Apoplastic Antioxidant Enzymes in the Leaves of Two Strawberry Cultivars and Their Relationship to Cold-Hardiness

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Abstract

In this study, apoplastic antioxidant enzymes in the leaves of two strawberry cultivars (‘Aromas’ and ‘Diamante’) and their relationship to cold-hardiness were investigated. Fully expanded, uniformly sized leaves from 1-year-old field plants of the cultivars were collected at the hardening (late autumn, in November and winter, in January) and de-hardening (summer, in July) stages. Leaf samples were exposed to low temperatures of 5, -5, -10, -20 and -30ºC for 12 h to determine their cold-hardiness (LT50; lethal temperature, where 50% of the plants were killed). Cold-acclimation produced a remarkable increase in cold-hardiness. It was found that ‘Diamante’ had higher cold-hardiness than ‘Aromas’. Moreover, malondialdehyde and total carotenoid content increased during the hardening stage and decreased during the de-hardening stage. The activities of catalase, peroxidase and ascorbate peroxidase in the leaf apoplast and nicotinamide adenine dinucleotide phosphate oxidase activity in the leaf tissue were correlated with changes in cold-hardiness. The activities of these enzymes were higher in the hardening stage than in the de-hardening stage. The activities of apoplastic catalase, peroxidase and ascorbate peroxidase varied significantly depending on the cold-acclimation stage and the cold-hardiness level of the cultivars. This study indicates that elevated apoplastic antioxidative enzymes may be determinants of cold-hardiness in the strawberry plant. The lower malondialdehyde content and higher total carotenoid and apoplastic enzyme activities in ‘Diamante’ indicated an enhanced cold-hardiness capacity of this cultivar, serving to protect the plant from oxidative damage.

Keywords: antioxidative enzymes, apoplast, cold-hardiness, Fragaria × ananassa, oxidative stress, strawberry

Abbreviation: APX=Ascorbate peroxidase; CAT=Catalase; DMSO=Dimethyl sulphoxide; EDTA=Ethylene diamine-tetraacetic acid; GR=Gluathione reductase; H2O2=Hydrogen peroxide; IWF=Intercellular washing fluid; K-phosphate=Potassium phosphate; MDA=Malondialdehyde; NAD[P]H=Nicotinamide adenine dinucleotide phosphate; O2-•=Superoxide; O2•-=Singlet oxygen; OH•=Hydroxyl; POX=Peroxidase; PVPP=Polyvinylpolypyrrolidone; ROS=Reactive oxygen species; SOD=Superoxide dismutase; TBA=Thiobarbituric acid; TCA=Trichloroacetic acid; TMB=3,3',5,5'-tetramethylbenzidine

Introduction

Cold temperatures limit the geographical locations where crop and horticultural plant species can be grown and periodically cause significant losses in plant productivity (Thomashow and Browse, 1999). The process whereby certain plants increase in freezing tolerance in response to low nonfreezing temperatures is known as cold-acclimation (or cold-hardiness) (Levitt, 1980; Thomashow, 2010). In temperate latitudes, cold-acclimation is established in the autumn, when the temperatures are low but positive and the photoperiod decreases, and it reaches a maximum in winter (Reulland et al., 2009). Strawberry is a semi-hardy evergreen and has a relatively low tolerance to cold temperatures (Palonen and Buszard, 1997). Cold-acclimation in strawberries begins when the days become shorter in late summer. Short days alone will trigger strawberries to develop tolerance to -2ºC or -3ºC. For further acclimation, plants must be subjected to cold temperatures, i.e., days of approximately 10 ºC and nights of approximately 0ºC (Fisher, 2004).

The apoplast is the first plant compartment to encounter environmental signals (Gao et al., 2004). The apoplast consists of extracellular, aqueous spaces outside the plasma membrane, including cell walls, spaces between the cells and xylem (Sakurai, 1998). As temperatures drop below freezing, ice forms primarily in the intercellular spaces (Levitt, 1980). Injury to the tissue may result from ice formation, either within the symplast or via ice growth into the symplast (Gusta et al., 2004).

