Available online at www.sciencedirect.com
SCIENCE DIRECT®
Plant Science 166 (2004) 711–719
PLANT SCIENCE
www.elsevier.com/locate/plantsci

MeSA and MeJA increase steady-state transcript levels of alternative oxidase and resistance against chilling injury in sweet peppers (*Capsicum annuum* L.)☆

Raymond W.M. Fung [a], Chien Y. Wang [a,*], David L. Smith [a], Kenneth C. Gross [a], Meisheng Tian [b]

[a] *Produce Quality and Safety Laboratory, Plant Sciences Institute, Beltsville Agricultural Research Center, US Department of Agriculture, Beltsville, MD 20705-2350, USA*
[b] *HortResearch, Mt Albert Research Centre, Private Bag 92169, Auckland, New Zealand*

Received 10 October 2003; received in revised form 12 November 2003; accepted 12 November 2003

Abstract

Methyl salicylate (MeSA) and methyl jasmonate (MeJA) vapors increased resistance against chilling injury in freshly harvested green bell pepper (*Capsicum annuum* L. cv Century). The period within 2 days of cold storage was considered the most critical because chilling injury symptom (surface pitting) was not apparent. The expression patterns of alternative oxidase (AOX) and seven other genes involved in defense against oxidative stress before and during the early chilling period suggested that pre-treatment of pepper fruit with MeSA or MeJA vapors increased preferentially the transcript levels of AOX. Overnight treatment with MeSA or MeJA vapors increased transcript levels of AOX (1.5 kb) even at room temperature of 25 °C, whereas no change was observed with untreated control. In addition to the expected 1.5 kb AOX transcript, RNA gel blot analysis revealed an extra 3.5 kb transcript that was induced only at 0 °C. At 0 °C, both AOX transcripts (1.5 and 3.5 kb) reached maximal levels firstly in MeSA treated fruit, secondly in MeJA treated fruit and lastly in controls. Compared with freshly harvested peppers, overnight treatment of wholesaler peppers with MeJA does not increase AOX transcript level and no differences in chilling injury symptom were observed between treated and control fruit. AOX transcript level in peppers from farm and wholesaler were maintained at a high level as long as the fruits were kept at 0 °C. Transcript levels of AOX (1.5 kb) were increased by storage at low temperature but the steady-state mRNA accumulation rate was faster at 0 than at 5 °C. Alternative respiratory pathway was proposed to mediate chilling injury. Here, we show that the increase in AOX transcript levels by MeJA or MeSA before cold treatment was correlated with reduced incidence of chilling injury.

Keywords: *Capsicum*; AOX; Methyl salicylate; Methyl jasmonate; Antioxidative enzyme; Chilling injury

1. Introduction

Chilling injury imposes a limitation on extended storage for many horticultural crops. The genus *Capsicum* originated in the American tropics with the non-pungent, sweet cultivars of *Capsicum annuum* (e.g. green bell pepper) being one of the most grown species [1]. It is consumed as a vegetable or fresh-cut produce. One of the most significant reasons for postharvest loss is due to bell peppers susceptibility to chilling injury when stored below 7 °C. Chilling induced symptoms in green bell peppers include: dot-pitting followed by sheet-pitting, development of alternaria rot on pods and calyxes, seed darkening and shrinkage due to moisture loss [2]. Damage caused by low temperature severely reduced the quality and storage life of bell pepper [3].

Chilling injury can result from oxidative stress caused by reactive oxygen species (ROS) via overproduction of photosynthetic electrons from chloroplasts in light [4,5]. In darkness, mitochondria were shown to be the main site for electron production and therefore the major source of superoxides in chilling-sensitive plant tissues where the

☆ The nucleotide sequence data reported appear in the EMBL, Genbank, DDBJ nucleotide sequence databases under the accession numbers AY250708 (AOX).

* Corresponding author. Tel.: +1-301-504-5981; fax: +1-301-504-5107.
E-mail address: wangc@ba.ars.usda.gov (C.Y. Wang).

0168-9452/$ – see front matter © 2003 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.plantsci.2003.11.009

cytochrome respiratory pathway was impaired by low temperature [6]. Scandalios [7] showed that several enzymes, e.g. superoxide dismutases, catalases and peroxidases were involved in the reduction of ROS in plants. Chilling resistance in plants was correlated with an increase of enzymatic activities in antioxidant systems [8,9]. Møller [10] suggested that defense against oxidative stress consisted of two lines of defense. The first line of defense is termed ROS avoidance genes includes alternative oxidase (AOX) and the second is termed as ROS scavenging genes includes manganese superoxide dismutase (MnSOD), catalase, the ascorbate/glutathione cycle, the glutathione peroxidase system and thioredoxin system.

The plant AOX pathway branches from the main respiratory electron transport chain, bypasses the final steps of the cytochrome respiratory pathway and catalyses the oxidation of ubiquinol. It was suggested that by maintaining the flow of mitochondrial electrons, AOX maintained activation of NAD(P)H dehydrogenase and proton-pumping NADH dehydrogenase [10] and helps in generation of sufficient ATP required for the rapid adaptation and the maintenance of plant growth rate homeostasis [11,12]. In this process, AOX is involved in reducing ROS by preventing electrons from reducing O_2 to O_2^- and thus reduces the level of O_2 in the mitochondria [10,13,14]. The concept of AOX acting as an antioxidant enzyme has been shown in isolated bell pepper mitochondria [15] and in intact tobacco cells [16].

Phylogenetic analysis suggests the presence of two discrete AOX gene subfamilies in plants with AOX family 1, but not family 2, existing in both monocot and dicot plant species [17–19]. AOX transcript, protein levels and activity were shown to respond to multiple developmental and environmental triggers (reviewed in [19]). For example, AOX transcript and protein can be increased by low temperature [17,20–24]. It was proposed that the alternative respiratory pathway mediated chilling injury by keeping the production of ROS in balance with the levels of antioxidants and active oxygen scavenging enzyme systems (reviewed by Purvis et al. [6]). In addition, mutated AOX gene was linked to a quantitative trait loci (QTL) for low temperature tolerance in a rice cultivar [25].

