

Antifreeze proteins promote the germination of low temperature-treated petunia seeds via regulation of antioxidant- and proline-related genes

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Abstract The involvement of antifreeze proteins (AFPs; type I and III) in the germination of low temperature-treated petunia seeds (cv. ‘Mirage Rose’) was investigated. The addition of AFPs (300 or 500 µg/l) in low-temperature treatment significantly promoted the germination of seeds compared with that in which AFPs were not added. Among all treatments, treatment with AFP I added at 300 µg/l showed the highest germination percentage and improved plant growth. The expression levels of antioxidant-related genes such as superoxide dismutase, peroxidase, and proline synthesis were associated with the germination of low temperature-treated seeds. Overall, this study demonstrated that AFP I may potentially function as a cold-protective agent for the germination of low temperature-treated seeds.

Keywords Germination, Gene expression, Low temperature, Plant growth, *Petunia hybrida*

Introduction

The influence of temperature in the mechanism of seed germination has been reported in several crops, including *Pittosporum*, *Salicornia rubra*, and tomato (Bewley 1982; Khan et al. 2000; Mobayen 1980; Moore et al. 1994; Kyu et al. 2019). *Pittosporum* seeds exposed to low temperatures (4°C) were shown to exhibit rapid germination (Moore et

al. 1994). Similarly, purple cauliflower seeds also exhibited improved germination when exposed to a low temperature (10°C; Wartidiningsih et al. 1994). However, inhibition of seed germination at low temperatures (5°C ~ 15°C) was observed in *S. rubra* (Khan et al. 2000). In case of tomato seeds, a specific temperature (11°C ~ 25°C) is required for seed germination, and temperatures below 10°C delay germination (Mobayen, 1980). Similarly, Foolad and Lin (2000) and Kyu et al. (2019) observed inhibition of tomato seed germination at low temperatures. Generally, low temperatures induce reactive oxygen species (ROS) in various cellular compartments of seeds (Scandalios 2005). It also negatively affects seed physiology (Bailly 2004), leading to seed deterioration (Bailly et al. 2008).

The effects of low temperature on seed germination have not been investigated in petunia, which is a popular ornamental bedding plant in landscape industries as well as a model crop in biotechnology research. In fact, both tomatoes and petunias belong to the family Solanaceae. It was therefore interesting to investigate the effects of low temperature on petunia seed germination. The protective role of antifreeze proteins (AFPs) against low temperatures was recently reported in various plant species (Jeon et al. 2015; Seo et al. 2018; Pe et al. 2019). Jeon et al. (2015) and Seo et al. (2018) reported that the use of AFP type III positively affected the cryopreservation efficiency of chrysanthemums and potatoes. The transcriptional variation of cold-responsive genes in plants through AFPs (type I and type III) was also observed (Pe et al. 2019). Kyu et al. (2019) recently reported that AFP-treated tomato seeds promoted tomato seed germination by regulating the expression levels of major antioxidant-related genes, including superoxide dismutase (*SOD*) and catalase (*CAT*). This minimizes abiotic stress-induced oxidative stress by scavenging ROS in cells (Mittler et al. 2004; Murshed et al. 2014; Wu et al. 2004; Xu et al. 2010). Therefore, investigating the

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function of AFPs in the germination mechanism of petunia seeds under low temperatures would be interesting.

In this study, we investigated the function of AFPs in the germination of petunia seeds subjected to a low temperature (4°C) for 5 days (d), followed by maintenance at a normal temperature (20°C) for 15 d. Germination percentages and transcript levels of the genes associated with germination were determined.

Materials and Methods

Materials

Petunia hybrida cv. ‘Mirage Rose’ seeds were used as source material for this experiment.

AFP treatment

The seeds were pretreated with various concentrations of two AFPs (type I and type III), which were derived from fish (A/F Protein Inc., Waltham, MA, USA), and sown for germination, as described by Kyu et al. (2019). Briefly, the seeds were immersed in water containing various concentration of AFPs (0, 300, and 500 µg/l) for 24 h. Seeds immersed in water only (without AFPs, 0 µg/l) were used as the control.

