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Phytochemistry 67 (2006) 710–715
PHYTOCHEMISTRY
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Effects of salicylic acid and cold treatments on protein levels and on the activities of antioxidant enzymes in the apoplast of winter wheat leaves

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Received 13 July 2005; received in revised form 2 December 2005
Available online 7 March 2006

Abstract

The effects of salicylic acid (SA) and cold on apoplastic protein levels and activities of apoplastic catalase (CAT), peroxidase (POX) and polyphenol oxidase (PPO) were investigated in winter wheat (*Triticum aestivum* cv. Dogu-88) leaves. The plants were grown with and without 10 μM SA treatment at both control (20/18 °C for 30 and 45-day) and cold (10/5 °C for 30-day and 5/3 °C for 45-day) acclimatisations. Molecular masses of the apoplastic polypeptides were shown ranging in size from 20 to 66 kDa on SDS–PAGE. Accumulation and pattern of the polypeptides were changed by both SA and cold. It is observed that CAT, POX and PPO activities at 45-day control leaves were higher than at 30-day. When the activities with SA and cold treatments are compared to their controls, CAT activities were decreased while POX and PPO activities were increased by both the treatments. When the activities with cold + SA treatment are compared to their cold treatments, CAT and POX activities were decreased while PPO activity was increased by SA. It is concluded that exogenous SA can be involved in cold tolerance by regulating apoplastic proteins and antioxidant enzyme activities.

Keywords: Apoplastic proteins; Catalase; Cold; Peroxidase; Polyphenol oxidase; Salicylic acid; *Triticum aestivum*; Winter wheat

1. Introduction

Low temperature is one of the most important stress factors limiting the growth and productivity of cereals (Janda et al., 2003). Plants produce several compounds to protect cells against fatal intracellular and intercellular ice formation. Many overwintering plants accumulate sugars, amino acids and antifreeze compounds including antifreeze proteins in apoplastic (extracellular) region (Atıcı and Nalbantoğlu, 1999, 2003; Griffith and Yaish, 2004). Growth at low temperature may increase the concentration of reactive oxygen species (ROS) (Wise and Naylor, 1987; Okuda et al., 1991). To alleviate or prevent low temperature-induced oxidative injury, plants have evolved mechanisms to scavenge these toxic and reactive species by antioxidant compounds and by enzymatic antioxidant systems (Atıcı and Nalbantoğlu, 2003; Kang et al., 2003). H_2O_2 is metabolised to H_2O by peroxidase (POX) and catalase (CAT) (Willekens et al., 1997). In addition to these enzymes, polyphenol oxidase (PPO) generally catalyzes the oxidation of phenolic compounds to quinones (Mayer, 1987; Mohammadi and Kazemi, 2002). Therefore, it is crucial that plants should maintain the activities of these enzymes in order to accommodate these oxidative stresses.

Under severe stress conditions, however, the antioxidant capacity may not be sufficient to minimize the harmful effect of oxidative injury. Therefore, the search for signal molecules that mediate the stress tolerance is an important step in our better understanding how plants acclimate to the adverse environment. Recent studies indicate that SA is a natural and hormone-like signal molecule for the activation of plant defenses (Klessig and Malamy, 1994).

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0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.01.022

Recently, several studies also supported a major role of SA in modulating the plant response to several abiotic and biotic stresses, such as ultraviolet light, drought, salt, chilling and heat (Janda et al., 1999; Senaratna et al., 2000; Chang-Kui et al., 2002; Kang et al., 2003; Taşgın et al., 2003; Ananieva et al., 2004). Different effects of SA on protective enzymes activities could be associated with H_2O_2 metabolism (Janda et al., 2003). However, the molecular events involved in SA signaling are not yet fully understood.

SA pretreatment could directly or indirectly change freezing tolerance and cellular antioxidant enzyme activities during chilling stress, which had a higher ability to withstand chilling-induced injury (Horvath et al., 2002; Kang et al., 2003; Taşgın et al., 2003). In the present study, the effects of both SA and cold acclimation on apoplastic proteins and apoplastic antioxidant enzyme (CAT, POX and PPO) activities in winter wheat leaves were studied.

