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# Protective role of salicylic acid applied before cold stress on antioxidative system and protein patterns in barley apoplast

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## Abstract

This study was carried out to better understand the role of salicylic acid (SA) applied before cold stress in the cold tolerance mechanism. Two barley (*Hordeum vulgare*) cultivars, cold-sensitive (Akhisar) and cold-tolerant (Tokak), were used and 0.1 mM SA was applied to 7-d-old barley seedlings growing under control conditions (20/18 °C). The seedlings were transferred to cold chamber (7/5 °C) at the age of 14, 21, and 28 d. After three days, the leaves were harvested to determine the activities of apoplastic antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX), ice nucleation activity and electrophoretic patterns of apoplastic proteins. Cold treatment decreased the activities of all enzymes in cold-sensitive cultivar, however, it increased CAT and POX activities in cold-tolerant cultivar. Exogenous SA increased enzyme activities in both cultivars. Ice nucleation activity increased by cold treatment, especially in 17-d-old seedlings of both cultivars. In addition, SA treatment increased ice nucleation activity in all examined samplings of both cultivars. SA treatment caused accumulation or *de novo* synthesis of some apoplastic proteins. The results of the present study show that exogenous SA can improve cold tolerance by regulating the activities of apoplastic antioxidative enzymes, ice nucleation activity, and the patterns of apoplastic proteins.

*Additional key words*: catalase, cold tolerance, *Hordeum vulgare*, ice nucleation activity, peroxidase, superoxide dismutase.

## Introduction

Low temperature is one of the major factors that limit crop production and reduce yield (Janda *et al.* 2003, Zhang *et al.* 2011). Plants differ in their resistance to chilling and freezing temperatures (Levitt 1980). Low temperatures cause severe deformation of cell membranes (Mahajan and Tuteja 2005), dehydration of intracellular spaces, and physical damage by forming ice crystals (Levitt 1980). Cold tolerance is the capacity to avoid intracellular ice formation, to withstand extracellular ice formation, and to decrease oxidative stress (Tasgin *et al.* 2003, Xu *et al.* 2006). Freezing-tolerant plants suffer from injury only under temperatures at which extracellular ice formation begins (Antikainen 1996). Plants produce several compounds to protect their cells against ice formation. Many overwintering plants accumulate sugars, amino acids, and antifreeze proteins in apoplastic region (Livingston and Henson 1998, Yu *et al.* 2001, Atici and Nalbantoglu 1999, 2003, Tasgin *et al.* 2003, 2006, Griffith and Yaish 2004, Belintani *et al.* 2012). Furthermore, cold sensitive plants may suffer from metabolic decomposition when exposed to cold stress and characteristically exhibit structural injuries (Kacperska 1989, Atici and Nalbantoglu 2003). On the other hand, many plants completing adaptation to cold conditions have evolved a mechanism to enhance their cold tolerance during exposure to low or nonfreezing temperatures in a process known as cold acclimation (Atici and Nalbantoglu 2003, Tasgin *et al.* 2006, Zhao *et al.* 2009). Effective antioxidant system has an important role in the response of plants to low temperatures as well as in decreasing cold damage. One of the most important parts of this system is represented by the antioxidant enzymes, such as superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POX, EC 1.11.1.7), and

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*Abbreviations*: CAT - catalase; POX - peroxidase; SA - salicylic acid; SDS-PAGE - sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SOD - superoxide dismutase.
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catalase (CAT, EC 1.11.1.6). Antioxidant enzymes allow the elimination of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide ($H_2O_2$). Excessive production of ROS during cold stress can cause oxidative damage to membrane lipids, proteins, and nucleic acids (Rout and Shaw 2001, Mutlu *et al.* 2009a,b). In plant cells subjected to stresses, initial events occur mostly in apoplastic space (Vanacker *et al.* 1998, Atici and Nalbantoglu 2003, Mutlu *et al.* 2009a). Some researchers studied the effects of environmental stresses on antioxidant system in apoplastic space and suggested that this compartment is important for plant response to biotic and abiotic stresses (Vanacker *et al.* 1998, Tasgin *et al.* 2006, Cakmak and Atici 2009, Mutlu *et al.* 2009a) although intracellular content of these enzymes is not less important (Ping and Rui 2007, Mutlu *et al.* 2009b, 2011, Hao *et al.* 2011, Mallik *et al.* 2011, Mutlu and Atici 2013).