Effect of AFPs on seed germination

The AFP-treated seeds were sown in a seedling tray filled with a soil-less mixture (BM7; Berger Co., Quebec, Canada). The tray was placed in a growth chamber set at a fixed temperature (4°C), with a 16 h photoperiod and 70% relative humidity, for 5 d. The seeds were allowed to grow at a normal temperature (20°C) for 15 d. Each treatment consisted of 30 seeds with three replications. For all treatments, the germination percentage was recorded at 10, 12, and 14 d after sowing (DAS). The number of leaves per plant and fresh weight were recorded at 14 DAS.

Expressional analysis of antioxidant-related genes

RNA was extracted from the leaves of 14-d-old petunia seedlings using an RNAqueous kit (Ambion Inc., Austin, TX, USA). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using ReverTra Ace-α (Toyobo Co., Ltd., Osaka, Japan). The expression levels of antioxidant-related genes (*SOD*, *CAT*, and peroxidase [*POD*])

and proline synthesis gene (*Osmotin*) were analyzed using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The tubulin gene was used as a reference gene for the normalization of gene expression levels. The primers and PCR conditions used for gene amplification are listed in Supplementary Table 1. Three different biological samples were used for the expression analysis.

Data analysis

Data were analyzed using SPSS version 11.09 (IBM Corporation, Armonk, NY, USA). The data represent the mean of three replications. Duncan’s multiple range test ($P < .05$) was used for statistical analyses.

Results

Petunia seeds that were sown under normal growth temperature (20°C) germinated at 5 DAS (data not shown). However, this was not observed at low temperature (4°C) for all treatments regardless of AFP treatment. When the seeds were transferred to normal growth conditions (20°C), germination was observed at 10 DAS and germination rates peaked at 12 DAS. The germination percentage of AFP-treated seeds was significantly higher than that of non-AFP-treated seeds (control), except for AFP III (500 µg/l) (Fig. 1). When the germination percentages were further assessed at 14 DAS, significant improvements were not observed in seeds treated with AFP I but were observed in seeds treated with AFP III (300 µg/l). In addition, the germination percentage between the two concentrations of AFP I was not significantly different at 10, 12, and 14 DAS. A significant difference was observed for AFP III (300 and 500 µg/l), especially at 14 DAS. However, in terms of growth performance of the germinated seedlings, those treated with AFP I (300 µg/l) showed the best growth, followed by those treated with AFP I (500 µg/l). The growth performance of seeds treated with AFP III and control was not different (Fig. 2). This was confirmed by measuring their fresh weights (Fig. 3). Significantly higher fresh weights were observed in the following order: [AFP I (300 µg/l) > AFP I (500 µg/l) > AFP III (300 µg/l), AFP III (500 µg/l), and control]. Therefore, AFP I (300 µg/l) is better suited for the low temperature-treated seed germination of ‘Mirage Rose’.