2. Results and discussion

The effects of both biotic and abiotic stress on apoplastic space have been studied by some authors. The reports suggest that apoplastic compartment could be important in the plant cells response to stresses (Vanacker et al., 1998a,b, 1999; Ranieri et al., 1996, 2000; Minibaeva et al., 2001). Therefore, in the present study, the effects of SA and cold on apoplastic protein levels and activities of apoplastic CAT, POX and PPO were investigated in winter wheat leaves. The plants were grown with and without 10 μM SA treatment at both control (20/18 °C for 30- and 45-day) and cold (10/5 °C for 30-day and 5/3 °C for 45-day) acclimatisations. It was shown that 10 μM SA was very relevant for thermotolerance in mustard seedlings (Dat et al., 1998) and cold tolerance in winter wheat leaves (Taşgın et al., 2003). Our previously study showed that 1000 μM SA increased freezing injury and 10 μM SA was better than 100 μM SA for decreasing of freezing injury (Taşgın et al., 2003). Therefore, 10 μM SA concentration was used and cold acclimatizations were also made in imitation of natural conditions (Table 1) (Taşgın et al., 2003).

2.1. Effect of salicylic acid and cold on apoplastic proteins by SDS–PAGE

The apoplastic proteins were concentrated and investigated by SDS–PAGE. Polypeptides of apoplastic proteins in control, control + SA, cold and cold + SA leaves were separated by comparing with standards. Molecular masses of the polypeptides were shown ranging in size from 20 to 66 kDa (Fig. 1). When 30 and 45-day control leaves are compared, accumulation of polypeptides was decreased at 45-day leaves. When polypeptides in control and control + SA leaves are compared, accumulation of polypeptides of 66, 64, 36 and 32 kDa was increased by SA at both 30 and 45-day leaves. When polypeptides in control and cold leaves are compared, accumulation of polypeptides of 66, 64, 45, 36, 32, 22 and 21 kDa was increased at both 10/5 and 5/3 °C, and 26 and 25 kDa was increased at only 10/5 °C. When polypeptides in cold and cold + SA leaves are compared, accumulation of polypeptides of 36 and 32 kDa was increased by SA at both 30 and 45-day leaves, accumulation of polypeptides of 66, 64, 22 and 21 kDa was increased while 26 and 25 kDa was decreased only at 30-day leaves, and accumulation of polypeptides of 25 and 23 kDa was increased only at 45-day leaves. These results are consistent with previously our study that both SA treatment and cold acclimation decreased freezing injury in winter wheat leaves and increased ice nucleation activity by apoplastic proteins (Taşgın et al., 2003). It was also found that the levels of apoplastic antifreeze proteins (Antikainen and Griffith, 1997) were highly correlated with frost tolerance in cereals. Therefore, both SA and cold can increase freezing tolerance in winter wheat leaves by increasing accumulation of apoplastic proteins including antifreeze proteins. It is also seen that exogenous SA can have an important role on regulation of apoplastic proteins.