Salicylic acid (SA), jasmonic acid (JA) and their methyl esters (MeSA, MeJA) are endogenous signal molecules that play essential roles in regulating stress responses and plant development [26–30]. SA has been shown to induce expression of AOX [31,32]. Although there are many methods to reduce chilling injury in various horticultural crops [33,34], we have shown MeSA and MeJA treatments are inexpensive, easy to set up and applicable to various fruit produce [35–37]. In particular, MeJA reduced chilling injury in bell pepper fruit [38] and fresh-cut pepper strips [39]. Because of the potential usefulness of MeSA and MeJA in extending postharvest longevity of produce, it is important to understand the process. In this study, we investigated how seven different ROS scavenging genes (MnSOD, Cu/ZnSOD, catalase, ascorbate peroxidases, peroxidase and thioredoxin peroxidase) and ROS avoidance genes (AOX) might be involved in the alleviation of chilling injury. We characterized the expression of the family 1 AOX gene at low temperature and its response to exogenous MeSA and MeJA vapors.

2. Methods and materials

2.1. Plant materials and postharvest treatments

For the chilling injury experiment, green bell pepper fruit (*C. annuum* L. cv "Century") were harvested from a local farm in the 2002 season. Field temperature during harvest was 30 °C. Within an hour after harvest, fruits were washed and blotted dry. A total of 180 fruits were divided into three lots. Each lot was kept in three airtight glass jars (20 fruit each in 19.4 l jar), together with 22.4 μl/l MeJA, 13.2 μl/l MeSA or water (for the control) spotted onto filter paper so that the final vapor concentration reached 10^{-4} M, for 1 day in darkness at 25 °C. After treatment, the glass jars were ventilated in a fume hood, covered with perforated parafilm and stored in darkness at 0 °C for 14 days. After the cold treatment, fruits were stored at 20 °C for 9 days.

Green bell pepper fruit used for both the temperature experiment and the methyl jasmonate concentration experiment were obtained from a local wholesaler. Fruits were harvested in Florida and had been stored at 7 °C for about 1 week. Fruits were divided into seven lots (20 fruit each) of which three lots were used for the MeJA experiment and four lots were used for the temperature experiment. For the MeJA experiment, each lot was placed into three airtight glass jars (19.4 l), together with MeJA spotted onto filter paper at the final vapor concentration of 0 (control), 10^{-5} or 10^{-4} M, then incubated for 1 day at 25 °C. After treatment, the glass jars were ventilated in a fume hood, stored at 0 °C for 13 days, followed by 20 °C for another 5 days. For the temperature experiment, fruits were kept in the original packing boxes, and were stored at 0, 5, 10 or 20 °C for 7 days and then 20 °C for 6 days. Samples were taken daily during the first week unless stated otherwise. At each time point, fruits were taken from the jars and equatorial slices were taken from each fruit, diced, frozen in liquid nitrogen and stored at −80 °C until used.

2.2. Evaluation of chilling and ripening characteristics

Chilling symptoms were evaluated during and after cold storage [37]. It appeared as surface pitting followed by a combination of both surface pitting and sheet-pitting after 2–3 days in 0 °C. The severity of the symptoms was assessed visually and the percentage of fruit surface covered by pitting was scored for each fruit. The average value and standard error value of each group of fruit at each time point was plotted. At the end of the experiment, the percentage of rotten blackened seeds to healthy white seeds was estimated from the remaining fruit.

2.3. Isolation of cDNA fragments of genes encoding AOX and antioxidative enzymes

Degenerate primers (MST1, MST2) of AOX were designed based on similar primers sequences used by Saisho et al. [40], with slight nucleotide modification to favor amplification of dicot plant species (Table 1). Gene-specific primer pairs of seven *C. annuum* genes encoding antioxidative enzymes were designed based on sequence information from the public sequence database. They included cytosolic ascorbate peroxidase, ascorbate peroxidase, thioredoxin peroxidase, catalase, peroxidase, Mn-superoxide dismutase and Cu/Zn-superoxide dismutase (Table 1). Complementary DNA was prepared with SuperScript™ reverse transcriptase (Invitrogen™) using odtRACE1 primer and total RNA extracted from pepper fruit as template. PCR conditions were as outlined in [41]. PCR products for AOX were cloned into pGEM-T vectors (Promega) and confirmed by sequencing. PCR products for other antioxidative genes were gel-purified and sequenced to confirm their identity before being used as probes for Northern analysis.

2.4. RNA gel blot analysis

Total RNA was extracted from *C. annuum* L. fruit tissues [42]. Electrophoresed RNA was transferred to Hybond N^+ membrane using 20× SSC, according to manufacturer's instructions. RNA gel blot hybridization was performed according to Virca et al. [43]. Probes were labeled with [α-^{32}P]-dCTP by DNA random prime labeling RTS system (Invitrogen). After an overnight hybridization at 65 °C, unbound probe was removed by washing the membrane in 0.2× SSC at 65 °C. Membranes were sealed in plastic bags and exposed to X-ray film. Membranes were then stripped and rehybridized with an 18S ribosomal DNA probe from apple (*Malus domestica*) (S. Pechous, personal communication). Hybridization signal of 18S rRNA was used as RNA loading control.