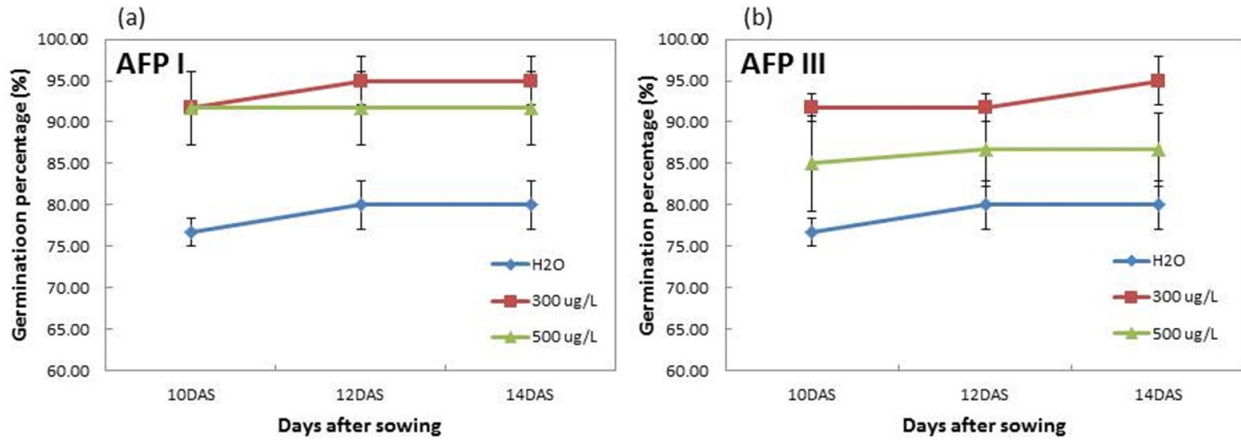


Fig. 1 Germination percentages of petunia seeds treated with or without antifreeze protein type I (a) and type III (b) under specific growing conditions (at 4°C for 5 d, followed by 20°C for 9 d). In control treatment, the seeds were soaked in water only. Data represent the means of the replications, and bars indicate standard errors of the three replications



Fig. 2 Illustration of the growth performance of seedlings germinated from petunia seeds treated with AFP I and III (300 and 500 µg/l) and control (0 µg/l)

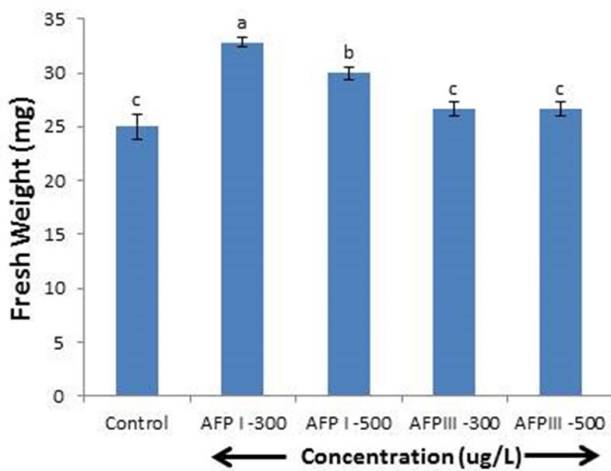


Fig. 3 Fresh weights of seedlings germinated from petunia seeds treated with AFP I and III (300 and 500 µg/l) and control (0 µg/l). Data represent the means of the replications, and bars indicate standard errors of three replications. Means with the same letters are not significantly different by DMRT ($p < 0.05$)

Transcriptional analysis of genes involved in seed germination

As shown in Figure 1, the germination percentages of the AFP-treated seeds varied depending on the type of AFPs and their concentrations. To determine the molecular mechanism underlying the variation in germination percentages among the AFP treatments, we investigated how AFPs affected the expression of the genes (*SOD*, *POD*, *CAT*, and *Osmotin*) associated with germination using RT-qPCR. As shown in Figure 4, the gene expression levels of the seedlings varied depending on the concentrations and types of AFPs. Specifically, *Osmotin* expression was upregulated in the seedlings germinated from control seeds (0 µg/l), with a transcript level significantly higher than that in seedlings germinated from seeds treated with AFPs. *SOD* expression patterns resembled those of *Osmotin*, with higher expression levels in control plants than in those treated with AFPs. However, *CAT* expression levels in both control

