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ORIGINAL ARTICLE

Proline induces calcium-mediated oxidative burst and salicylic acid signaling

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Abstract Although free proline accumulation is a well-documented phenomenon in many plants in response to a variety of environmental stresses, and is proposed to play protective roles, high intracellular proline content, by either exogenous application or endogenous over-production, in the absence of stresses, is found to be inhibitory to plant growth. We have shown here that exogenous application of proline significantly induced intracellular Ca^{2+} accumulation in tobacco and calcium-dependent ROS production in Arabidopsis seedlings, which subsequently enhanced salicylic acid (SA) synthesis and *PR* genes expression. This suggested that proline can promote a reaction similar to hypersensitive response during pathogen infection. Other amino acids, such as glutamate, but not arginine and phenylalanine, were also found to be capable of inducing *PR* gene expression. In addition, proline at concentration as low as 0.5 mM could induce *PR* gene expression. However, proline could not induce the expression of *PDF1.2* gene, the marker gene for jasmonic acid signaling pathway. Furthermore, proline-induced SA production is mediated by NDR1-dependent signaling pathway, but not that mediated by PAD4. Our data provide evidences that exogenous proline, and probably some other amino acids can specifically induce SA signaling and defense response.

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Introduction

Proline accumulation in higher plant in response to various environmental stresses is a widespread phenomenon, and is believed to play adaptive roles in stress tolerance (Mattioli et al. 2009; Trovato et al. 2008; Verbruggen and Hermans 2008; Szabados and Savour 2010). During stresses, proline was proposed to act as a compatible osmolyte, carbon and nitrogen storage, pH stabilizer, cell redox balancer (Hare and cress 1997), and stress-related growth-regulating signal (Maggio et al. 2002; Khedr et al. 2003). However, the presence of excessive amount of proline in the absence of stress, either by exogenous application or by endogenous over-production, appears to be inhibitory for plant growth and development. Application of 20 mM proline can cause visible growth inhibition of Arabidopsis seedlings (Mani et al. 2002) and lesions on the leaves of plantlet (Deuschle et al. 2004). Transgenic Arabidopsis over-expressing Δ^1-pyrroline-5-carboxylate synthetase (P5CS) and hence over-producing proline exhibited retarded growth and various developmental abnormalities (Mattioli et al. 2008).

The inhibitory effects of proline were found to be associated with the generation of reactive oxygen species (ROS) (Deuschle et al. 2004; Hellmann et al. 2000), which subsequently induces cell death. However, to achieve significant growth inhibition, supra physiological concentration of proline is usually to be applied. It is still controversial as to whether proline toxicity is due to the proline itself or due to its degradation intermediate pyrroline-5-carboxylate (P5C). The results of Mani et al. (2002) showed that antisense-suppression of proline

dehydrogenase (PDH), the rate-limiting enzyme in proline degradation, conferred enhanced sensitivity to proline in Arabidopsis, suggesting that proline itself is responsible for the toxicity. Characterization of T-DNA insertional mutant of *atpdh1* further supported this notion (Nanjo et al. 2003). However, by studying *p5cdh* mutant with elevated level of P5C, Deuschle et al. (2004) proposed that glutamic-γ-semialdehyde (GSA)/P5C rather than proline accounts for proline toxicity.

In mammalian cells, proline metabolism and its regulation were also given special attention. PDH is a mitochondria inner membrane enzyme catalyzing the first step of proline degradation, it was proposed to be capable of transferring electron resulted from oxidizing proline into electron transport chain via its cofactor flavine adenine dinucleotide (FAD). Therefore, proline degradation is a direct donor of electron for mitochondria electron transport chain to generate adenosine-triphosphate (ATP) or ROS. Furthermore, *PDH* was found to be highly induced by p53, a critical tumor suppressor. Therefore, proline oxidation is potentially involved in bioenergetics in some special circumstances and p53-induced apoptosis (Liu et al. 2008).

Recently, it has been reported that the proline cycle which was well characterized in mammalian cells is also present in plant cell (Miller et al. 2009). That is the P5C generated from proline oxidation in mitochondria can be transported to cytosol, and there it can be converted back to proline by P5C reductase. This cycle will help to maintain the intracellular proline to P5C ratio. When high level of externally supplied proline is taken up into the plant cell, the activity of proline cycle will increase, causing the overflow of electrons into the generation of ROS.

Transcriptomic analysis reveals that treatment of proline induces the expression of defense or detoxification-related genes and it represses the expression of genes involved in photosynthesis (Deuschle et al. 2004; Hellmann et al. 2000; Nanjo et al. 2003), suggesting that high concentration of proline in plant may also affect the function of chloroplast.

Previously, it has been reported that several mutants and transgenic plant affecting salicylic acid (SA) content or SA signaling, such as *eds1*, *eds8*, *ndr1*, *NahG* transgenic plant and ecotype Cvi-0 (Cape Verde Islands) demonstrated altered proline sensitivity (Deuschle et al. 2004), implying that proline toxicity might be related to SA signaling.

In this report, we have further characterized the inhibitory effects of proline in Arabidopsis, and found that exogenous proline can induce calcium-mediated generation of H_2O_2 and provoke salicylic acid signaling transduction pathway, but not jasmonic acid signaling pathway. Our results also indicated that this effect occurs at a concentration as low as 0.5 mM and other amino acids, such as arginine and phenylalanine, cannot have this effect at similar concentration.

Table 1 The inhibitory effect of proline on the germination and seedling survival of Arabidopsis (100 seeds were used in each treatment, $n = 3$)

Concentration (mM)	0 mM	20 mM	50 mM	75 mM	100 mM
Germination (%)	98	98	95	97	94
Survival (%)	100	20	1	0	0

Results

Proline-induced growth inhibition and ROS production in Arabidopsis seedlings

To characterize the inhibitory effects of exogenous proline on plant growth, Arabidopsis seeds were plated on 1/2 MS medium containing 0–100 mM L-proline. The germination was not significantly affected (Table 1), but the subsequent seedling growth was inhibited in a dose-dependent manner. Root elongation was impaired and leaf was bleached by proline at concentration higher than 20 mM. As shown in Fig. 1a, on 100 mM proline the root length was only 15% of that of the control plate with 0 mM proline. The expansion of true leaves was virtually stopped shortly after their emergence and chlorophyll content was dramatically decreased by proline treatment (Fig. 1b).