Table 1
Growth condition for cold acclimation

Time (day)	Day/night temperature (°C)
1–7	20/18
8–15	15/10
16–30	10/5
31–45	5/3

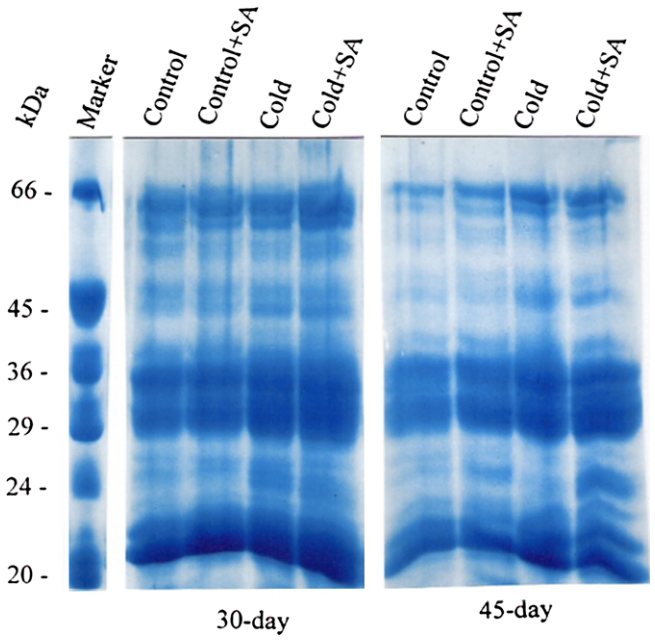

Fig. 1. SDS–PAGE of apoplastic proteins obtained from winter wheat leaves growing at control (20/18 °C for 30 and 45-day), control + SA, cold (10/5 °C for 30-day and 5/3 °C for 45-day) and cold + SA conditions. Three days before leaf cutting, 10 μM SA solution was sprayed on the leaves of both control and cold acclimatised plants.

2.2. Effect of salicylic acid and cold on apoplastic antioxidant enzyme activities

Under both normal and stress conditions, the contribution to the total ROS content by numerous intracellular and extracellular ROS producers, such as enzymes, is important (Schraudner et al., 1998; Vanacker et al., 1998a, 1999; Hernandez et al., 2001). Experimental data suggest that the enzyme systems located at the cell surface, that is, in the plasmalemma and cell wall, are the principal sources of superoxide and H_2O_2 during the oxidative burst in plant cells. In addition to cellular CAT, POX and PPO activity studies under SA treatment and cold stress (Schneider and Ullrich, 1994; Hodges et al., 1997; Janda et al., 1999; Horvath et al., 2002; Shim et al., 2003; Kang et al., 2003), a few researches were reported on apoplastic CAT and POX activities under pathogen attack and ozone stress (Ranieri et al., 1996, 2000; Vanacker et al., 1998a, 1999; Patykowski and Urbanek, 2003). Until now, any study has been reported on apoplastic PPO activity. It was found that cellular antioxidant enzymes (Janda et al., 2003) were highly correlated with frost tolerance in cereals. In the present study, it was hypothesized that SA and cold also can affect apoplastic antioxidant enzyme activities in the winter wheat leaves.

Apoplastic CAT activity was determined in some plants. A secreted CAT activity from maize roots was found in extracellular spaces (Salguero and Bottger, 1995). CAT activity was also found in apoplastic region of oat, barley and tomato leaves (Vanacker et al., 1998a,b, 1999; Patykowski and Urbanek, 2003) and of onion roots (Cordoba-Pedregosa et al., 2003). In addition, a high molecular weight CAT isoform was observed in intercellular compartment of overwintering barley leaves (Baek et al., 2000). The results indicated that the found CAT activity could be involved in the control regulation of H_2O_2 in extracellular spaces (Salguero and Bottger, 1995; Cordoba-Pedregosa et al., 2003; Patykowski and Urbanek, 2003). In the present study, apoplastic CAT activity was also determined in winter wheat leaves and the values of the CAT activity were similar to values of apoplastic CAT activity in tomato leaves (Patykowski and Urbanek, 2003). When apoplastic CAT activities at 30 and 45-day the control leaves are compared, the activity was increased 70% at 45-day. It is seen that the CAT activities have the different behaviours at the different ages. In the leaves growing at SA, it was observed that apoplastic CAT activities were decreased 21% and 40% by SA at 30 and 45-day leaves, respectively (Fig. 2). SA generally also caused a decrease in cellular CAT activity (Hodges et al., 1997; Janda et al., 1999; Horvath et al., 2002; Shim et al., 2003). It has been reported that SA can cause an accumulation of H_2O_2 by blocking a substantial portion of CAT activity in several plant species (Raskin, 1992). In the leaves growing at cold, it was also observed that apoplastic CAT activity was decreased 65% at 5/3 °C, while not changed at 10/5 °C (Fig. 2). Cold generally caused a decrease in cellular CAT activity (Feng et al., 2003; Janda et al., 2003; Shim et al., 2003). In the leaves growing at cold + SA, it was also observed that apoplastic CAT activities were decreased 40% by cold (10/5 °C) + SA and 91% by cold (5/3 °C) + SA (Fig. 2). It is also seen that SA decreases the activity at both cold treatments (Fig. 2). Under cold stress, SA decreased cellular CAT activity (Horvath et al., 2002; Kang et al., 2003). It has been reported that SA may be the compound causing CAT inhibition and induction of disease resistance by the signaling of H_2O_2 and activation of the defense genes. Therefore, it might be possible that SA is produced during oxidative stresses and causes the fall in CAT activity in oxidatively-stressed plants (Shim et al., 2003). However, it is still necessary to confirm whether the CAT inhibition depends only on an increase in SA (Shim et al., 2003). It remains to be seen whether this could be accomplished by reducing the SA biosynthesis chemically or genetically (Shim et al., 2003).