3. Results

3.1. MeJA and MeSA reduced chilling injury of bell pepper

Fruits were harvested from a local farm when the field temperature was around 30 °C. These fruits were treated overnight with MeJA or MeSA at concentration of 10^{-4} M before cold storage. Chilling injury severity was estimated for untreated fruit, MeJA-treated fruit and MeSA-treated fruit as shown in Fig. 1. Apparent chilling injury symptoms were visible as pitting or sheet-pitting within 2 days of storage at 0 °C and progressed rapidly to maximal level within 9 days. MeJA and MeSA treatments delayed the onset of chilling injury by about 4 days during the 13 days of storage at 0 °C (Fig. 1, days 2–14). By the end of 2 weeks at 0 °C, the percentage of surface pitting in treated fruit approached that of untreated control. Following the rewarming period at 20 °C, severe mold, rotting and decay developed on the surface of both treated and untreated fruit. By day 23, 58% of seeds in control fruits were blackened, whereas only 28 and 17% of seeds in MeSA- and MeJA-treated fruit, respectively, were blackened. Both MeJA and MeSA vapors

Table 1
Oligonucleotide primers used to isolate genes of AOX and other antioxidative enzymes from *C. annuum* and primers used to carry out gene-specific RT-PCR

Gene (abbreviation) (Genbank accession no.)	Oligo sequence	Expected size (bp)
Cytosolic ascorbate peroxidase (Cys APX) (X81376)	Forward 5′-ATGGTATTGACATTGCTCTC-3′; reverse 5′-AATTCAGAGAGCTTCAAGTG-3′	520
Ascorbate peroxidase (APX) (AF442387)	Forward 5′-TGTGCTCCTATCATGCTTCG-3′; reverse 5′-GTAAACCCTAGCTCAGACAG-3′	650
Thioredoxin peroxidase (Thio PX) (AF442385)	Forward 5′-ACTTCGACGAGCAAGATCAG-3′; reverse 5′-TGGTATATACATTTCAGAAG-3′	500
Catalase (Cat) (AF227952)	Forward 5′-GTGCCAGTGCCAAGGGCTTC-3′; reverse 5′-CTAATAGCTTCTTCCTCCGT-3′	520
Peroxidase (PX) (AF442386)	Forward 5′-GGCGCCAGGATTGCTGACAA-3′; reverse 5′-GTGGACATAATCCTCGAAGC-3′	520
Mn-superoxide dismutase (MnSOD) (AF036936)	Forward 5′-ATCATCAGACTTACATAACA-3′; reverse 5′-CAAGGGCATTCTTTCTCGTA-3′	520
Cu/Zn-superoxide dismutase (Cu/ZnSOD) (AF009734)	Forward 5′-CCATGGTGAAGGCTGTCGCCGTCCTTAG-3′; reverse 5′-AATCTTCAGCGCACGGGAGT-3′	480
Alternative oxidase (AOX)		450
MST1	5′-ACWGTRGCWGCWGTVCCYGGRATGGT-3′	
MST2	5′-GGTTKACATCWCGRTGRTGWGCCTC-3′	
RFAOX3	5′-ACTCCACTCTCCGCGATGTTGTCTTG-3′	
odtRACE1[a]	5′-GACTCGAGTCGACATCGA-$(T)_{17}$-3′	
RACE1	5′-GACTCGAGTCGACATCG-3′	

[a] Parentheses indicate the number of nucleotide repeats used in the odtRACE1 primer.

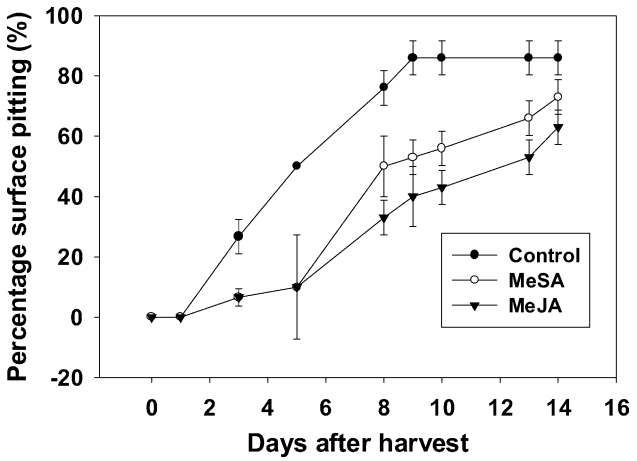


Fig. 1. Effect of methyl salicylate (MeSA) and methyl jasmonate (MeJA) on chilling injury of green bell pepper stored at 0 °C. Fruits were harvested manually from a local farm (field temperature was 30 °C, day 0) and treated at 25 °C overnight with MeJA (10^{-4} M), MeSA (10^{-4} M) or with air as a control (day 1). Fruits were then stored at 0 °C for 13 days (days 2–14). After cold treatment, fruits were rewarmed at 20 °C for another 9 days. Only data from days 0–14 are shown. Chilling injury evaluation was performed based on the percentage of fruit surface area covered by pitting. Vertical bars represent S.E.; $n = 30$. S.E. value was 0 for control at 6 days after harvest.

were efficient to delay short-term external (surface pitting) and long-term internal (blackened seeds) chilling injury in pepper fruit stored at 0 °C.

3.2. AOX transcript levels increased dramatically in response to MeJA and MeSA treatments

We examined the transcript levels of AOX and seven other genes encoding antioxidative enzymes in pepper fruit treated with MeSA or MeJA vapors. Since chilling injury symptoms were visible within the first 2 days of cold storage, this critical early period was investigated by Northern analysis.

Total RNA was extracted from pepper fruit freshly harvested from the local farm (day 0). The transcripts of AOX (1.5 kb) and antioxidative enzymes were all detectable at harvest (Fig. 2). After overnight incubation at 25 °C with MeJA or MeSA vapors, transcript levels of antioxidative enzymes MnSOD, Cu/ZnSOD, catalase and cytosolic ascorbate peroxidase were higher than control. During the 0 °C storage period, transcript levels of MnSOD maintained at level higher than harvest timepoint but that of Cu/ZnSOD decreased slightly. Transcript levels of catalase and cytosolic ascorbate peroxidase seem to follow the treatments with higher transcript levels in MeSA-treated fruit especially at day 3. However, transcript levels of ascorbate peroxidase, peroxidase and thioredoxin peroxidase did not change with the treatments or the temperatures.