To examine whether the sensitivity of Arabidopsis seedlings to proline inhibition is age-dependent, the seedlings were first germinated on normal 1/2MS medium for different time before being transferred to medium containing 45 mM. As shown in Fig. 1c, the later the seedlings were transferred onto proline-containing medium, the higher their relative fresh weight became. 13-day-old seedlings showed only 25% of growth reduction as compared to the 75% reduction of 1-day-old seedlings, indicating that the seedling was more sensitive to proline toxicity at early growth stage. As the seedling got older, their sensitivity to proline was gradually decreased.

To understand whether the inhibitory effects of proline is reversible, seedlings that have been grown on proline-containing medium were moved back to normal 1/2MS medium. All the seedlings that were transferred before 8 days on L-proline turned green and grew further. The earlier they were removed from proline-containing medium, the better they were recovered. None of the seedlings that were transferred after 10 days on L-proline recovered (data not shown). Thus, there is an accumulating effect of proline toxicity before a threshold is reached, beyond which the seedlings cannot recover.

To compare the toxicity of proline with that of other amino acids, nine amino acids at 10 mM were tested for their effects on seedling growth. All the examined amino acids were found to significantly inhibit the seedling

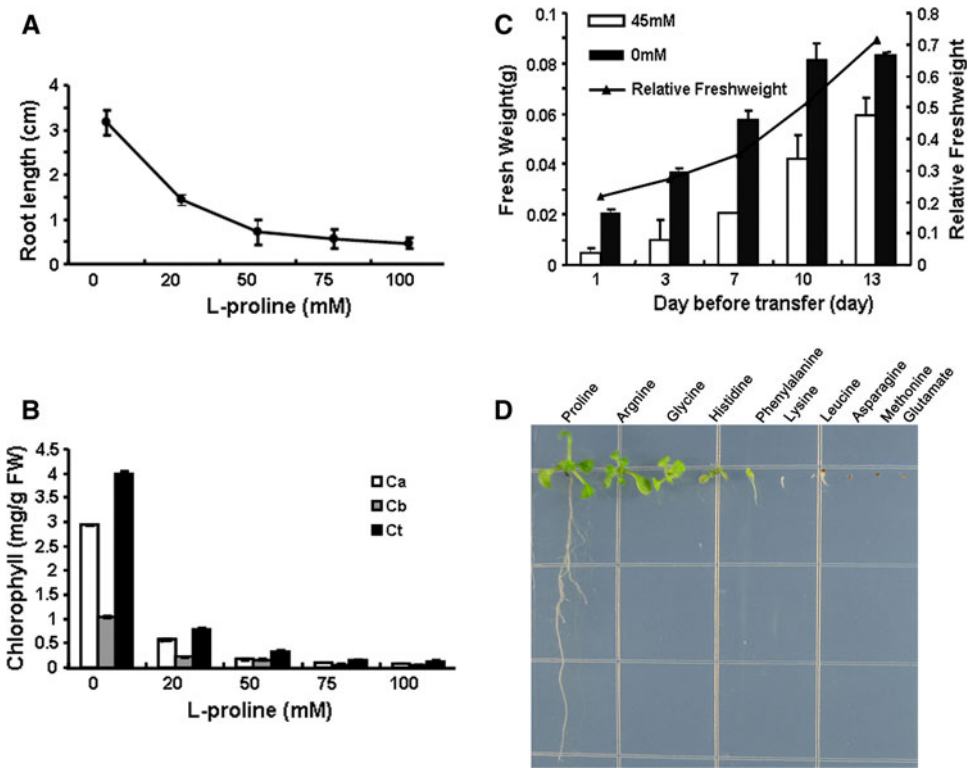

Fig. 1 Proline inhibits the growth of Arabidopsis seedlings. Arabidopsis (ecotype Columbia) seeds were germinated on media containing different concentrations of proline. The length of primary root (**a**) and chlorophyll a (Ca), chlorophyll b (Cb) and total chlorophyll (Ct) content (**b**) was measured14 days after germination (±SEM; $n = 5$–6). **c** Proline sensitivity of Arabidopsis seedlings of different age. Arabidopsis seedlings were transferred to plates with or without 45 mM L-proline at different time after germination. Fresh weight of six seedlings was measured 14 days after transfer. **d** Inhibitory effect of different amino acids at 10 mM on the growth of Arabidopsis seedlings

growth at this concentration (Fig. 1d). Proline was the weakest growth inhibitor compared with these amino acids.

Proline treatment may inhibit plant growth by disturbing the balance in total amino acid pool. To explore this possibility, the free amino acid content of the seedlings treated with 45 and 0 mM proline for 24 h was compared. The data (Table 2) showed that proline content increased nearly 100-fold after proline treatment. Significant change (one-way ANOVA, $P < 0.05$) was also detected in the level of glycine, alanine, serine, and asparagine in seedlings treated with 45 mM proline, in comparison with that in control, but with an increase less than 5-fold. The level of other amino acids, including glutamate, the product of proline degradation, is relatively unchanged. Therefore, this result suggested that proline treatment can (to some extent) disturb the total amino acid pool. However, whether these relatively minor changes are responsible for the growth inhibition needs to be further studied.

It has been reported that proline treatment can induce ROS production, resulting in programmed cell death (Deuschle et al. 2004). To further characterize the proline-induced ROS production, we have measured the hydrogen peroxide (H_2O_2) level in 14-day-old seedlings after treatment with 45 mM proline for 24 h by 3,3-diaminobenzidine (DAB) staining. The level of H_2O_2 was increased by proline treatment (Fig. 2c).