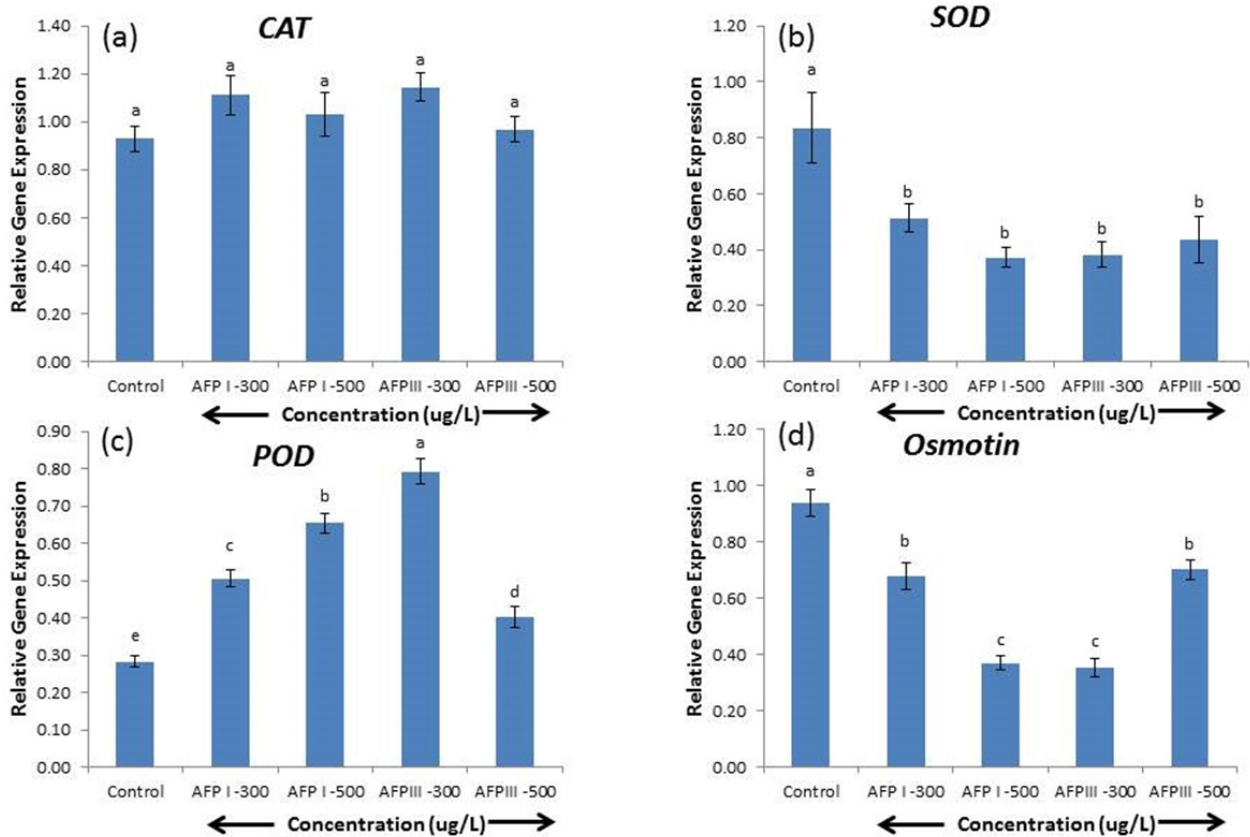


Fig. 4 Expression levels of antioxidant-related genes *CAT* (a), *SOD* (b), and *POD* (c) and proline synthesis gene *Osmotin* (d) expressed in seedlings germinated from seeds treated with or without antifreeze proteins (AFPs) type I and III under low temperature conditions (at 4°C for 5 d, followed by 20°C for 9 d). Control indicates seeds soaked in water only. Data represent the means of the biological replicates, and bars indicate standard errors of three replicates. Means with the same letters are not significantly different by DMRT ($p < 0.05$)

seedlings and AFP-treated seedlings were not significantly different. In addition, *POD* expression was upregulated in seedlings treated with AFPs, with transcript levels significantly higher than those of control seedlings (Fig. 4).

Discussion

Temperature plays an important role in seed germination, but the optimum temperature varies depending on the plant species. For example, lower temperatures (4°C and 10°C) are optimal for the germination of *Pittosporum* and purple cauliflower seeds, respectively (Moore et al. 1994; Wartidiningsih et al. 1994). Conversely, temperatures of 5°C ~ 15°C were found to significantly inhibit germination of *S. rubra* seeds (Khan et al. 2000). Similarly, inhibition of seed germination at low temperatures was observed in tomatoes (Foolad and Lin 2000), for which higher temperatures (11°C ~ 25°C) are required for seed germination (Mobayen, 1980). Kyu et al (2019) recently reported that AFP I could

be used as a cold-protective agent for tomato seed germination at low temperatures. In addition, a similar role of AFPs in cryopreservation of chrysanthemums and potatoes was reported (Jeon et al. 2015; Seo et al. 2018). Despite investigations on the role of AFPs in other Solanaceae crops, such as tomatoes and potatoes, its role has not been tested in petunias. Therefore, the role of AFPs in the germination of petunia seeds exposed to a low temperature (4°C) for 5 d, followed by 20°C for 15 d, was investigated.