In contrast to the mild variation in transcript levels of other antioxidative enzymes, AOX transcript levels were increased

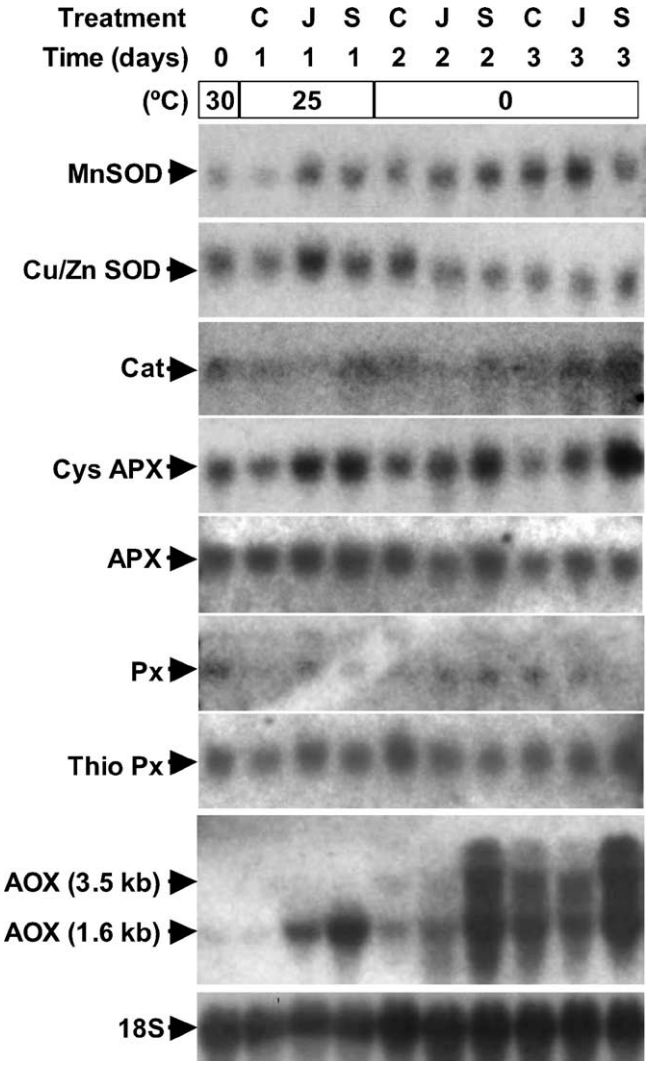


Fig. 2. Expression profiles of seven different genes of antioxidative enzymes and AOX gene in response to MeSA (S), MeJA (J) in chilled green pepper fruits (abbreviation of each genes is shown in Table 1). Untreated control fruit is represented with (C). Pepper fruits were harvested from a local farm (field temperature was 30 °C, day 0) and treated as described in Fig. 1. Fruit from the first 3 days of experiments were used for Northern analysis. RNA gel blot analysis was performed as described in the materials and methods section. 18S ribosomal fragment was used as probe for loading control and a typical blot is shown.

strongly by MeJA and to a higher extent by MeSA during day 1 (Fig. 2). Unexpectedly, two AOX transcripts were detected after fruits were transferred to 0 °C. In plants, the expected size of AOX transcript is around 1.5–1.6 kb. Both AOX transcripts (1.5 and 3.5 kb) accumulated and reached maximum levels within 24 h at 0 °C in MeSA-treated fruit (day 2, treatment 'S' in Fig. 2). For the MeJA-treated fruit and control fruit, it took 3–4 days for AOX transcript levels to reach their maximum (data not shown). Both MeJA and MeSA vapors increased the accumulation of AOX transcripts in pepper fruit at room temperature (day 1) and to very high levels during cold storage.

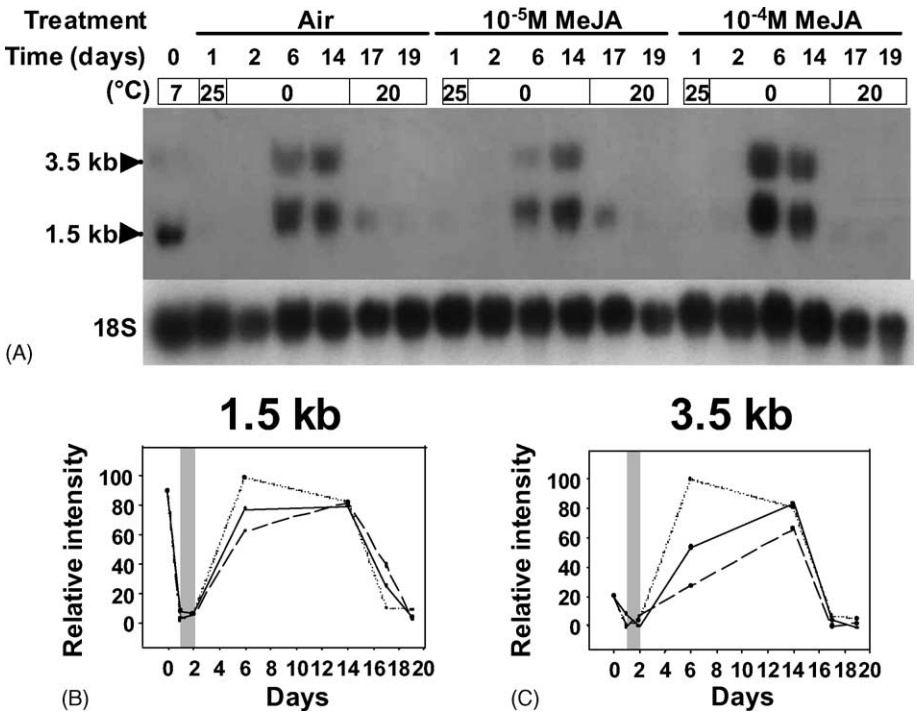


Fig. 3. Effect of different concentrations of MeJA on expression of AOX transcript at 0 °C. Fruits were obtained from a local wholesaler (day 0) and treated at 25 °C overnight with two different concentrations of methyl jasmonate (10^{-5} or 10^{-4} M) or with air as a control (day 1). Fruits were then stored at 0 °C for 13 days (days 2–14) before being returned to 20 °C for another 5 days (days 14–19). (A) RNA gel blot analysis was performed as described in Fig. 2. Relative AOX transcript levels, 1.5 and 3.5 kb, were quantified by densitometry and normalized to hybridization signals with an 18S ribosomal probe for each time point. Graphical representation of relative amount of 1.5 kb (B) and 3.5 kb (C) transcripts were plotted against time (days). Sampling time point during the treatment at air control (solid line), 10^{-5} M MeJA (long dash line) and 10^{-4} M MeJA (dotted line) are shown in gray as indicated. Expression levels were expressed as a ratio to the time point with the highest intensity, which is set to 100.