Table 2 Free amino acid contents

Amino acid	Concentration (mg/g FW)	
	Control	Proline treatment
Asp	0.109 ± 0.013	0.081 ± 0.000*
Thr	–	–
Ser	3.533 ± 0.323	6.832 ± 1.416*
Glu	0.114 ± 0.010	0.166 ± 0.056
Gly	0.036 ± 0.005	0.164 ± 0.028**
Ala	0.233 ± 0.026	0.697 ± 0.208*
Cys	0.042 ± 0.001	0.041 ± 0.002
Val	0.111 ± 0.004	0.126 ± 0.028
Met	0.022 ± 0.002	0.026 ± 0.008
Ile	0.023 ± 0.001	0.024 ± 0.009
Leu	0.044 ± 0.002	0.049 ± 0.018
Tyr	0.022 ± 0.001	0.021 ± 0.011
Phe	0.041 ± 0.002	0.054 ± 0.020
His	0.121 ± 0.004	0.135 ± 0.016
Lys	0.037 ± 0.001	0.033 ± 0.013
Arg	0.469 ± 0.031	0.383 ± 0.299
Pro	0.045 ± 0.001	4.430 ± 0.946**
Proline/Total amino acids (%)	2.9723	68.4244

Arabidopsis seedlings treated with 45 mM proline for 24 h. Values marked by asterisk show significant differences between the treatments (one-way ANOVA, $P < 0.05$)

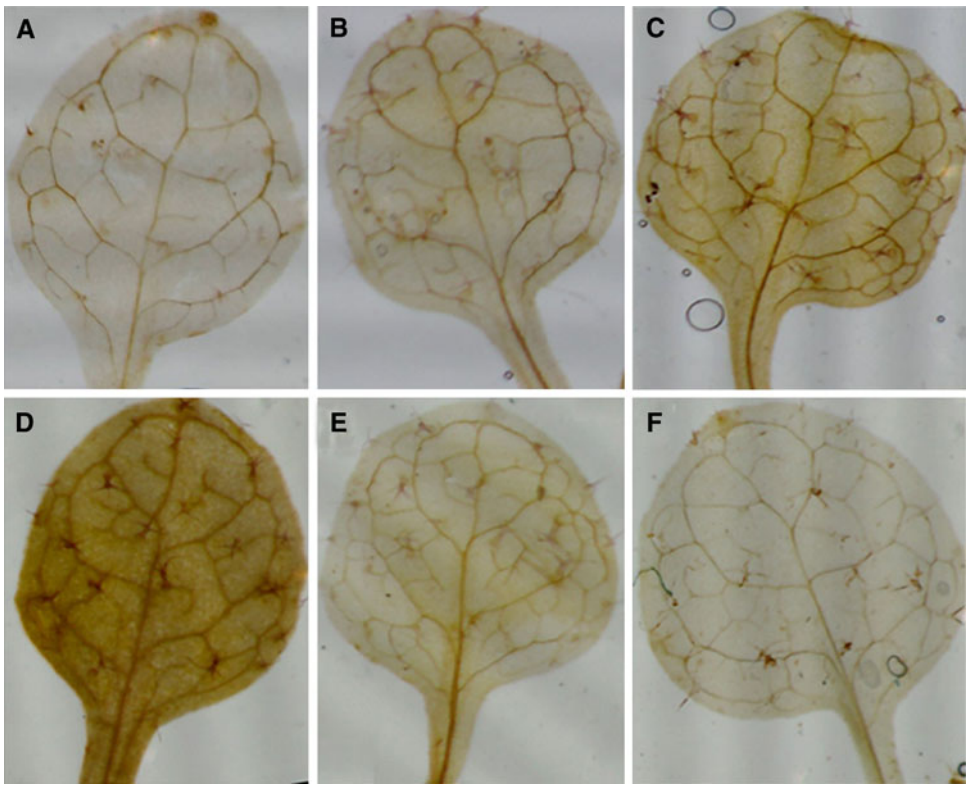

Fig. 2 Effect of calcium on proline inhibition on seedling growth. **a** Calcium but not magnesium enhanced the proline toxicity. Result shown is a representation of at least three repeats. **b** Fresh weight of Arabidopsis seedlings treated with or without proline under different concentrations of $CaCl_2$ (mean ± SD, $n = 3$). **c** Fresh weight of Arabidopsis seedlings treated with or without proline under different concentrations of $MgCl_2$ (mean ± SD, $n = 3$). **d** Proline content of Arabidopsis seedlings treated with 45 mM proline for 4, 24 and 72 h with or without 10 mM calcium (mean ± SD, $n = 3$). **e** RT-PCR analysis of *AtPDH1* expression in Arabidopsis seedlings treated with 45 mM proline with or without 10 mM calcium. *ACTIN7* was used as an internal control. Result shown is a representation of at least three repeats. **f** Proline treatment induces the concentration of cytosolic calcium in the Bright Yellow-2 (BY-2) cells. Confocal images of BY-2 cells loaded with Fluo-3/AM and treated with 45 mM proline for 0, 3 and 6 min. *Bar* = 40 μm. Result shown is a representation of at least three repeats

Calcium is involved in proline-induced ROS generation

Calcium, an important and ubiquitous second messenger, can influence many cellular processes, including ROS generation (Chen and Li 2001; Lecourieux et al. 2006; Ma et al. 2008; White and Broadley, 2003). To understand whether calcium can also affect proline toxicity, Arabidopsis seedlings were cultured on 0, 5, 10 and 20 mM calcium in the presence of 45 mM proline. Increasing calcium concentrations led to decreased relative growth and relative fresh weight under proline treatment, while calcium alone at these concentrations didn't show any visible effect on growth (Fig. 3a, b), indicating that calcium treatment can increase proline toxicity. Similar stimulating effect on proline toxicity was not observed by magnesium treatment (Fig. 3a, c), suggesting that proline toxicity can be enhanced specifically by calcium treatment.

To investigate whether the effect of calcium on proline toxicity is due to its effect on increasing ROS production, we treated 14-day-old seedlings with 45 mM proline with or without addition of 10 mM $CaCl_2$. Treatment with proline alone increased the H_2O_2 level compared to the control (Fig. 2a, c). But when the seedlings were treated with proline and $CaCl_2$ together, H_2O_2 accumulated to a much higher level than that in the sample treated with proline alone (Fig. 2d). Ca^{2+} channel blocker $LaCl_3$ could effectively block the proline-induced H_2O_2 production in leaves of Arabidopsis (Fig. 2e). Treatment with NADPH oxidase inhibitor, diphenylene iodonium (DPI), also completely blocked the increase in H_2O_2 level (Fig. 2f), suggesting that NADPH oxidase may be involved in proline-induced generation of ROS.

