samples were homogenized in 1 ml of 90% methanol and extracted using 1 ml of 100% methanol. The extracts were mixed and dried at 40°C, and the residues were dissolved in 4 ml of water at 80°C for 15 min. One milliliter of each extract was used for quantifying free and total SA. For total SA quantification, 1 ml of β-glucosidase (Merck, Darmstadt, Germany) solution (3 units/ml) was prepared with 0.1 M sodium acetate buffer, added to 1 ml of extract and incubated at 37°C for 4–6 h. For free SA quantification, 1 ml of 0.1 M sodium acetate buffer was added to 1 ml of extract. Subsequently, 2.5 ml of ethyl acetate–cyclohexane (1:1) and 50 µl of concentrated HCl were added and mixed intensely. The upper layer was dried at 35°C and dissolved in 1 ml of 20% methanol in 20 mM sodium acetate buffer. This solution was used for HPLC analysis (JASCO FP-1520S) using the CAPCELL PAK C18 MG column (5 µm, 150 mm × 2 mm) with 20% methanol in 20 mM sodium acetate buffer at a flow rate of 1 ml/min. Detection was performed at an excitation wavelength of 295 nm and an emission wavelength of 370 nm.

Trypan blue staining and quantification

Trypan blue staining was performed as previously described (Koch and Slusarenko 1990, Fukuda et al. 2016) with some modifications. Briefly, shoots were soaked in ethanol for 1 min and incubated in lactophenol-trypan blue solution (lactic acid:phenol:trypan blue:glycerol:ethanol:distilled water = 5 ml:5 g:5 mg:5 ml:20 ml:5 ml) at 70°C for 10 min. The samples were transferred to chloral hydrate [chloral hydrate:distilled water = 5:2 {w/w)], incubated at 22°C overnight and photographed.

Fiji software (Schindelin et al. 2012) and custom macros were used for the quantification of trypan blue staining results. The stained area (%) was calculated by dividing the cell death area by the leaf size. The cell death area was determined by increasing the color threshold until the leaf veins (xylem) were selected, as veins were stained in all leaves at a level similar to that of the cell death region. Leaf size was measured by selecting the edges of the leaves using the Polygon Sections function of the Fiji software.

Supplementary Data

Supplementary data are available at *PCP* online.

Data Availability

The data underlying this article are available in the article and online supplementary material. Information on the mutants and primers is available in Supplementary Tables.

Funding

The Japan Society for the Promotion of Science KAKENHI Grant-in-Aid for Doctoral Course (DC) Research Fellowships (17J06965) to Y.S., Grant-in-Aid for Scientific Research (S) (19H05637) to T.F., and Grant-in-Aid for Scientific Research (B) to T.K. (21H02087).

Acknowledgments

We thank Atsushi Ishikawa for kindly providing *pad4-1* and *npr1-1*.

Author Contributions

S.H., Y.S., T.F. and T.K. designed the study. S.H., Y.S., M.K. and H.N. performed the experiments. S.H. analyzed the data. S.H., Y.S., T.F. and T.K. wrote the manuscript.

Disclosures

The authors have no conflicts of interest to declare.

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