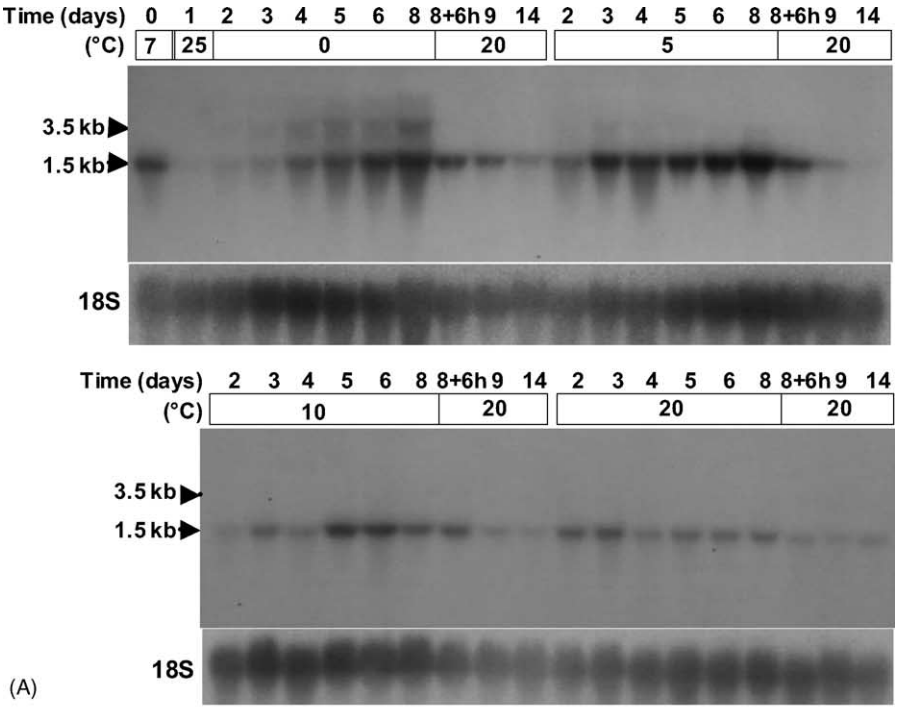
3.3. AOX transcript levels were increased by MeJA at concentration of 10^{-4} M

Due to the significant induction of AOX transcript by cold storage, we decided to study AOX in more detail. Bell pepper fruit from the wholesaler that had been kept at 7 °C were treated with two different concentrations of MeJA in order to determine the effects on expression of AOX at 0 °C (Fig. 3A). Fruit obtained from farm and wholesaler were found to behave differently in response to the MeJA treatment. Compared with fruit obtained freshly from the farm, high levels of AOX 1.5 kb transcripts were detected from wholesaler fruit that had been in storage for 1 week at 7 °C (Fig. 3A and B, day 0). This AOX 1.5 kb transcripts were, however, disappeared after overnight storage at 25 °C with MeJA. In fact, no increased in AOX transcript levels were detected after the first day of 0 °C storage (Fig. 3A and B, day 2). AOX transcripts (1.5 and 3.5 kb) accumulated to maximum levels by day 6 for fruit treated with 10^{-4} M MeJA and day 14 for the control fruit and fruit treated with lower concentration of 10^{-5} M MeJA (Fig. 3B and C). Upon returning to 20 °C, the 1.5 kb transcript disappeared completely at day 17 for fruit treated with 10^{-4} M MeJA, but was still detectable in control fruit and fruit treated with 10^{-5} M MeJA. No significant differences in chilling injury symptoms were observed among treated and untreated fruits (see Section 4 for details), though AOX transcript levels increased and decreased faster in fruit treated with 10^{-4} than 10^{-5} M MeJA and air control.

3.4. Higher levels of 1.5 kb AOX transcript were expressed at lower temperature but the 3.5 kb AOX transcript levels were induced only at 0 °C

Transcript levels of AOX at 0, 5, 10, and 20 °C were determined. Bell pepper fruit from wholesaler coldstore were kept at 25 °C overnight. They were then transferred to storage at four different temperatures for 1 week before being stored at 20 °C for 6 days. For fruit stored at 20 °C during the first week, approximately 20% of the fruit showed signs of ripening judged by appearance of red coloration by the end of the total 2-week storage period. No ripening occurred among fruit that were stored at 0, 5 or 10 °C.