To distinguish whether the stimulating effects of calcium on proline toxicity is due to its effect on increasing proline uptake, decreasing degradation or increasing sensitivity to proline toxicity, we have measured intracellular proline content when treated with proline in the presence of 10 mM calcium. The result indicated that the presence of calcium did not significantly increase the proline content (Fig. 3d). Furthermore, the expression of *AtPDH1*,

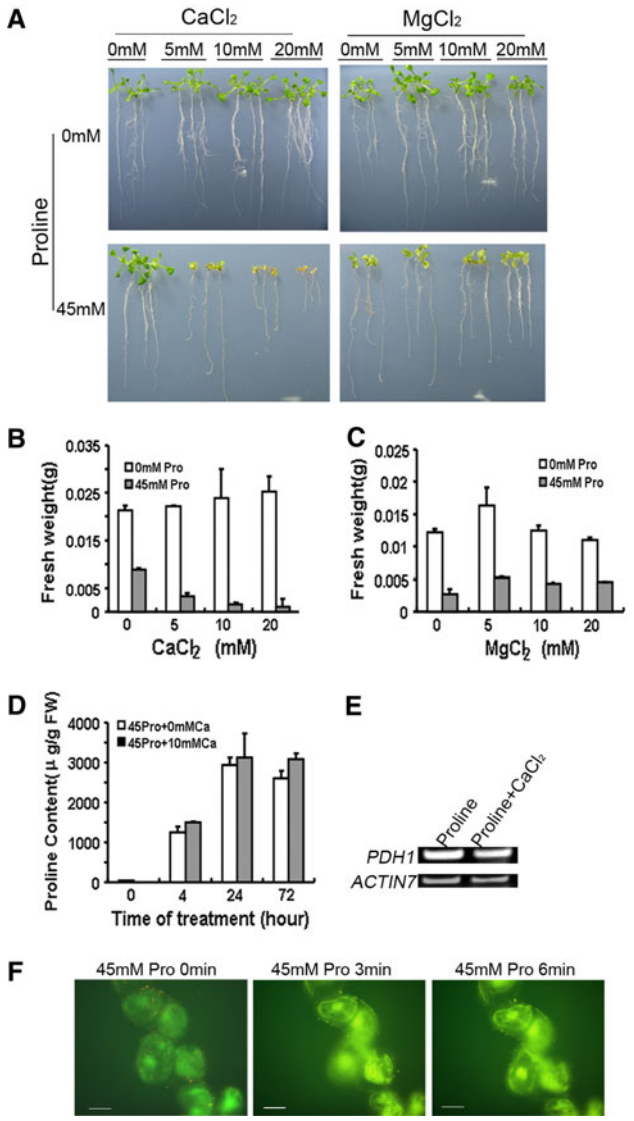

Fig. 3 Histochemical detection of H_2O_2 by 3,3-diaminobenzidine (DAB) staining in leaves of Arabidopsis seedlings treated with liquid 1/2MS as a control (**a**) 10 mM $CaCl_2$ (**b**) 45 mM proline (**c**) 45 mM proline plus 10 mM $CaCl_2$ (**d**) 45 mM proline plus 1μM DPI (**e**) 45 mM proline plus 2 mM $LaCl_3$ (**f**). Experiments were performed with three replicas. At least six seedlings were examined per replica with similar results. Leaves at same position were photographed

encoding the rate-limiting enzyme in proline degradation pathway, did not seem to be affected either (Fig. 3e), suggesting that calcium treatment may increase the sensitivity of seedlings to proline.

To further substantiate that calcium is indeed a component mediating proline toxicity in vivo, we have examined the cytosolic calcium concentration in response to exogenous proline in tobacco (*Nicotiana tabacum*) bright yellow-2 cells by Fluo-3/AM loading. As shown in Fig. 3f, treatment with 45 mM proline led to an increase in the fluorescence intensity of the suspension cultures. This result indicates that intracellular calcium is probably involved in proline-induced ROS generation growth inhibition.

Proline induce salicylic acid accumulation

It has been previously reported that the sensitivity of Arabidopsis seedlings to proline toxicity was affected by SA signaling; therefore, we evaluated the effects of SA on the plant response to proline toxicity. Lower concentration (22.5 mM) of proline was used to better illustrate the stimulating effect of SA on proline toxicity. The growth of Col-0 seedlings was more severely inhibited by 22.5 mM proline if 0.01 mM SA is present (Fig. 4a), indicating that exogenous SA can enhance the proline toxicity.

To understand the role of endogenous SA in proline toxicity, we examined the proline sensitivity of SA deficient mutant *sid2* and NahG transgenic plant. *SID2* is a SA synthetic enzyme, while NahG catalyses the degradation of SA to catechol (Gaffney et al. 1993). As shown in Fig. 5a, both *sid2* and NahG plants were more resistant to proline than WT, suggesting that endogenous SA is involved in proline toxicity.

The above result prompted us to measure level of SA in Arabidopsis seedlings in response to proline treatment. As shown in Fig. 4b, the amount of free SA is 2.3 and 3.7 folds as high as that in control when treated with 10 and 45 mM proline, respectively, for 24 h.

PR genes are commonly used as molecular markers for SA signaling, so we examined whether treatment with proline can up-regulate their expression. As shown in Fig. 4c, treatment with 10 mM proline for 24 h can induce expression of *PR1* and *PR2*. However, in *sid2* mutant and NahG plant, the inducibility of *PR1* gene expression by proline is abolished and that of *PR2* is also greatly reduced, indicating that proline-regulated *PR* genes expression is mediated by SA. On the other hand, the expression of *PDF1.2* is not affected by proline even at 45 mM (Fig. 4d), suggesting that jasmonic acid signaling is probably not involved in proline toxicity.

To test the ability of other amino acid in inducing *PR* genes expression, we treated Arabidopsis seedlings with proline, glutamate, arginine, and phenylalanine. As shown in Fig. 4c, at 10 mM, only treatment with proline and glutamate can induce *PR1* expression, whereas arginine and phenylalanine have no effect on the *PR1* gene expression. This result suggested that not all the amino acids could activate SA signaling, even though they are more toxic than proline.

Our above result showed that proline treatment could induce both H_2O_2 generation and SA production. To investigate whether proline-induced SA production is mediated by ROS, we treated Arabidopsis seedlings with proline in the presence of DPI, a NADPH oxidase inhibitor. As shown in Fig. 4c, DPI completely abolished the induction of *PR* genes