Some studies indicate that salicylic acid (SA) plays a role in the activation of plant defences (Hayat and Ahmad 2007). Many researchers examined the role of SA in plant growth and development, flowering, ion uptake, stomatal opening, and photosynthesis (Pancheva *et al.* 1996, Popova *et al.* 1997, Uzunova and Popova 2000). Several studies also supported the major role of SA in modulating the plant response to several abiotic and biotic stresses, such as UV radiation, drought, salinity, extreme temperatures, heavy metals, and plant pathogenes (Senaratna *et al.* 2000, Ananieva *et al.* 2004, Mahdavian *et al.* 2008, Wen *et al.* 2008, Guo *et al.* 2009, Mutlu *et al.* 2009a, Fu *et al.* 2011, Hao *et al.* 2011, Kadioglu *et al.* 2011, Mutlu and Atici 2013, Saruhan *et al.* 2012, Song *et al.* 2012). Recent studies have also reported the effects of SA on cold tolerance. These studies demonstrated that SA treatment increased chilling tolerance in maize (Janda *et al.* 1999, Horvath *et al.* 2002), tomato (Ding *et al.* 2002), banana (Kang *et al.* 2003), winter wheat (Tasgin *et al.* 2003, 2006), grapevine (Li *et al.* 2005), *Brassica juncea* (Setia *et al.* 2006), cucumber (Xia *et al.* 2007, Lei *et al.* 2010), radish (Biao 2006), *Zoysia matrella* (Wang *et al.* 2009a), rice (Wang *et al.* 2009b), eggplant (Chen *et al.* 2011), and barley (Mutlu *et al.* 2013). However, molecular events involved in SA signalling are not known yet.

The present study, therefore, examined the effects of exogenous SA before cold stress on cold tolerance in two barley cultivars. The activities of apoplastic antioxidative enzymes, the ice nucleation, and the protein patterns in apoplast were determined at various times after treatments.

## Materials and methods

Seeds of two barley (*Hordeum vulgare* L.) cultivars, cold-tolerant Tokak and cold-sensitive Akhisar, were provided from the Institute of East Anatolian Agricultural Research, Erzurum, Turkey. They were planted in sand in 15-cm pots and maintained at day/night temperatures of 20/18 °C, 75 % relative humidity, photon flux density of 400 μmol $m^{-2}$ $s^{-1}$ (photosynthetic active radiation), and a 12-h photoperiod for 7 d. After germination, 0.1 mM salicylic acid (SA) solution was sprayed on the leaves of plants. Distilled water of the same pH was used to spray the control leaves. At three different times (14, 21, and 28 d), the plants (with and without SA treatment) were transferred to cold chamber (7/5 °C). Standard nutrient solution (Hoagland) was once added to all pots. After 3 d in cold chamber, the plant leaves were harvested (on days 17, 24, and 31).

Apoplastic proteins were extracted as described Hon *et al.* (1994). Harvested fresh leaves (7 g) were carefully cut with a sharp bistoury into 2 cm lengths and rinsed in distilled water to remove proteins from the cut ends. At the end of each rinsing, the amount of removed cellular proteins was calculated by measuring absorbance at 280 nm. The leaves were then vacuum-infiltrated in 20 mM ascorbic acid and 20 mM $CaCl_2$ solution for 15 min. The leaves were blotted dry and placed vertically in a 20 $cm^3$ syringe. The syringes were placed in centrifuge tubes. The apoplastic extract was collected from the bottom of the tubes after the leaves were centrifuged at 1 500 *g* for 20 min. Proteins from apoplastic extracts were precipitated by adding 1.5 times the volume of ice-cold methanol containing 1 % (m/v) acetic acid and incubated overnight at -28 °C. After centrifugation at 3 500 *g* for 20 min, the protein pellets were washed with 100 % ice-cold ethanol and then 70 % (v/v) ice-cold ethanol. Contamination of apoplastic extract by cytoplasm constituents, as monitored by the activity of glucose-6-phosphate dehydrogenase, was always less than 1 % in relation to the catabolic fraction (Mutlu *et al*. 2009a, 2013).