At 0 °C, the 1.5 kb transcript of AOX accumulated gradually throughout the 7-day storage period and declined to lower levels after the fruits were returned to 20 °C (Fig. 4A and B). The 3.5 kb AOX transcript appeared 2 days later than the 1.5 kb transcript and also accumulated gradually during 0 °C storage. The 3.5 kb transcript, however, disappeared completely within 6 h of rewarming at 20 °C (Fig. 4A



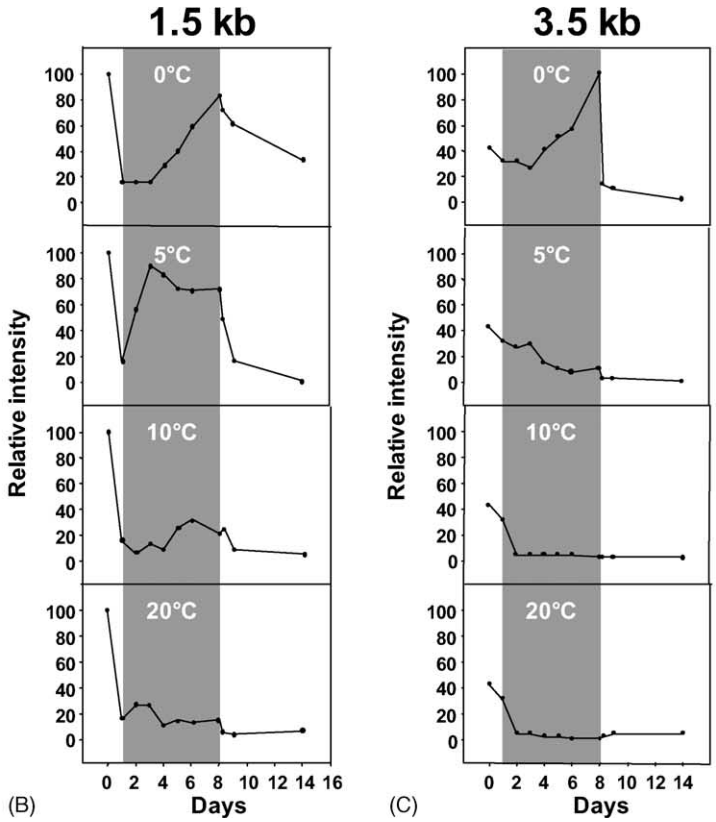


Fig. 4. Effect of various temperatures on transcript levels of AOX. Fruit (*C. annuum*) obtained from a local wholesaler (day 0) were kept at 25 °C overnight (day 1) and then stored separately at four different temperatures (0, 5, 10 and 20 °C) for 1 week (days 2–8). Fruits were then transferred to 20 °C for 1 week (days 8–14). (A) RNA gel blot analysis was performed as described in Fig. 2. Relative AOX transcript levels (1.5 and 3.5 kb fragments) were quantified by densitometry and normalized to hybridization signals with an 18S ribosomal probe for each time point. Graphical representation of relative amount of 1.5 kb (B) and 3.5 kb (C) transcript were plotted against time (days). Sampling time point during the treatment at four different temperatures of 0, 5, 10 and 20 °C are shown in gray from top to bottom as indicated. Expression levels were expressed as a ratio to the time point with the highest intensity, which is set to 100.

and C). At 5 °C, the 1.5 kb transcript levels reached maximal level at day 3 and stayed at the same high levels until rewarming to 20 °C (Fig. 4A and B). During 10 and 20 °C storage, the 1.5 kb AOX transcripts were expressed at lower level with subtle variation compared with fruit stored at 0 and 5 °C. The 3.5 kb transcripts were not detected at storage temperatures of 5, 10 or 20 °C (Fig. 4A and C, see Section 4 for details). RNA gel blot analysis indicated that transcript level of AOX transcripts at 1.5 kb were increased at 5 and 0 °C, whereas the 3.5 kb transcript was induced only at 0 °C.

4. Discussion

Expression levels of genes involved in oxidative stress defense mechanisms were compared in bell pepper fruit under conditions leading to either chilling tolerance or sensitivity. During the period before chilling injury symptoms (pitting) were visible, transcript levels of several genes encoding antioxidative enzymes varied slightly and showed little correlation with the degree of chilling resistance. However, levels of AOX transcripts increased significantly when pepper fruits were stored at low temperature. In addition, AOX transcript levels were dramatically increased in pepper fruit, which were treated with MeSA or MeJA vapors.

Although none of the antioxidative genes other than AOX change significantly in their transcript levels, it is possible that some gene family member(s) were missed under the conditions tested. This possibility was recognized when the sequences of the pepper clones were used to search the tomato EST database (TIGR). Tomato EST sequences with equivalent-coding regions to the pepper probes were selected for analysis. In tomato, individual tomato ESTs sharing nucleotide identity of at least 64% (e.g. Cu/ZnSOD) and up to 87% (e.g. catalase) were found, suggesting the presence of multigene families for the selected antioxidative enzymes in the tomato genome. Based on the high similarity between green pepper and tomato genomes [44], there is the potential that the pepper gene of antioxidative enzymes tested in this study may (or may not) hybridize to transcripts of other family members, due to high (or low) shared nucleotide identity. Further studies are needed to determine the transcript profiles of other gene family members of each antioxidative enzyme with respect to their response in chilling tolerant and chilling-sensitive pepper fruit.

In this study, an AOX cDNA fragment was isolated from bell pepper that shared 89% identity at the nucleotide level and 97% identity at amino acid level with a tobacco AOX clone (accession no. X79768) [45]. This fragment is also highly similar to the Genbank EST sequences from *C. annuum* (accession no. CB164923) and *L. esculentum* (accession no. BM408876) with 98 and 89% nucleotide identity. This pepper AOX clone belongs to the family 1 group of AOXs in plants classified by Considine et al. [19] and was designated as CaAOX1. The putative amino acid sequence of CaAOX1 is similar to other AOX proteins and this conservation includes iron binding motifs and their surrounding helical regions [46,47]. In addition, the CaAOX1 clone shares 73% nucleotide identity and 81% amino acid identity with the AOX subfamily 2 clone, CaAOX2 from pepper (Tian et al., unpublished data). No cross-hybridization with family 2 transcripts was expected using the CaAOX1 cDNA as probe, due to the lower degree of nucleotide homology between family 1 and 2 CaAOX sequences.