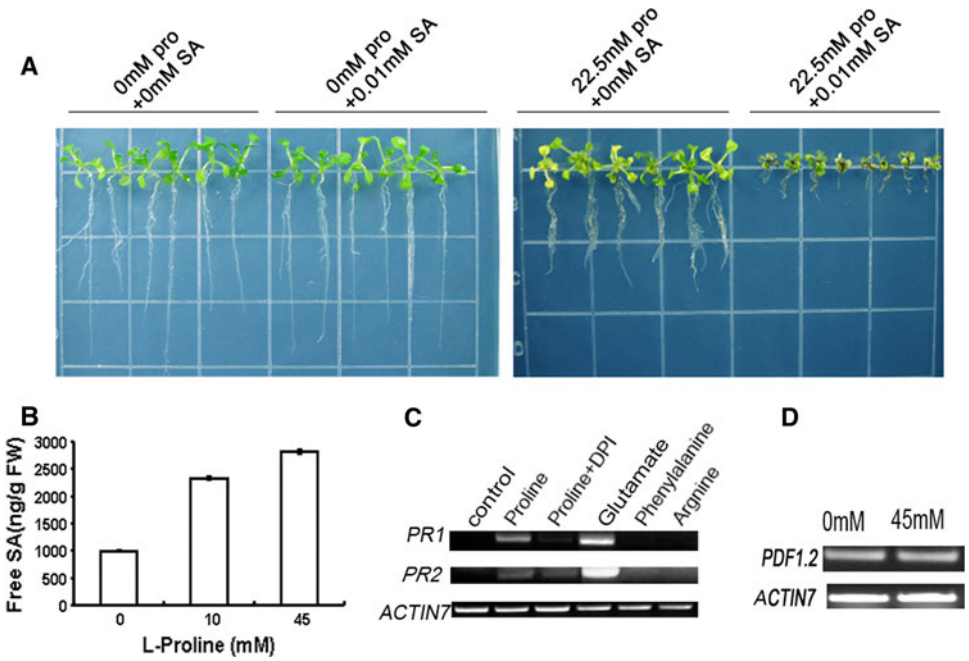

Fig. 4 **a** Exogenous SA enhances proline toxicity. **b** Exogenous proline induces SA accumulation. Free SA levels in wild type Col-0 plants treated with 10 and 45 mM proline (mean ± SD, $n = 3$). **c** RT-PCR analysis of *PR1* and *PR2* expression in Arabidopsis seedlings treated with liquid 1/2MS, 10 mM proline, 10 mM proline plus 10 μM DPI, 10 mM glutamate, 10 mM phenylalanine and 10 mM arginine. **d** Effect of proline on *PDF1.2* expression. Result shown is a representation of at least three repeats

by exogenous proline, suggesting that ROS generated by NADPH oxidase is upstream of SA induction.

Proline-induced SA production is mediated by NDR1-dependent signaling transduction pathway

It has been demonstrated that proline treatment can induce cell death resembling hypersensitive response during pathogen infection (Deuschle et al. 2004). Therefore, proline-induced SA synthesis may share some common mechanism with pathogen-induced SA synthesis. It has been documented that upon avirulent bacterial pathogen infection, SA synthesis can be activated by two major signal transduction pathways, depending on the subset of R gene involved. These two pathways are regulated by PAD4 and NDR1, respectively. To understand which pathway is responsible for proline-induced SA synthesis, we treated *pad4* and *ndr1* with 45 mM proline for 24 h. As shown in Fig. 5b, c, *PR* gene expression in *pad4* is not affected, whereas that in *ndr1* is attenuated, suggesting that the normal function of NDR1, but not that of PAD4 is required for full induction of SA signaling by proline.

The above results indicate that exogenous application of proline significantly induced both ROS generation and SA production, and the ROS generation is calcium-dependent. To investigate whether proline-induced SA production and *PR* gene induction are also calcium-dependent, we treated Arabidopsis seedlings with proline in the presence of $LaCl_3$, a Ca^{2+} channel blocker, to inhibit the elevation of cytosolic calcium concentration. As shown in Fig. 5f, $LaCl_3$ significantly inhibited the proline-induced expression of *PR1* and *NDR1*, suggesting that calcium generated by exogenous application of proline is upstream of SA induction.

Above results suggested that proline may specifically induced NDR1-mediated pathway leading to the production of SA, which subsequently amplifies ROS level. Therefore, we tested the proline sensitivity of *pad4* and *ndr1* mutants. As expected, *ndr1* has reduced proline sensitivity, while the proline sensitivity of *pad4* is not reduced (Fig. 5a, b).

To determine the lowest concentration of proline that is capable of inducing *PR* gene expression, Arabidopsis seedlings was vacuum-infiltrated with different concentrations of proline. As shown in Fig. 5e, f, treatment of Col-0 plants with proline at a concentration of 0.5 mM or higher could induce *PR1* expression.

Pre-treatment with proline protects Arabidopsis against damage from salt stress

It has been previously reported that exogenous SA could ameliorate the adverse effects of abiotic stresses (Horváth et al. 2007). To investigate whether proline-induced ROS and SA signaling have similar effects, 7-day-old Arabidopsis seedlings were pretreated with 0.5 and 5 mM proline for 1 day before transfer to media plus 150 mM NaCl. As shown in Fig. 6a, the leaves of seedlings which have not been pretreated with proline are severely bleached by 150 mM NaCl, whereas the leaves of seedlings which received the proline pretreatment are still green. Quantitative measurement of chlorophyll content revealed a dramatic decrease in chlorophyll level in NaCl-treated seedlings, however, proline-pretreated samples were less bleached