Plants respond to low temperature with a number of physiological, biochemical and developmental changes...
(Shinozaki and Yamaguchi-Shinozaki, 2000). One of the biochemical changes occurring when plants are subjected to low-temperature stress is the production of reactive oxygen species (ROS), such as the superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl (OH) radicals and singlet oxygen (O$_2^*$) (Foyer and Noctor, 2005; Karpinski et al., 2002; Suzuki and Mittler, 2006). ROS can severely disrupt normal metabolism through oxidative damage to lipids, protein and nucleic acids (Rout and Shaw, 2001). Plants contain complements of enzymatic and non-enzymatic antioxidants that play a role in regulating the levels of ROS. Antioxidative enzymes, such as superoxide dismutase (SOD: EC 1.15.1.1), catalase (CAT: EC 1.11.1.6), peroxidase (POX: EC 1.11.1.7), ascorbate peroxidase (APX: EC 1.11.1.11), and glutathione reductase (GR: EC 1.6.4.2), are the most important components in the scavenging system for ROS (Mc Kersie and Leshem, 1994; Noctor and Foyer, 1998). In addition, the antioxidant enzymes in the apoplastic spaces of plants under environmental stresses have been shown to play an important role in the regulation of the stress response (Mutlu et al., 2009; Taşgın et al., 2006). Non-enzymatic antioxidants, such as ascorbic acid, glutathione, flavonoids, carotenoids and tocopherols, also work in concert to control the cascades of uncontrolled oxidation and to protect plant cells from oxidative damage through the scavenging of ROS (Gill and Tuteja, 2010). Recent studies have shown that ROS could also play a key role in mediating important signal transduction events (Suzuki and Mittler, 2006). Thus, ROS, such as O$_2^-$, are produced by nicotinamide adenine dinucleotide phosphate oxidase [NAD(P)H oxidases: EC 1.6.3.1] during abiotic stress to activate stress-response pathways and induce defense mechanisms (Suzuki and Mittler, 2006).

Strawberry exposed to low temperature can increase cold resistance through an increase in the activity of antioxidative enzymes (Gülen et al., 2008; Zhang et al., 2008). In addition, acid invertase and sucrose synthase enzymes in the apoplast are regulated by cold-acclimation and de-acclimation in strawberry plants (Turhan, 2012). There is, however, no information available on the apoplastic enzymes involved in antioxidative metabolism in strawberry during cold-acclimation. The aim of the present study was to investigate changes in lipid peroxidation, total carotenoid content, NAD(P)H oxidase activity and apoplastic antioxidative enzyme (CAT, POX and APX) activities under hardening and de-hardening stages in ‘Aromas’ and ‘Diamante’ strawberry cultivars with contrasting levels of cold-hardiness.

Materials and methods

**Plant material**

Uniformly sized plants of approximately 1-year-old ‘Aromas’ and ‘Diamante’ cultivars were used. Both cultivars are day-neutral and were originally licensed by the University of California. The two cultivars are adapted to different temperature zones. Although ‘Aromas’ has broader environmental tolerance than ‘Diamante’ (Anonymous, 2012), it has been found that ‘Diamante’ is relatively more tolerant to low temperature than ‘Aromas’ (Turhan et al., 2011). Fully expanded, uniformly sized leaves from introduced strawberry cultivars were collected from plants in Ekşişehir, Turkey (39°47’ N, 30°31’ E) in summer (in July), late autumn (in November) and winter (in January). The monthly mean, maximum and minimum temperatures in the experimental area are shown in Fig. 1. Leaf samples were collected randomly from twenty plants of each cultivar, packed on ice and taken to the laboratory. Parts of the leaves from each treatment group were then processed to determine cold-hardiness. Fresh tissue was also used immediately for all malondialdehyde (MDA) and total carotenoid determination assays and for the preparation of intercellular washing fluid (IWF).

**Controlled freezing test**

Leaves from each cultivar were wrapped (10 leaves per temperature in each replication) in aluminum foil along with moistened paper and placed in a manually controlled low-temperature freezer. The plant tissue temperature was monitored with a copper-constant thermocouple (Testo 925, Omni Inst., Scotland, UK) inserted in the foil pouch. The temperature was decreased stepwise at approximately 1.5°C/h to -5°C and 5°C/h thereafter to a final value of -30°C. The samples were exposed to low temperatures of 5, -5, -10, -20 or -30 °C for 12 h. The samples were then removed from the freezer at each temperature and placed at 4°C overnight for slow thawing.

**Cold-hardiness determination**

At each temperature, the cold-hardiness of the leaf tissues was estimated by assaying freezing injury using the electrolyte leakage method of Arora et al. (1992). Briefly, leaf discs (10 mm diameter) were cut from the leaves; they were then lightly rinsed in distilled water, gently blotted with paper and placed in test tubes (one disc per tube). Distilled water (10 mL) was added to the test tubes, which were then vacuum-infiltrated to allow uniform diffusion of the electrolytes. The tubes were shaken on an orbital shaker (250 rpm) (Unimax 2010, Heidolph Instruments, Germany) for 4 h at room temperature. The electrical con-
ductivity of each sample was measured using a conductivity meter (YSI 3200, USA). Electrical conductivity was measured once more after the tubes were autoclaved (0.12 MPa, 120°C, 20 min) and cooled. Proportional injury at each temperature was calculated from ion leakage data with the following equation:

Proportional injury = \frac{[\text{proportional } L(t) - \text{proportional } L(c)]}{(100 - \text{proportional } L(c))} \times 100

where proportional \( L(t) \) and proportional \( L(c) \) are the proportional ion leakage data for the treatments and control samples, respectively (Arora et al., 1992). In addition, the survival of the plants was determined for \( L_{50} \) estimation (lethal temperature, where 50% of the plants were killed). The \( L_{50} \) was defined as the temperature causing half-maximal % injury, calculated as the midpoint between the maximum injury and the control.

Preparation of intercellular washing fluid (IWF)

Apoplastic IWF was extracted from leaf tissues according to Fecht-Christoffers et al. (2003). Freshly harvested leaves were cut into small pieces (approximately 5 mm in width) and rinsed for 2 min with tap water. Leaves (approximately 10 g) were immersed in distilled water for 1 min, then vacuum infiltrated for 5 min under a weak vacuum (-40 kPa) (KNF Neuberger, Germany). The infiltrated tissue was then gently surface-dried and centrifuged at room temperature for 1 min at 1,200 × g. The resulting fluid (apoplast) was collected and dispensed equally (200 µL) to Eppendorf tubes, placed in liquid N\(_2\) and kept at -80°C prior to further analyses.

Lipid peroxidation

Lipid peroxidation was estimated by determining the malondialdehyde (MDA) content in the leaves according to the method of Rajinder et al. (1981). One hundred milligrams of fresh leaf samples was homogenized in 5 mL of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 × g for 5 min at 4°C (Beckman Coulter Allegra 64R, USA). An aliquot of 0.3 mL of supernatant was mixed with 1.2 mL of 0.5% thiobarbituric acid (TBA) prepared in 20% TCA and incubated at 95°C for 30 min. After stopping the reaction in an ice bath for 5 min, the samples were centrifuged at 10,000 × g for 10 min at 25°C. The supernatant absorbance at 532 nm was then measured with a spectrophotometer (Perkin Elmer Lambda 25, USA). The absorbance of the extract at 460 nm was measured with a spectrophotometer (Perkin Elmer Lambda 25, USA).

\( \text{NAD(P)H oxidase activity assay} \)

NAD(P)H oxidase was extracted with a mortar and pestle from 1.0 g of leaf tissue at 4°C with 1.0% polyvinylpyrrolidone (PVPP) and 5 mL of the following extraction solution: 100 mM Potassium phosphate (K-phosphate) buffer at pH 7.0, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1% Triton. The extract was centrifuged at 15,000 × g for 20 min (Beckman Coulter Allegra 64R, USA). The supernatant was used for the enzymatic assay. NAD(P)H oxidase was assayed according to Cakmak and Marschner (1988). The reaction mixture (1.5 mL) contained 0.1M K-phosphate buffer, pH 7.0, 0.1 mM EDTA and 50 mM NADPH or NADH. The reaction was initiated by adding 200 µL of the enzyme extract, and the oxidation of NADPH or NADH was recorded with a spectrophotometer at 340 nm. The total soluble protein content of the crude enzyme extracts was determined with the Bradford assay method (Bradford, 1976).

Determination of apoplastic enzyme activity

The activities of CAT, POX and APX in the apoplastic fractions were determined spectrophotometrically. CAT was assayed by monitoring the consumption of H\(_2\)O\(_2\) in a spectrophotometer (Perkin Elmer Lambda 25, USA) at 240 nm (Rao et al., 1996). The activity was calculated using an extinction coefficient of 39.4 mM/cm for H\(_2\)O\(_2\). Apoplastic peroxidase activity was measured according to Andrews et al. (2000). Aliquots of 100 µL IWF were added to 2 mL reaction mixtures containing 100 mM Na-acetate-citrate buffer pH 6.0 and 10 µg 3,3′,5,5′-tetramethylbenzidine (TMB), solubilized initially in dimethyl sulfoxide (DMSO). The reaction was initiated by adding 10 µL 6% H\(_2\)O\(_2\), and incubation was performed for 30 min at 25°C. To stop the reaction, 0.5 mL 0.6 M H\(_2\)SO\(_4\) was added, and the optical density of the yellow color was determined at 450 nm with a UV/VIS spectrophotometer (Perkin Elmer Lambda 25, USA). Enzyme activity was expressed relative to a standard curve obtained with horseradish peroxidase (Sigma Chemical Co. Ltd.). APX activity was determined by measuring the decrease in absorbance of the oxidized ascorbate at 290 nm, according to Nakano and Asada (1980). The concentration of oxidized ascorbate was calculated with the extinction coefficient \( \varepsilon = 2.8 \text{ mM/cm} \); one unit of APX was defined as 1 µmol/mL ascorbate oxidized per minute.