Apoplastic SOD, CAT, and POX activities were measured spectrophotometrically (*Shimadzu UVmini-1240*, Japan). The SOD activity was estimated by recording the decrease in absorbance of nitro-blue tetrazolium chloride at 560 nm according to Dhindsa *et al.* (1981), and one unit of enzyme activity was taken as the amount of enzyme that reduced the absorbance reading to 50 % in comparison with tubes lacking enzyme. The CAT activity was measured by monitoring the decrease in absorbance at 240 nm in 50 mM phosphate buffer (pH 7.5) containing 20 mM $H_2O_2$. One unit of CAT activity was defined as the amount of enzyme that used 1 μmol of $H_2O_2$ per min (Upadhyaya *et al.* 1985). The POX activity was measured by monitoring the increase in absorbance at 470 nm in 50 mM phosphate buffer (pH 5.5) containing 1 mM guaiacol and 0.5 mM $H_2O_2$. One unit of POX activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01 per min (Mutlu *et al.* 2009a,b, 2011, 2013).

Apoplastic proteins were used to determine ice nucleation activity (Atici and Nalbantoglu 1999). The

dried protein pellets in the Eppendorf tubes were dissolved in 1 $cm^3$ of HPLC-grade water and the tubes were then positioned in the freezing bath. After equilibration at -1 °C for 30 min, the temperature was lowered stepwise by 0.2 °C intervals. The tubes were allowed to equilibrate at each temperature for 5 min. The tubes were then removed from the freezing bath after the apoplastic protein solution in each tube had been frozen. The freezing temperature was used as a threshold for ice nucleation activity (Mutlu *et al.* 2013).

The dried apoplastic protein pellets (7 g) were dissolved in equal volumes of sample buffer and proteins were separated in 12.5 % (m/v) SDS-polyacrylamide gel (SDS-PAGE) at 110 V (Laemmli 1970). The gel was stained with Coomassie brilliant blue (Laemmli 1970, Tasgin *et al.* 2006).

All measurements were performed 3 times and the means of the values were used. Statistical analysis was performed using a two-way analysis of variance (*ANOVA*) and means were compared by Duncan´s multiple range test at $P < 0.05$.

## Results and discussion

Numerous studies have suggested that enzymes located at cell surface or in the apoplast are sources of superoxide ($O_2^{\cdot-}$) and $H_2O_2$ during stress-induced oxidative burst in plant cells (Hernandez *et al.* 2001, Minibaeva and Gordon 2003, Tasgin *et al.* 2006, Mutlu *et al.* 2009a). Antioxidant enzymes in apoplast of plants under different stresses, such as pathogen attack, ozone, salt, and drought have important roles in regulating stress responses (Ranieri *et al.* 1996, Hernandez *et al.* 2001, Minibaeva and Gordon 2003, Patykowski and Urbanek 2003, Mutlu *et al.* 2009a, Saruhan *et al.* 2012). Although there are many studies on cellular activities of CAT, POX, and SOD in plants affected by salicylic acid or cold (*e.g.* Kang *et al.* 2003), a limited number of research papers described activities of antioxidative enzymes in apoplast under cold stress or after SA treatment. Recently, Tasgin *et al.* (2006), using leaves from winter wheat, have reported that exogenous SA treatment could be involved in cold tolerance by regulating apoplastic antioxidant enzyme activities. The present study is the first to examine the time-dependent effect of SA on alleviating cold damage at two barley cultivars (tolerant and sensitive) through determining the activity of apoplastic antioxidant enzymes (SOD, CAT, and POX), the ice nucleation activity of apoplastic proteins, and the electrophoretic patterns of apoplastic proteins.