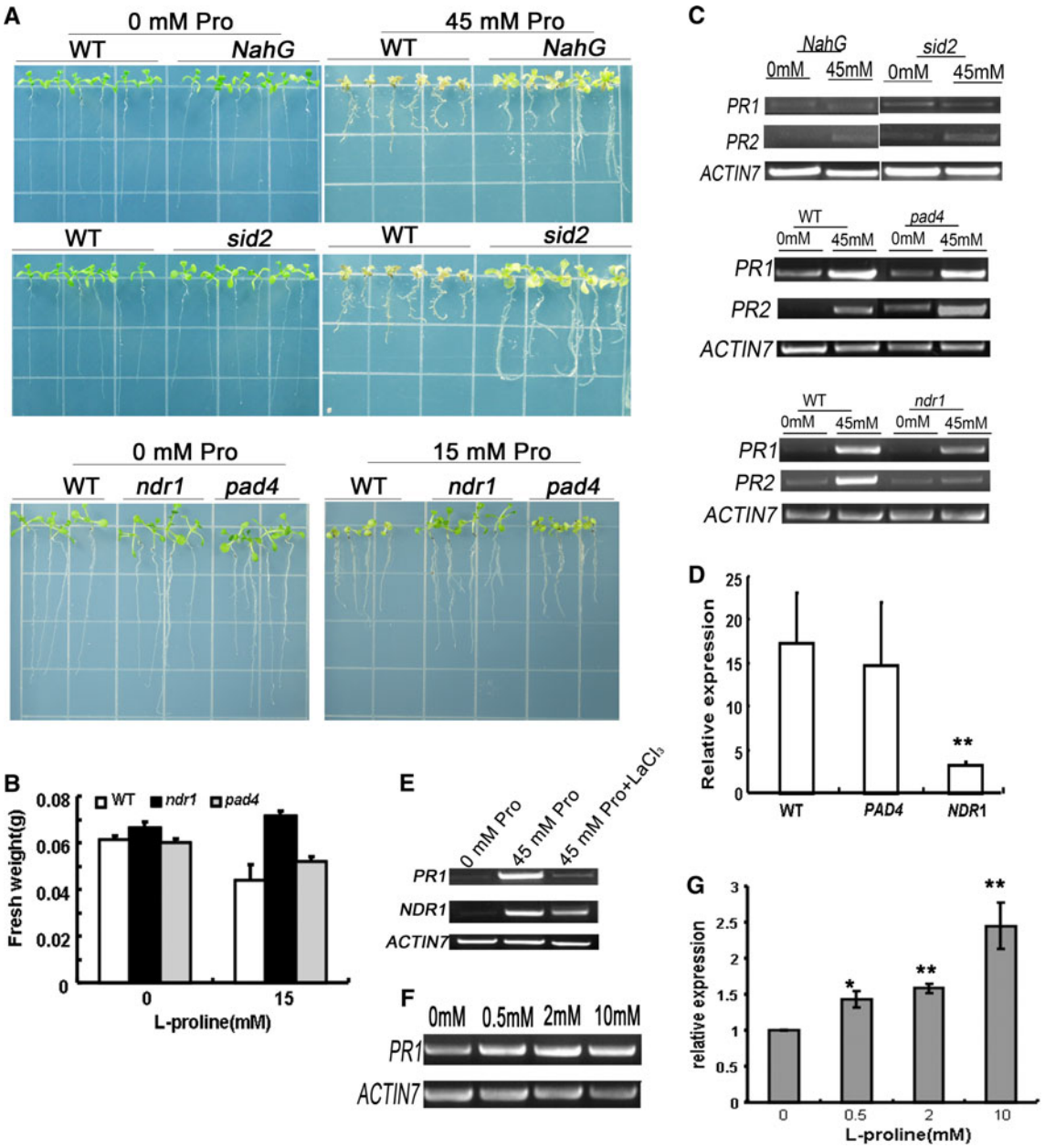

Fig. 5 **a** Proline sensitivity of wild type Col-0, NahG, *sid2*, *ndr1*, and *pad4*. **b** Fresh weight of wild type Col-0, *ndr1*, and *pad4* treated with or without proline. Ten seedlings were pooled for fresh weight measurement (mean ± SD, $n = 3$). **c** RT-PCR analysis of *PR1* and *PR2* transcript level in WT, NahG, *sid2*, *pad4*, and *ndr1*. *ACTIN7* was used as an internal control. **d** Quantitative results of the induction of *PR1* expression by proline treatment in *pad4* and *ndr1* (mean ± SD, $n = 4$). **e** RT-PCR analysis of *PR1* and *NDR1* expression in Arabidopsis seedlings treated with liquid 1/2MS, 45 mM proline, 45 mM proline plus 2 mM $LaCl_3$. **f** RT-PCR analysis of *PR1* transcript level in WT treated with different concentrations of proline. *ACTIN7* was used as an internal control. **g** Quantitative results of *PR1* transcript level induced by different concentrations of proline (mean ± SD, $n = 3$)

than control, suggesting that pre-treatment with proline can increase the salt tolerance of Arabidopsis seedlings.

Discussion

In this report, we have demonstrated that exogenous applied proline is capable of inducing calcium-dependent generation of ROS, which subsequently induces the production of salicylic acid probably via NDR1-mediated signaling transduction pathways, supporting previous notion that proline treatment induces a symptom strongly reminiscent of hypersensitive response during pathogenesis (Deuschle et al. 2004). Therefore, we speculate that the inhibitory affect of proline treatment on seedling growth may be the consequence of inducing an ROS and SA

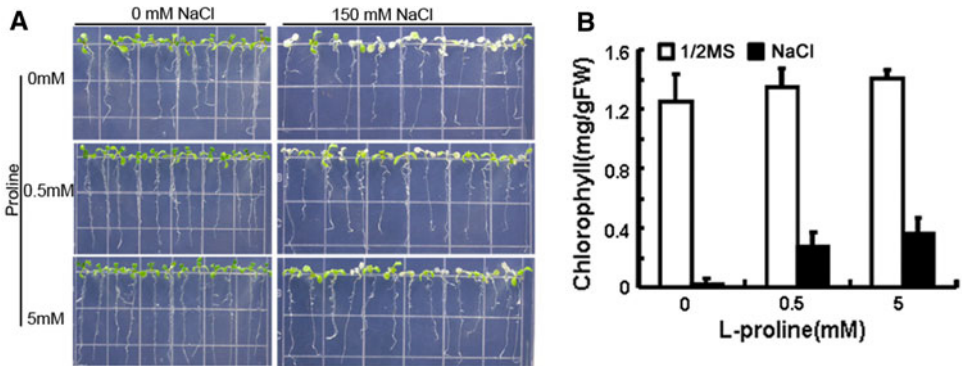

Fig. 6 Pre-treatment with proline can protect Arabidopsis against damage from the salt stress. Arabidopsis seedlings were pre-treatment with 0, 0.5 and 5 mM proline before being incubated under 150 mM NaCl, photos were taken (**a**) and chlorophyll content was measured (mean ± SD, $n = 3$) (**b**) after 10 days. Result shown is a representation of at least three repeats

signaling pathway that leads to cell death. High concentration of SA could be phytotoxic and inhibitory to plant growth. However, proline-induced SA production might not be the major cause of proline-induced growth inhibition, since as shown in Fig. 5a, NahG plant and *sid2* mutant only partially resist to proline inhibition.

The inhibition of amino acids on plant growth has been well-documented previously. In *Nicotiana silvestris* suspension cells, 19 out of 20 amino acids tested have been found inhibitory to cell growth, except for glutamine (Bonner et al. 1996; Bonner and Jensen, 1997). Although the underlying molecular mechanism was not understood, the programmed cell death triggered by abnormally high amino acid to glutamine ratio, established in vivo after exogenous application of individual amino acid, was suggested to be one of the possibilities (Bonner et al. 1996).