Total carotenoid content

The total carotenoid content was quantified according to Lichtenhaler and Wellburn (1983), with some modifications. Fresh leaf tissues were homogenized in ice with a homogenizer (Miccra D-1, Germany). Extracts were shaken at 120 rpm for three hours in the dark. Fifteen minutes later, the absorbance of the extract at 460 nm was measured.
Results and discussion

Cold-hardiness

Cold-hardiness, expressed as the LT$_{50}$ of leaf tissue from two strawberry cultivars, is shown in Fig. 2. The cold-hardiness of the cultivars increased in the hardening stages (late autumn: average LT$_{50}$ -18.9ºC; winter: average LT$_{50}$ -23.4ºC) and decreased in the de-hardening stage (summer: average LT$_{50}$ -13.2ºC). Cold-acclimation produced an increase in the freezing tolerance of the cultivars (by lowering LT$_{50}$). The greatest increase was observed in 'Diamante' (-19.5ºC). In contrast, 'Aromas' had the lowest cold-hardiness (-17.6ºC). A two-way ANOVA revealed significant effects of sampling stage and cultivar on cold-hardiness but no significant interaction between sampling stage and cultivar (LT$_{50}$; Tab. 1).

Winter cold-hardiness is an important component of cultivars developed for colder climates. In our study, a clear increase in cold-hardiness was observed in the leaf tissues of 'Aromas' and 'Diamante' strawberry cultivars in response to decreasing temperature during the late autumn and winter (Fig. 2). Increases in cold-hardiness were reported during cold-acclimation in strawberry (Marini and Boyce, 1977; Palonen and Lindén, 2002; Paquin et al., 1989), as well as in many other plant species such as wheat (Apostolova et al., 2008), peach (Arora et al., 1992) and olive (Cansev et al., 2009; Eris et al., 2007). Palonen and Buszard (1997 b) stated that cultivar differences in cold-hardiness are only revealed after acclimation. The results of the current study showed that 'Diamante' had higher cold-hardiness than 'Aromas'. In addition, Turhan et al. (2011) recently investigated the occurrence of cell membrane injury in these cultivars in response to low temperatures. 'Diamante' was found to be relatively more tolerant to low temperatures than 'Aromas'.

Lipid peroxidation

The changes in MDA content in the leaf tissues are shown in Fig. 3. MDA content increased during the hardening stages (during late autumn and winter) and decreased during the de-hardening stage (summer). According to the average values, the maximum MDA content occurred in winter (195.2 nmol g$^{-1}$ FW) and late autumn (165.2 nmol FW).
Carotenoid is a lipid-soluble antioxidant that plays a multitude of roles in plant metabolism, including that of oxidative stress tolerance (Gill and Tuteja, 2010). Similarly, Turan and Ekmekci (2011) found that chilling temperatures produced a gradual increase in carotenoid content in ‘Canitez 87’ and ‘Gökçe’ chickpea cultivars. However, the same author remarked that this increase was more significant in the ‘Canitez 87’ cultivar, which is more sensitive to chilling temperatures.

NAD(P)H oxidase activity

In general, the average NAD(P)H oxidase activity in the hardening stage (during late autumn: 176.6 nmol/mg protein/min; winter: 232.8 nmol/mg protein/min, Fig. 5) was significantly higher than that in the de-hardening stage (summer: 88.6 nmol/mg protein/min, Fig. 5). NAD(P)H oxidase activity in summer and in winter was higher in cv. ‘Diamante’ than in cv. ‘Aromas’. Based on the average values of data from the three stages, ‘Diamante’ (168.2 nmol/mg protein/min) had higher NAD(P)H oxidase activities than ‘Aromas’ (163.8 nmol/mg protein/min). A two-way ANOVA revealed a significant effect of sampling stage on NAD(P)H oxidase activity but no significant effect of the cultivar or of the interaction between sampling stage and cultivar (Tab. 1).

Apoplastic enzyme activity

The changes of CAT activity in the apoplast of leaf tissues are shown in Fig. 6A. In general, the average CAT activity in the hardening stage (during winter: 52.4 nmol/mL IWF, during late autumn: 33.9 nmol/mL IWF) was significantly higher than that in the de-hardening stage (summer: 19.2 nmol/mL IWF). CAT enzyme activity was higher in the leaf apoplast of ‘Diamante’ than in ‘Aromas’ in all sampling stages. A two-way ANOVA revealed significant effects of sampling stage, cultivar and the interaction between sampling stage and cultivar (Tab. 1).
tion between sampling stage and cultivar on CAT activity (Tab. 1).