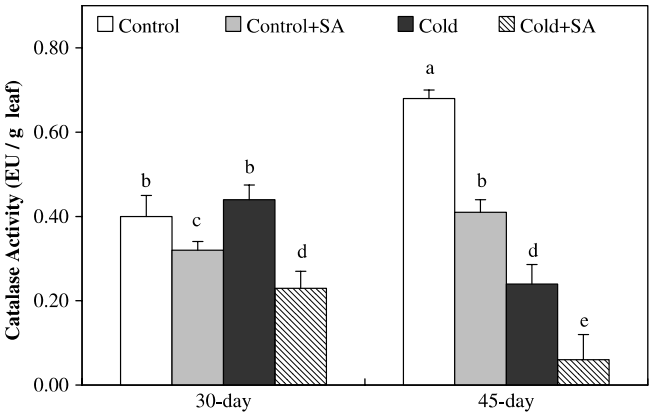

Fig. 2. Apoplastic catalase activity in winter wheat leaves growing at control (20/18 °C for 30 and 45-day), control + SA, cold (10/5 °C for 30-day and 5/3 °C for 45-day) and cold + SA conditions. Three days before leaf cutting, 10 μM SA solution was sprayed on the leaves of both control and cold acclimatised plants. Values in a group followed by the same letter are not statistically different at $P < 0.05$ level as determined by Duncan's multiple range test.

When apoplastic POX activities at 30 and 45-day the control leaves are compared, the activity was increased 95% at 45-day. It is seen that the POX activities have the different behaviours at the different ages. Apoplastic peroxidases in the cell wall could play a critical role in regulating the wall stiffening process during cell differentiation by interfering with the activity of secretory peroxidases (De Pinto and De Gara, 2004). In the leaves growing at SA, apoplastic POX activities were increased 96% and 20% by SA at 30 and 45-day, respectively (Fig. 3). It was found only a research about apoplastic POX activity induced by exogenous SA stimulating the $O_2^{\cdot-}$ synthesis by root cells (Minibaeva et al., 2001). SA might function as a weak detergent and change the electric charge of the cell surface, facilitating secretion of soluble POX isoforms into the apoplast (Minibaeva and Gordon, 2003). In the leaves growing at cold, it was also observed that apoplastic POX activities were increased 554% at 10/5 °C and 254%