Calcium has long been recognized as a signal to regulate cellular ROS homeostasis in response to environmental stimuli, such as pathogen attack (Choi et al. 2009), mechanical stimulation (Monshausen et al. 2009), and ABA treatment (Hu et al. 2006). In these processes, the calcium signal is perceived by calmodulin to modulate the down-stream target. It was reported that calcium/calmodulin has dual roles in regulating ROS homeostasis (Yang and Poovaiah 2002). On one hand, calcium/calmodulin could activate NAD(H) kinase to provide NADP(H), the substrate for ROS production by NADPH oxidase, which itself can be activated by calcium. On the other hand, calcium/calmodulin can also bind and stimulate catalase activity to reduce ROS accumulation. Actually, amino acid induced calcium signaling has also been observed. Glutamate, glycine, alanine, serine, asparagine, and cysteine were reported to induce rapid rise in cytosolic calcium level and concomitant membrane depolarization, and is probably mediated by plant homolog of neuronal ionotropic glutamate receptors (Qi et al. 2006).

It is interesting to ask what the physiological significance of exogenous proline treatment on SA signaling is. It was speculated that an increase in apoplast amino acids resulted from surrounding dead cell during pathogen infection could initiate calcium-mediated pathogenic response (Ma and Berkowitz 2007). In our system, the minimum concentration of proline capable of inducing *PR* gene expression is 0.5 mM, similar to the minimal effective concentration of pyridine nucleotides (NAD and NADP), which was propose to function as a signal in extracellular compartment to activate plant defense responses (Zhang and Mou 2009). Although the apoplastic concentration of amino acid is not known, it might fall within a similar range to that of pyridine nucleotides. Moreover, our result also demonstrated that proline is not the only amino acid, nor is it the best amino acid that could induce *PR* gene expression. Other amino acids, such as glutamate also activates *PR* gene expression. So it is reasonable to suggest that amino acids might also act as a signal to activate plant defense responses. But detailed characterization of the effect of amino acid on plant response to pathogen infection is needed to address this question.

The unique feature of proline is that it accumulates in response to a variety of environmental stimuli, including pathogen infection (Fabro et al. 2004). Therefore, during stress the level of proline may surpass that of other amino acids to become the dominant amino acid, suggesting that proline is a major player in signaling. It was reported that infection with avirulent *Pseudomonas syringae* pv. tomato DC3000 (*avrRpm1 and avrRpt2*), but not with isogenic virulent strain, could induce proline accumulation via up-regulation of *At-P5CS2* (Fabro et al. 2004). *avrRpm1 and avrRpt2* are known to interact with R gene of cc-NBS-LRR subclass and subsequently activate signal cascade involving NDR1. This coincides with our result that proline could specifically activate NDR1-mediated pathway for SA synthesis. Further investigation is warranted to elucidate the relationship between proline accumulation and SA signaling.

Although prolonged exposure to proline higher than 20 mM significantly inhibited the seedling growth, a short pre-treatment with proline seemed to be beneficial during subsequent imposition of salt stress. It is possible that

proline pre-treatment invokes some defense mechanisms, such as ROS and SA signaling, and, therefore, prepares seedlings for upcoming stress condition. The beneficial effects of exogenous salicylic acid on plant tolerance to abiotic stresses, including heavy metals, temperature, water, ozone, UV irradiance, drought and salinity stresses, have been well documented (Horváth et al. 2007; Hayata et al. 2010). In Arabidopsis, SA was also found to potentiate the response of the germinating seedling to salt and osmotic stresses by mediating the generation of ROS (Borsani et al. 2001). Whether SA is indeed required for exogenous proline-induced salt resistance needs to be further investigated using nahG plant or other SA deficient mutants. Given the fact that transgenic plant overproducting proline often exhibits both stress tolerance and retarded growth, it is possible that SA signaling may also been activated by over-produced proline.

Interestingly, our result showed that proline does not upregulate the expression of *PDF1.2*, the marker gene in jasmonic acid signaling pathway, indicating that proline only specifically induces SA signaling pathway. This is in agreement with previous notion that SA and JA pathway are generally antagonistic to each other (Takahashi et al. 2004). It would be interesting to characterize the difference between proline-induced SA signaling and that induced by pathogen infection. In addition, screening and characterizing the mutants resistant to proline inhibition during seedling growth stage may shed some new lights on proline-induced ROS generation and SA signaling.

In summary, our working model for the signaling pathways activated by proline treatment is shown in Fig. 7. Proline can activate Ca^{2+} influx, possibly via glutamate receptor, and induces ROS production by NADPH oxidase. ROS generated can further induce SA and be amplified by SA signaling involving NDR1, resembling the positive feedback loop of SA and ROS in hypersensitive response after avirulent pathogen attack.

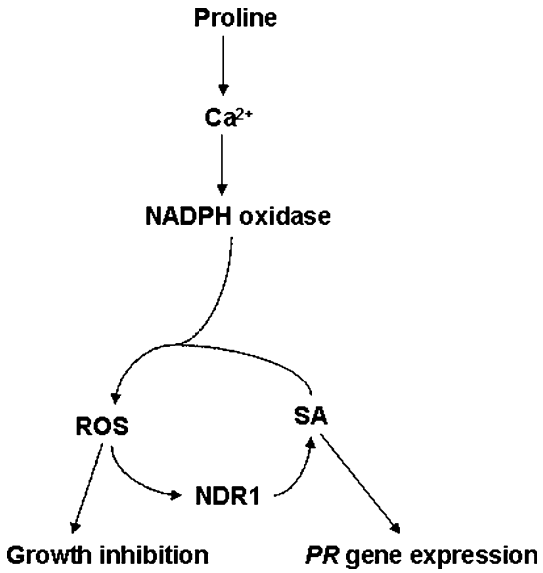

Fig. 7 Proposed model for the signal transduction pathway mediating proline toxicity. Proline induces cytosolic Ca^{2+} elevation, which enhances the generation of reactive oxygen species through NADPH oxidase. ROS subsequently leads to the accumulation of SA, and be amplified by SA signaling involving NDR1, resulting in programmed cell death

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana Columbia (Col) wild type, and mutant *sid2*, *pad4*, *ndr1*, and *NahG* on Columbia background were used in this study. Arabidopsis seeds were surface-sterilized by rinsing in 75% ethanol for 30 s and sequentially soaked in 10% sodium hypochlorite for 12 min with shaking, followed by rinsing 7–8 times with sterile distilled water. Seeds were grown on half-strength MS (Murashige and Skoog, 1962) (1% w/v sucrose) agar plates in a growth chamber under 16/8 h light/dark cycle of fluorescent light at 22°C. BY-2 tobacco (*Nicotiana tabacum*) suspension culture was grown in constant darkness in liquid culture.