In this study, ice nucleation activity in a cold-tolerant barley cultivar was increased by cold treatment in 17-d-old seedlings whereas it was not affected in 22-d-old seedlings and it was reduced in 31-d-old seedlings. However, the ice nucleation activity of cold-sensitive barley was also induced by cold in 24-d-old seedlings whereas it remained unchanged in 17- and 31-d-old seedlings (Table 1). This result may indicate that the barley plants tried to adaptate to cold stress by changing the composition of apoplastic proteins. In plants treated with SA before cold stress, the ice nucleation activity in both cultivars was generally increased. The SA treatment was more effective in Tokak than in Akhisar. These results are consistent with the previous study in which SA treatment caused a significant increase in ice nucleation activity in winter wheat leaves under cold stress (Tasgin *et al.* 2003). However, the mechanism of SA action is not currently known.

Cold treatment alone decreased apoplastic SOD activity in both cultivars and in all samplings (17-, 24-, and 31-d-old seedlings) compared to the respective controls (Table 1). In addition, SOD activity was gradually increased with age in the control plants of the both cultivars. Some researchers have shown decreased apoplastic SOD activity in pepper under salinity (Turhan *et al.* 2006), however, an increased SOD activity after pathogen attack in both resistant and sensitive barley cultivars (Vanacker *et al.* 1998) and due to Cd toxicity in *Phaseolus aureus* and *Vicia sativa* leaves (Zhang *et al.* 2009). Under cold conditions, SA treatment significantly increased SOD activity in the both barley cultivars (Table 1). This result indicates that SA can play a significant role in responding to cold conditions. Although the effect of exogenous SA on apoplastic SOD under cold has not been explained yet, it has been reported that under salt stress, the activity of apoplastic SOD was increased by SA treatment (Turhan *et al.* 2006, Mutlu *et al.* 2009a).

The stimulation of apoplastic CAT activity by biotic and abiotic stresses is a phenomenon that occurs in many kinds of plant species (Gosset *et al.* 1994, Vanacker *et al.* 1998, Hernandez *et al.* 2000, Patykowski and Urbanek 2003, Tasgin *et al.* 2006, Mutlu *et al.* 2009a) and contributes to better removal of $H_2O_2$ from apoplastic environment (Patykowski and Urbanek 2003). Apoplastic CAT activity increased in 17-, 24-, and 31-d-old Tokak plants when they were exposed to cold stress compared to the respective controls. In contrast, cold treatment reduced CAT activity in cv. Akhisar (Table 1). Some researchers suggested that the stress-tolerant genotypes have a better radical scavenging ability (Mutlu *et al.* 2009a, Mallik *et al.* 2011, Zhang *et al.* 2011). For example, salt treatment increases CAT activity in salt-tolerant cultivars and decreases it in salt-sensitive cotton (Gosset *et al.* 1994) and pea cultivars (Hernandez *et al.* 2000). These results support the findings in the present study: the cold tolerant cultivar had higher CAT activity under cold stress than the sensitive one. However, cold treatment decreased apoplastic CAT activity in winter wheat (Tasgin *et al.* 2006). SA treatment before cold conditions significantly increased apoplastic CAT activity in 17-, 24-, and 31-d-old seedlings of the both cultivars (Table 1). On the contrary, Tasgin *et al.* (2006)

Table 1. The effects of cold and cold + 0.1 mM salicylic acid (SA) on apoplastic antioxidant enzyme (SOD, CAT, and POX) activities and ice nucleation activities (INA) of apoplastic proteins in the leaves of barley, cold-tolerant cv. Tokak and cold-sensitive cv. Akhisar. Means ± SE, $n$ = 10. Values in a group followed by the same letter are not statistically different at $P < 0.05$ as determined by Duncan's multiple range test.