The size of AOX transcripts is commonly 1.5–1.6 kb in plants. In addition to the expected 1.5 kb band, the CaAOX1 probe used here detected a 3.5 kb transcript but only after fruits were stored at 0 °C. Interestingly, Ito et al. [17] also detected two AOX transcripts (1.5 and 2.9 kb) in rice seedlings but only after 3 days at 4 °C. By using intron DNA as a probe for Northern analysis, the 2.9 kb band was confirmed as an unprocessed transcript containing the AOX intron region [17]. Therefore, the 3.5 kb CaAOX1 transcript in pepper fruit observed here is also likely to be an unprocessed transcript due to altered RNA splicing or processing at 0 °C. For the genes tested other than CaAOX1, we observed only one transcript size before and during 0 °C storage.

Family 1 AOX genes typically occur as multigene families in plants, e.g. *Arabidopsis*, mango, rice and soybean [17,40,48,49]. Except for soybean [50], AOX family 2 genes occur as single copies in plants [19] including pepper (Tian et al., unpublished data). Therefore, there is a potential that the CaAOX1 probe used in this study may cross-hybridize with closely related gene members of AOX family 1.

Closely related AOX members are expected to differ enzymatically. An AOX protein with a single amino acid residue change has been shown to have altered specificity of organic acid activation [22]. Karpova et al. [51] reported differential expression of three family 1 AOX members by using 3′ untranslated regions as gene-specific probes, suggesting unique roles among the closely related AOX genes. For example, Moore et al. [11] proposed that differential expression of multiple AOX isozymes is needed to recover respiratory activity and maintain phosphorylation potential and homeostasis of plants. The function of AOX is generally regarded as scavenging of ROS [15,21]. However, a unique function has never been assigned to any of the AOX family 1 isozymes.

Expression of AOX family 1 gene(s) in pepper was tightly associated with low temperature stress, suggesting that family 1 genes play a role in stress-induced conditions in plants (summarized in [19,51]). Within 6 h of rewarming, AOX transcripts (3.5 and 1.6 kb) in pepper fruit are depleted suggesting the presence of a high turnover rate for AOX transcripts. Low levels of CaAOX1 transcripts are detected in fruit stored at 20 °C and freshly harvested. This suggests that the CaAOX1 transcript is constitutively expressed throughout fruit development and corresponds to basal levels necessary for alternative respiration. This supported the idea that alternative pathway operates actively and the cytochrome and alternative respiration are constantly competing with each other for respiratory electrons [47,52].

CaAOX1 transcripts (1.5 kb) reach high levels within 2 days at 5 °C but fruit at 0 °C takes up to 7 days to attain the same level (Fig. 4). This is consistent with the findings from Purvis [53] that higher AOX protein levels were found in mitochondria from pepper fruit stored at 4 °C than at 1 °C. The delay of CaAOX1 transcript accumulation at 0 °C can be overcome by application of MeJA or MeSA vapors (Figs. 2 and 3). Both MeSA and MeJA reduce short-term external surface pitting and long-term internal seed blackening symptoms in pepper fruit (Fig. 1). Though MeSA induced higher and earlier expression of CaAOX1 transcripts, MeJA (10^{-4} M) was more effective than MeSA (10^{-4} M) at alleviating chilling injury. It is possible that the beneficial effects for chilling resistance from enhancement of alternative oxidase might be compromised by the death-inducing properties of MeSA [54].

Some differences were noticed between fruit from the wholesaler and from the farm. Fruit from the farm responded with faster induction of AOX transcript levels during the overnight MeJA treatment at room temperature, while no overnight induction was observed in the wholesaler fruit (compare day 1 in Figs. 2 and 3). This might be due to varied properties among different cultivars of *C. annuum* L. Fruit from the wholesaler had been kept at 7 °C, and this might have induced protection against chilling temperature as observed in cucumber [55]. Therefore, the temperature variation prior to cold storage might explain our observation that fruit from the wholesaler showed less chilling injury symptoms and no differences between treatments. On the other hand, freshly harvested fruit showed severe chilling injury and alleviation was observed in MeJA and MeSA treated fruit (Fig. 1).

The ROS scavenging system and the ROS avoidance system was found to work co-operatively [16]. Rizhsky et al. [56] suggested that the plant genome has a high degree of plasticity in reducing ROS level and protects plants from oxidative stress by expressing alternative survival strategies when environmental challenges go beyond the normal range. When compared with seven other ROS scavenging genes (MnSOD, Cu/ZnSOD, Cat, CysAPX, etc.), the ROS avoidance gene (AOX) was the most responsive and was dramatically induced by MeSA and MeJA vapors and by low temperature stress.

The work presented here points to the possibility to use plant signal molecules as cues for activating the ROS defense system and these treatments may be of particular value to increase resistance against chilling injury in bell pepper and a broad range of other tropical crops.

Acknowledgements

We thank David Spaulding and Hilarine Repace for technical assistance during the course of this research.