Proline treatment of Arabidopsis plants

Proline treatment was performed by transferring 2-week-old seedlings to 1/2 MS liquid medium supplemented with or without 45 mM proline for various time. After treatment, the seedlings were then collected and frozen in liquid nitrogen, and stored at −80°C for subsequent analysis.

Measurement of proline content

Sample frozen in −80°C was ground in liquid nitrogen and then free proline content was measured as described by Bates et al. (1973).

Measurement of chlorophyll content

The procedure was carried out mainly as described (Lichtenthaler and Buschmann 2001). Leaf samples (50 mg) were mashed with a mortar and pestle and extracted with 80% acetone (v/v) overnight at 4°C in the dark. The extract was centrifuged at 12,000 rpm for 5 min. The supernatant was collected and read at 663 and 647 nm for chlorophyll a and chlorophyll b, respectively. The contents for chlorophyll a, chlorophyll b, and the total leaf chlorophyll were calculated according to the equations proposed by Lichtenthaler and Buschmann (2001).

Amino acid analysis

For total amino acid analysis, the seedlings were ground to power in liquid nitrogen and then dehydrated in a freeze dryer. The dried tissue was then extracted with 80% ethanol as described (Chiang and Dandekar 1995). The supernatant was then dried down in a Speed Vac followed by resuspending in 200 mM lithium citrate buffer (pH 2.2). Finally, amino acids were analyzed using a SYKAM 433-D Amino Acid Analyzer.

Staining procedures and detection

H_2O_2 was visually detected in leaves of plants using 3,3-diaminobenzidine (DAB). Briefly, whole plants were treated with proline and immersed in a 1 mg/ml DAB solution (pH 4) for 10 h in the dark at 21°C. The staining was terminated by rinsing in warm ethanol (80%) until they were decolorized except for the deep brown polymerization product produced from DAB in the presence of H_2O_2.

Measurement of $[Ca^{2+}]i$

For measurement of proline-induced cytosolic Ca^{2+} concentration, BY-2 cells were loaded with Ca^{2+}-sensitive fluorescent dye Fluo-3/AMester (Molecular Probes, Eugene, OR, USA) and were observed under confocal laser scanning microscopy (CLSM) according to the method described by Zhang et al. (1998). Fluo-3/AM was added from a stock solution of 1 mM in DMSO, and the incubation solution contained 20 μM Fluo-3/AM ester, 0.5 M mannitol, 4 mM MES (pH 5.7), and 20 mM KCl. After incubation at 25°C for 1 h in the dark, 45 mM proline was added to the incubation buffer for proline treatment. Pictures were taken at 0 min, 3 min and 6 min after proline addition. Fluorescent probe was excited with a 488 nm laser, and emission fluorescent was filtered by a 515 nm filter to eliminate the autofluorescence of chlorophyll.

SA measurement

Free SA level was measured by GC–MS analysis. 2 μl samples were injected in hexane in the splitless mode at 280°C. GC was operated on a DB-35 capillary, 30 m × 0.32 mm inner diameter, 25 μm film at constant flow of 1.2 ml/min. The temperature program started with 2 min isocratic at 80°C, followed by temperature ramping at 14°C/min to a final temperature of 290°C which was held for 6 min. Data acquisition was performed on a Pegasus® IV TOF mass spectrometer (LECO, St. Joseph, MI) with an acquisition rate of 30 scans/sec (Morgenthal et al. 2005).

Isolation of RNA and semi-quantitative RT-PCR

Total RNA was extracted from 10-day-old seedlings as described by Hua et al. (2001). Twenty to twenty-five seedlings were pooled for total RNA extraction. First-strand cDNA was synthesized by Superscript III reverse transcriptase following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). PCR, using the first-strand cDNA as a template, was performed using the following primers: 5′-CGCCAGTCCACGACACAA-3′ (forward) and 5′-GCAAGTCTCCCCGAATCAG-3′ (reverse) for *PDH1* (accession no. At3g30775); 5′-TCGTCTTTGTAGCTCTTGTAGGTG-3′ (forward) and 5′-TAGATTCTCGTAATCTCAGCTCTC-3′ (reverse) for *PR1* (accession no. At2g14610); 5′-CGTTGTGGCTCTTTACAAACAACAAAAC-3′ (forward) and 5′-GAAATTAACTTCATACTTAGACTGTCGAT-3′ (reverse) for *PR2* (accession no. At3g57260); 5′-GCTAAGTTTGCTTCCATCATCACCCTT-3′ (forward) and 5′-AACATGGGACGTAACAGATACACTTGTG-3′ (reverse) for *PDF1.2* (accession no. At5g44420); 5′-CAAACCCAAATGCTCAATCCA-3′ (forward) and 5′-ACCTGAAAACAGCCGATCCAT-3′ (reverse) for *NDR1* (accession no. At3g20600); and 5′-TGCACAAGTCATAACCATCGG-3′ (forward) and 5′-TGTGAACAATCGATGGACCTGAC-3′ (reverse) for *ACTIN7* (accession no. At5g09180). PCR amplification of all genes was performed by an initial denaturation at 94°C for 3 min followed by 25 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 60 s, and a final extension at 72°C for 10 min.

Quantitative real-time PCR

Quantitative real-time RT-PCR was carried out with actin gene as the internal standard. The gene-specific primers were as follows; *PR1* (forward, 5′-GCGAGAAGGCTAACTACAACT-3′; reverse, 5′-TTACACCTCACTTTGGCACAT-3′); and Tubulin (forward, 5′-AGGCAAAATGAGCACGAAAGA-3′; reverse, 5′-TCAGACCTGTTGGTGGAATGTCAC-3′). Real-time PCR was performed using a Stratagene Mx3000P (Stratagene, LaJolla, CA). Amplification was started at 95°C for 10 min as the first step, followed by 40 cycles of PCRs: at 95°C for 25 s, at 57°C for 25 s, and at 72°C for 30 s. A melting curve was run after PCR cycles. Detection of real time RT-PCR products was done using the SYBR Green Real-time PCR Master Mix (Toyobo, Tokyo, Japan) following the manufacturer's recommendations. Reactions were repeated four times for each sample.

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