Despite observations of petunia seed germination at 5 DAS under normal growth conditions, seeds treated with or without AFPs did not germinate even when they were transferred from a low-temperature condition (4°C) to normal growing condition for a few days. It is possible that mitotic cell division was inhibited by the low temperature (Simon et al. 1976) because the inactivation of mitotic cell division inhibits seed germination and early seedling growth (Masubelele et al. 2005). As observed for petunias, inhibition of seed germination by low temperatures was reported in other Solanaceae crops, including eggplant, pepper, and tomato

(Wilcox and Pfeiffer, 1990).

Seed germination started on day 5 (10 DAS) after being transferred to 20°C for all treatments. During the germination period, the germination percentages for the AFP treatments (300 and 500 µg/l) were significantly higher than those for the control. Reduced germination percentages of the control seeds could be attributed to cell injury caused by low-temperature stress. The higher germination percentages of AFPs -treated seeds could be a result of the cold-protective role of AFPs because AFPs could prevent cell damage due to low temperatures. In this study, the germination initiation date was the same for both AFP I and AFP III treatments, and both played similar roles in seed germination. This was not consistent with the result of Kyu et al. (2019), who reported a more positive role of AFP I in tomato seed germination than AFP III. Kyu et al. (2019) further observed that the germination percentage of AFP III-treated seeds was lower than that of control seeds (0 µg/l) at 20 DAS. This discrepancy could be due to differences in the nature of tomato and petunia seeds. However, the plant growth performance observed with AFP I treatment was distinctly better than that observed with AFP III treatment, in which the growth performance was similar to that of the control. Although AFP III treatment could enhance seed germination, it may exert toxic effects on the seeds, resulting in slow seedling growth. Some adverse effects of AFP III have also been reported in previous studies (Naing and Kim 2019). Furthermore, AFP I has been shown to have more effective utilization than AFP III in cryopreservation (Naing and Kim 2019).

Abiotic stress-induced ROS production in plant cells has been reported (Scandalios 2005). In addition, the role of enzymatic antioxidants such as SOD, POD, and CAT and proline in the reduction of oxidative stress by scavenging ROS in plant cells has also been reported (Mittler et al. 2004; Murshed et al. 2014; Naing et al. 2017; Wu et al. 2004; Xu et al. 2010). In the present study, the expression levels of *SOD* and *Osmotin* were linked to seed germination. Downregulation of the gene expression levels with AFP treatments compared with that in the control treatment could be due to the fact that these genes were strongly induced by the control treatment to protect the cell membrane from oxidative damage under cold stress, and moderate transcript expression levels induced by AFP treatments might be due to the cold-protective role of AFPs. However, the expression level of *CAT* did not differ significantly among the AFP treatments and the control, suggesting no involvement of *CAT* in seed germination of petunias. In a study on tomatoes, *SOD* and *CAT* were found to be involved

in seed germination (Kyu et al. 2019). In the present study, *POD* expression levels were higher in the AFP treatments than in the control. This could be attributed to the inhibitory effect of low temperature on peroxidase activity in the seeds because inhibition of *POD* activity in the endosperm of wheat seeds at low temperatures has been reported (Bakalova et al. 2004). Thus, higher *POD* expression in the AFP treatments could be due to the cold-protective effects of AFPs during the germination of the cold-treated seeds. Collectively, these results highlight how cold stress may affect seed germination of petunias and indicate the potential of AFPs as cold-protective agents in the germination of cold-treated petunia seeds.

Conclusion

This study demonstrated the inhibition of petunia seed germination under low-temperature conditions and possible utilization of AFPs as cold-protective agents during seed germination. AFP I was found to have a greater positive effect on seed germination and seedling growth than AFP III. The effects of AFPs on seed germination were associated with the expression levels of antioxidant-related genes (*SOD* and *POD*) and proline synthesis gene (*Osmotin*). This study suggests a greater viability of AFP I as a cold-protective agent in the germination of cold-treated seeds. It also provides a better understanding of how AFPs promote seed germination via the reduction of cold injury during seed germination.

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