| Activities | Time [d] | Tokak control | cold | cold + SA | Akhisar control | cold | cold + SA |
|---|---|---|---|---|---|---|---|
| SOD | 17 | 0.08 ± 0.009b | 0.05 ± 0.006c | 0.15 ± 0.020a | 0.30 ± 0.026b | 0.26 ± 0.023c | 0.50 ± 0.061a |
| [U $g^{-1}$(f.m.)] | 24 | 0.46 ± 0.058a | 0.30 ± 0.026c | 0.33 ± 0.024b | 0.81 ± 0.073b | 0.65 ± 0.071c | 1.08 ± 0.098a |
| | 31 | 0.46 ± 0.061a | 0.14 ± 0.017c | 0.25 ± 0.031b | 0.69 ± 0.059a | 0.35 ± 0.043c | 0.49 ± 0.052b |
| CAT | 17 | 0.12 ± 0.016c | 0.17 ± 0.021b | 0.31 ± 0.033a | 0.12 ± 0.009b | 0.06 ± 0.005c | 0.15 ± 0.018a |
| [U $g^{-1}$(f.m.)] | 24 | 0.12 ± 0.019c | 0.15 ± 0.015b | 0.26 ± 0.031a | 0.46 ± 0.058a | 0.16 ± 0.012c | 0.33 ± 0.028b |
| | 31 | 0.19 ± 0.025c | 0.32 ± 0.028b | 0.38 ± 0.036a | 0.46 ± 0.047a | 0.14 ± 0.010c | 0.25 ± 0.030b |
| POX | 17 | 3.35 ± 0.242c | 4.33 ± 0.328b | 4.80 ± 0.341a | 3.51 ± 0.435a | 1.11 ± 0.102c | 2.41 ± 0.308b |
| [U $mg^{-1}$(f.m.)] | 24 | 2.67 ± 0.196c | 4.25 ± 0.523b | 5.06 ± 0.643a | 7.49 ± 0.596a | 5.58 ± 0.426c | 6.72 ± 0.449b |
| | 31 | 2.52 ± 0.281c | 3.46 ± 0.403a | 2.80 ± 0.260b | 4.89 ± 0.532a | 3.43 ± 0.403c | 4.37 ± 0.511b |
| INA | 17 | -7.3 ± 0.43a | -8.0 ± 0.58b | -8.9 ± 0.47c | -7.3 ± 0.61a | -7.5 ± 0.52a | -8.5 ± 0.49b |
| [°C] | 24 | -6.4 ± 0.51a | -6.3 ± 0.62a | -7.1 ± 0.48b | -6.0 ± 0.38a | -6.8 ± 0.46b | -7.5 ± 0.49c |
| | 31 | -7.1 ± 0.42a | -6.5 ± 0.38a | -7.9 ± 0.53b | -7.5 ± 0.43b | -7.5 ± 0.40b | -6.7 ± 0.33a |

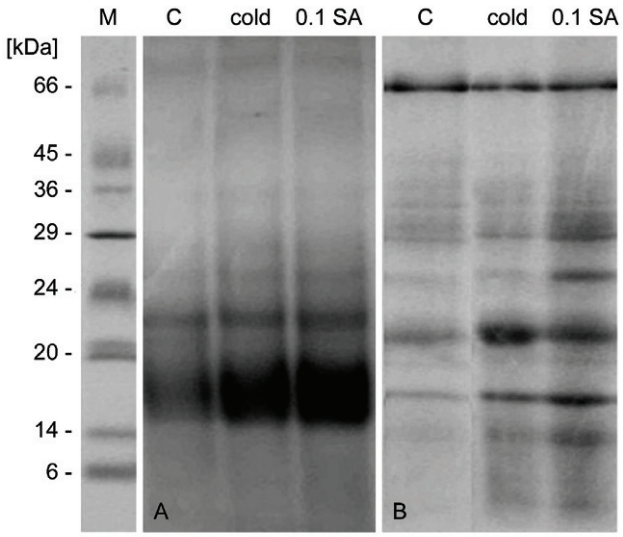


Fig. 1. SDS-PAGE of apoplastic proteins obtained from 24-d-old barley leaves grown at control (C), cold, and cold + 0.1 mM SA (0.1 SA) conditions. Cold-tolerant cv. Tokak (*A*) and cold-sensitive cv. Akhisar *(B).*

determined that SA treatment caused a decrease in apoplastic CAT activity in winter wheat under cold stress. Such contradictory findings related to SA treatment on CAT activity can be found in literature because the effect of SA on CAT can be dose-dependent and vary according to plant species (He *et al.* 2005). Hence, a significant increase in the apoplastic activity of CAT can be observed in only low SA concentration (0.1 mM) in the both cultivars grown under cold stress. In an inconvenient concentration, SA itself can also be a stress factor.