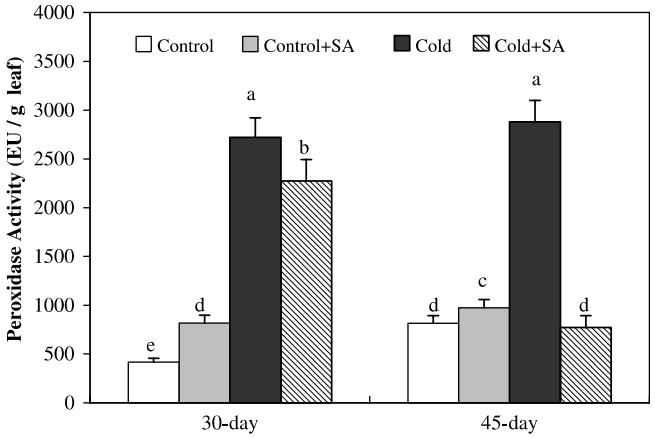

Fig. 3. Apoplastic peroxidase activity in winter wheat leaves growing at control (20/18 °C for 30 and 45-day), control + SA, cold (10/5 °C for 30-day and 5/3 °C for 45-day) and cold + SA conditions. Three days before leaf cutting, 10 μM SA solution was sprayed on the leaves of both control and cold acclimatised plants. Values in a group followed by the same letter are not statistically different at $P < 0.05$ level as determined by Duncan's multiple range test.

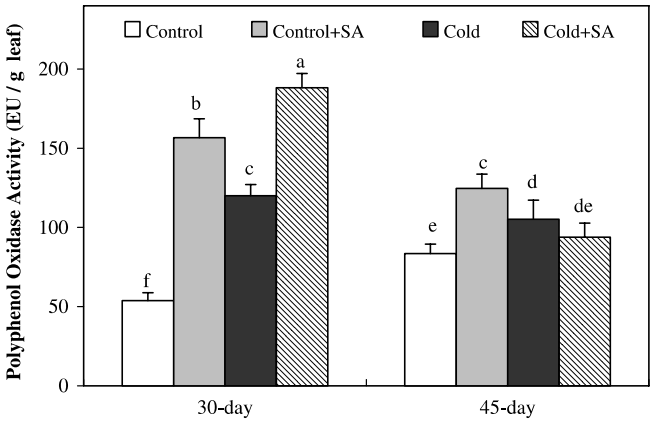

Fig. 4. Apoplastic polyphenol oxidase activity in winter wheat leaves growing at control (20/18 °C for 30 and 45-day), control + SA, cold (10/5 °C for 30-day and 5/3 °C for 45-day) and cold + SA conditions. Three days before leaf cutting, 10 μM SA solution was sprayed on the leaves of both control and cold acclimatised plants. Values in a group followed by the same letter are not statistically different at $P < 0.05$ level as determined by Duncan's multiple range test.

at 5/3 °C (Fig. 3). Under stress condition, some soluble POX isoforms can be easily released from the cell wall and go around inside the apoplast of the intact plant (Minibaeva and Gordon, 2003). Thus, POX protects cells against damaging effects of H_2O_2 during an oxidative-burst response (Westhuizen et al., 1998; Bolwell et al., 1999). In the leaves growing at cold + SA, it was also observed that apoplastic POX activity was increased 447% by cold (10/5 °C) + SA while not changed by cold (5/3 °C) + SA (Fig. 3). It is also seen that SA decreases the activity at both cold treatments (Fig. 3).

When apoplastic PPO activities at 30 and 45-day the control leaves are compared, the activity was increased 64% at 45-day. It is seen that the PPO activities have the different behaviours at the different ages. In the leaves growing at SA, apoplastic PPO activities were increased 191% and 49% by SA at 30 and 45-day, respectively (Fig. 4). Cellular PPO activity was also increased by SA (Schneider and Ullrich, 1994). In the leaves growing at cold, apoplastic PPO activity was increased 122% at 10/5 °C and 25% at 5/3 °C (Fig. 4). Cellular PPO activity was decreased by cold (Jiang and Li, 2003; Leja et al., 2003). In the leaves growing at cold + SA, it was also observed that apoplastic PPO activity was increased 248% by cold (10/5 °C) + SA while not changed by cold (5/3 °C) + SA (Fig. 4). It is also seen that SA increases the activity only at 10/5 °C. The apoplastic PPO activity results can be important at cold stress respond since it was studied for the first time in apoplastic region.