In plant cells subjected to stresses, the initial events occur primarily in the apoplastic space (Atıcı and Nalbantoglu 2003, Taşgün et al. 2003, 2006). In plants, H$_2$O$_2$ is destroyed primarily by APX and catalases (Asada, 1992). Although the catalases are restricted to the peroxisomes and perhaps to the mitochondria (Asada, 1992; Jimenez et al., 1997), CAT activity was also found in the apoplastic region of barley (Vanacker et al., 1998), tomato (Patykowski and Urbanek, 2003) and wheat leaves (Çakmak and Atıcı, 2009; Mutlu et al., 2009). The significant increase in CAT activity during the hardening stages in both strawberry cultivars (Fig. 6A) observed in this study may be a result of the greater tolerance of the cultivars to cold conditions. This result suggests that CAT activation is directly correlated with the degree of cold-hardiness in strawberry plants. In addition, ‘Diamante’, which showed greater cold-hardiness in the present study, exhibited greater apoplastic CAT activity than did ‘Aromas’. The association of apoplastic CAT with cold tolerance found by the present study is in good agreement with several previous studies that have demonstrated the important role of apoplastic CAT in increasing oxidative tolerance in plant tissues (Çakmak and Atıcı, 2009; Mutlu et al., 2009; Patykowski and Urbanek, 2003).

POX activity in the apoplast of leaf tissues in the sampling stages is shown in Fig. 6B. Apoplastic POX activity of both strawberry cultivars was significantly higher in winter (0.25 units/mL IWF) than in late autumn (0.12 units/mL IWF) and summer (0.11 units/mL IWF). Apoplastic POX activity was higher in cv. ‘Diamante’ than in cv. ‘Aromas’ at all sampling stages. A two-way ANOVA found significant effects of sampling stage and cultivar on POX enzyme activity in the apoplast of leaf tissues but no significant interaction between sampling stage and cultivar (Tab. 1).

The most common biosynthetic pathway in the apoplast is lignin biosynthesis. In this pathway, phenylpropanoid precursors of lignin are cross-linked by H$_2$O$_2$ in reactions initiated by peroxidases (Gross, 1980). In our study, ‘Diamante’, with a high level of cold-hardiness, had significantly higher apoplastic POX activity than ‘Aromas’, with a low level of cold-hardiness, in both the hardening and the de-hardening stages (Fig. 6B). Thus, a higher apoplastic POX activity was associated with greater cold-hardiness of the cultivars. This POX activation profile is in good agreement with the data for apoplastic POX enzyme activity in winter wheat leaves (Çakmak and Atıcı, 2009). However, a significant increase in the activity of this enzyme occurred during the hardening stage. This result suggests that this increase in enzyme activity may be related to cold-acclimation. POX activity in the leaf apoplast increased during the hardening stage in both cultivars. Several previous reports have suggested that apoplastic peroxidase is involved in crosslinking cell-wall components (Passardi et al., 2004), increasing cellular resistance to abiotic stresses.

The changes of APX activity in the apoplast of the leaf tissues are shown in Fig. 6C. APX activity increased during the hardening stage and decreased during the de-hardening stage. Based on the average values, the maximum APX activity occurred in winter (~ 429.0 nmol/mL IWF) and late autumn (~ 429.0 nmol/mL IWF), whereas the minimum APX activity occurred in summer (201.2 nmol/mL IWF). Apoplastic APX activity was higher in ‘Diamante’ than in ‘Aromas’ at all sampling stages. A two-way ANOVA found a significant effect of sampling stage and cultivar on APX activity in the apoplast of leaf tissues but no significant interaction between sampling stage and cultivar (Tab. 1).

One of the principal H$_2$O$_2$-scavenging enzymes in leaves is APX, which is located in the chloroplast, cytosol and apoplast (Asada, 1992). It was found that APX activity in the apoplast of two strawberry cultivars was significantly higher in the hardening stage than in the de-hardening stage (Fig. 6C). In addition, apoplastic APX activity was higher in ‘Diamante’, which had greater cold-hardiness than ‘Aromas’ at all stages. This result is in good agreement with previous results on apoplastic APX enzyme activity relative to increasing oxidative tolerance in barley (Vanacker et al., 1998) and tomato (Patykowski and Urbanek, 2003).
will be necessary to repeat the experiment with more cultivars to obtain precise information and further results.

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References