It is a well-known fact that POX protects cells against the damaging effects of $H_2O_2$ during an oxidative-burst under stress conditions (Levine *et al.* 1994) and detoxifies $H_2O_2$ also in the apoplast. In this study, cold treatment alone stimulated apoplastic POX activity in tolerant cultivar in all examined samplings whereas generally decreased it in sensitive cultivar compared to the respective controls (Table 1). The increase in POX activity obviously suggests that the cold-resistant cultivar can better scavenge the $H_2O_2$ excessively produced during cold stress. Cold stress also results in higher POX activity in apoplastic area of winter wheat leaves (Tasgin *et al.* 2006). Some studies have shown that also pathogen attack (Vanacker *et al.* 1998) and cadmium toxicity (Zhang *et al.* 2009) increased apoplastic POX activity in leaves of barley, *Phaseolus aureus*, and *Vicia sativa*. SA application before cold stress significantly increased apoplastic POX activity in the leaves of the both cultivars (Table 1) but the effect was more pronounced in sensitive cultivar in which POX activity was decreased by cold

alone. Similarly, SA treatment resulted in a higher activity of apoplastic POX in wheat plants under cold stress (Tasgin *et al.* 2006). SA-induced increase in POX activity is also known to be effective in the processes related to the biosynthesis of lignin and suberin which strengthen a cell wall (Sakhabutdinova *et al.* 2004).

The molecular masses of apoplastic proteins were between 16 and 50 kDa (Fig. 1*A*,*B*). Cold treatment increased the accumulation of 16-kDa polypeptides in 24-d-old seedlings of the cold-tolerant cultivar but it caused the accumulation of 14, 16, 20, and 29-kDa polypeptides in the cold-sensitive cultivar. The polypeptides of low molecular mass (from 15 to 32 kDa) were accumulated during the cold acclimation in leaf apoplast of winter rye (Marentes *et al.* 1993) and spruce (Jarzabek *et al.* 2009). These proteins probably increase cold tolerance by impeding freezing point in apoplastic space during cold stress. SA treatment caused the accumulation of 23 kDa polypeptide in 24-d-old Tokak plants and also the synthesis of a new polypeptide with an approximately 24 kDa in cv. Adhisar which also increased accumulation of polypeptides of 14, 16, and 29 kDa (Fig. 1*A*,*B*). Similar effects were observed by Antikainen and Griffith (1997), who determined that apoplastic antifreeze proteins were highly correlated with frost tolerance in winter rye, and Tasgin *et al.* (2003), who determined that SA treatment increased accumulation of apoplastic proteins and decreased freezing injury in winter wheat leaves.

Freezing is known to take place firstly in the apoplastic space of plant leaves when the protein concentration in intracellular area is sufficient to prevent freezing which could cause a significant irreversible damage to cell membranes. Therefore, the freezing occurring in apoplast should be managed. Proteins are important elements that impede freezing and regulate the formation of ice in apoplast (Yu *et al.* 2001, Griffith and Yaish 2004, Atici and Nalbantoglu 2003). In the present study, exogenous SA treatment applied before cold stress to the leaves of barley increased ice nucleation activity of apoplastic proteins and the accumulation of some apoplastic polypeptides and also caused *de novo* synthesis of some of them. SA applied before cold stress improves cold tolerance by regulating the apoplastic proteins especially in cold-sensitive cultivar.

In conclusion, cold treatment caused a regular decrease in all the apoplastic antioxidant enzymes (SOD, CAT, and POX) in the cold-sensitive cultivar of barley whereas it increased CAT and POX activities in the cold-tolerant cultivar. SA applied to the sensitive and tolerant cultivars of barley before the exposure to cold stress increased the activities of the apoplastic antioxidant enzymes. SA treatment also increased the ice nucleation activity of apoplastic proteins and caused the accumulation or *de novo* synthesis of some apoplastic polypeptides.

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