3. Conclusions

The antioxidant cycle enzymes located at cell surface can have important physiologic roles since they are the principal sources of superoxide and H_2O_2 during the oxidative burst in plant cells. The enzymes can function to remove apoplastic H_2O_2 with ascorbate and glutathione derived from the cytoplasm (Vanacker et al., 1998b). Therefore, it is crucial that plants should maintain the activities of these enzymes. In the present study, for the first time, our results showed that apoplastic proteins and apoplastic CAT, POX and PPO activities were significantly affected by both SA and cold acclimatisations. In apoplastic region of winter wheat leaves, it seems that developing an accumulation of proteins, a low CAT activity and a high POX and PPO activities can be a better strategy to tolerate freezing temperature. It is concluded that exogenous SA can be involved in cold tolerance by regulating apoplastic proteins including antifreeze proteins and antioxidant enzyme activities. However, the role of endogenous SA during the development of frost tolerance is still unknown and requires further research (Janda et al., 2003).

4. Experimental

4.1. Plant material

Winter wheat (*Triticum aestivum* cv. Dogu-88) seeds were planted in sand in 15 cm pots. They were maintained in a 20/18 °C (day/night) growth chamber with a 12 h day length for 7 days to initiate germination. After 7 days, control plants were maintained at 20/18 °C for another 38 days. The remaining plants were transferred for cold acclimation for another 38 days during which growth conditions were changed slowly from 20/18 to 5/3 °C (Table 1). The plants were watered routinely with Hoagland solution. Three days before leaf cutting, SA solution (10 μM, pH 5) was sprayed on the leaves of both control and cold acclimatised plants (Taşgın et al., 2003). The 30- and 45-day leaves were cut to extract apoplastic proteins.

4.2. Extraction of apoplastic proteins

Apoplastic proteins were extracted as described in Hon et al. (1994). Briefly, fresh leaves (7 g) were cut into 2 cm lengths, and rinsed in six changes of water to remove cellular proteins from the cut ends. Cellular proteins in the rinsing water were controlled by measuring at a wavelength of A_{280}. Then, the leaves were then vacuum-infiltrated for 30 min in 20 mM ascorbic acid + 20 mM $CaCl_2$ solution. The leaves were blotted dry and placed vertically in a 20 ml 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 1500*g* for 20 min. Proteins were precipitated from apoplastic extracts by adding 1.5 times the volume of ice-cold methanol containing 1% acetic acid and incubating the samples overnight at −20 °C. After centrifugation for 20 min at 3500*g*, the protein pellets were washed with 100% ice-cold ethanol and 70% ice-cold ethanol (Taşgın et al., 2003). The dried protein pellets were used to study protein electrophoresis and enzyme activities. Contamination of apolastic 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 (Patykowski and Urbanek, 2003; Cordoba-Pedregosa et al., 2003).

4.3. Protein electrophoresis

The dried apoplastic protein pellets obtained from the leaves (7 g) were dissolved in equal volumes of sample buffer. Polypeptides of apoplastic proteins were separated in 12.5% SDS–polyacrylamide gel (PAGE) at 110 V (Laemmli, 1970). The gel was stained with Coomassie brilliant blue.

4.4. Determination of enzyme activities

The dried apoplastic protein pellets obtained from the leaves (7 g) were dissolved in 1 ml phosphate buffers. CAT, POX and PPO enzyme activities in the apoplastic solution were measured spectrophotometrically. 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 H_2O_2/min (Gong et al., 2001). 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 (Janda et al., 2003). One unit of POX activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01/min. The PPO activity was measured by monitoring the increase in absorbance at 420 nm in 200 mM phosphate buffer (pH 7) containing 25 mM cathecol. One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001/min (Shi et al., 2002).

4.5. Statistical analysis

All experiments were performed six times and the average of the values was used. The data were analysed by analysis of variance, and means were compared by Duncan's multiple range test.

Acknowledgements

This work was supported by TUBITAK and BAS Grant No: TBAG-U/23(101T119).

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