References

[1] B. Pickersgill, Genetic resources and breeding of *Capsicum* spp, Euphytica 96 (1997) 129–133.
[2] R.E. Hardenburg, A.E. Watada, C.Y. Wang, The commercial storage of fruits, vegetables, and florist and nursery stocks, US Dep. Agri. Agri. Handb. 66 (1986) 65.
[3] R.E. Paull, Chilling injury of crops of tropical and subtropical origin, in: C.Y. Wang (Ed.), Chilling Injury of Horticultural Crops, CRC Press, Boca Raton, FL, 1990, pp. 17–36.
[4] P.R. van Hasselt, Light-induced damage during chilling, in: C.Y. Wang (Ed.), Chilling Injury of Horticultural Crops, CRC Press, Boca Raton, FL, 1990, pp. 113–128.
[5] B. Alam, J. Jacob, Overproduction of photosynthetic electrons is associated with chilling injury in green leaves, Photosynthetica 40 (2002) 91–95.
[6] A.C. Purvis, R.L. Shewfelt, J.W. Gegogeine, Superoxide production by mitochondria isolated from green bell pepper fruit, Physiol. Plant. 94 (1995) 743–749.
[7] J.G. Scandalios (Ed.), Molecular genetics of superoxide dismutases in plants, in: Oxidative Stress and the Molecular Biology of Antioxidant Defenses, Cold Spring Harbor Laboratory Press, 1997, pp. 527–568.
[8] M.A. Walker, B.D. McKersie, Role of ascorbate–glutathione antioxidant system in chilling resistance of tomato, J. Plant Physiol. 141 (1993) 234–239.
[9] S. Dipierro, S.D. Leonardis, The ascorbate system and lipid peroxidation in stored potato (*Solanum tuberosum* L.) tubers, J. Exp. Biol. 48 (1997) 779–783.
[10] I.M. Møller, Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species, Annu. Rev. Plant Physiol. Plant Mol. Biol. 52 (2001) 561–591.
[11] A.L. Moore, M.S. Albury, P.G. Crichton, C. Affourtit, Function of the alternative oxidase: is it still a scavenger? Trends Plant Sci. 7 (2002) 478–481.
[12] L.D. Hansen, J.N. Church, S. Matheson, V.W. McCarlie, T. Thygerson, R.S. Criddle, B.N. Smith, Kinetics of plant growth and metabolism, Thermochim. Acta 388 (2002) 415–425.
[13] A.M. Wagner, K. Krab, The alternative respiration pathway in plants: role and regulation, Physiol. Plant. 95 (1995) 318–325.
[14] R. Mittler, Oxidative stress, antioxidants and stress tolerance, Trends Plant Sci. 7 (2002) 405–410.
[15] A.C. Purvis, Role of alternative oxidase in limiting superoxide production by plant mitochondria, Physiol. Plant. 100 (1997) 165–170.
[16] D.P. Maxwell, Y. Wang, L. McIntosh, The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells, Proc. Natl. Acad. Sci. USA 96 (1999) 8271–8276.
[17] Y. Ito, D. Saisho, M. Nakazono, N. Trutsumi, A. Hirai, Transcript levels of tandem-arranged alternative oxidase genes in rice are increased by low temperature, Gene 203 (1997) 121–129.
[18] H. Saika, K. Ohtsu, S. Hamanaka, M. Nakazono, N. Tsutsumi, A. Hirai, AOX1c, a novel rice gene for alternative oxidase; comparison with rice AOX1a and AOX1b, Genes Genet. Syst. 77 (2002) 31–38.
[19] M.J. Considine, R.C. Holtzapffel, D.A. Day, J. Whelan, A.H. Millar, Molecular distinction between alternative oxidase from monocots and dicots, Plant Physiol. 129 (2002) 949–953.
[20] G.C. Vanlerberghe, L. McIntosh, Lower growth temperature increases alternative pathway capacity and alternative oxidase protein in tobacco, Plant Physiol. 100 (1992) 115–119.
[21] G.C. Vanlerberghe, L. McIntosh, Alternative oxidase: from gene to function, Annu. Rev. Plant Physiol. Plant Mol. Biol. 48 (1997) 703–734.
[22] I. Djajanegara, P.M. Holtzapffel, P.M. Finnegan, M.H.N. Hoefnagel, D.A. Berthold, J.T. Wiskich, D.A. Day, A single amino acid change in plant alternative oxidase alters the specificity of organic acid activation, FEBS Lett. 454 (1999) 220–224.

[23] M.A. Gonzàlez-Meler, M. Ribas-Carbo, L. Giles, J.N. Siedow, The effect of growth and measurement temperature on the activity of alternative respiratory pathway, Plant Physiol. 120 (1999) 765–772.
[24] M. Ribas-Carbo, R. Aroca, M.A. Gonzàlez-Meler, J.J. Irigoyen, M. Sánchez-Díaz, The electron partitioning between the cytochrome and alternature respiratory pathways during chilling recovery in two cultivars of maize differing chilling sensitivity, Plant Physiol. 122 (2000) 199–204.
[25] F. Abe, K. Saito, K. Miura, K. Toriyama, A single nucleotide polymorphism in the alternative oxidase gene among rice varieties differing in low temperature tolerance, FEBS Lett. 527 (2002) 181–185.
[26] I. Raskin, Role of salicylic acid in plants, Annu. Rev. Plant Physiol. Plant Mol. Biol. 43 (1992) 439–463.
[27] D.F. Klessig, J. Malamy, The salicylic acid signal in plants, Plant Mol. Biol. 26 (1994) 1439–1458.
[28] B.J. Feys, J.E. Parker, Interplay of signalling pathway in plant disease resistance, Trends Genet. 16 (2000) 449–455.
[29] L. Li, C. Li, G.A. Howe, Genetic analysis of wound signalling in tomato. Evidence for a dual role of jasmonic acid in defense and female fertility, Plant Physiol. 127 (2001) 1414–1417.
[30] J.G. Turner, C. Ellis, A. Devoto, The jasmonate signal pathway, Plant Cell Suppl. 14 (2002) 153–164.
[31] D.M. Rhoads, L. McIntosh, Salicylic acid regulation of respiration in higher plants: alternative oxidase expression, The Plant Cell 4 (1992) 1131–1139.
[32] D.M. Rhoads, L. McIntosh, The salicylic acid-inducible alternative oxidase gene aox1 and gene encoding pathogenesis-related proteins share regions of sequence similarity in their promoters, Plant Mol. Biol. 21 (1993) 615–624.
[33] C.Y. Wang (Ed.), Alleviation of chilling injury of horticultural crops, in: Chilling Injury of Horticultural Crops, CRC Press, Boca Raton, FL, 1990, pp. 281–320.
[34] C.Y. Wang, Approaches to reduce chilling injury of fruits and vegetables, Hort. Rev. 15 (1993) 83–95.
[35] C.Y. Wang, G. Buta, Methyl jasmonate improves quality of stored zucchini squash, J. Food Qual. 22 (1999) 663–670.
[36] G.A. Gonzalez-Aguilar, J. Fortiz, R. Cruz, R. Baez, C.Y. Wang, Methyl jasmonate reduces chilling injury and maintains postharvest quality of mango fruit, J. Agric. Food Chem. 48 (2000) 515–519.
[37] C.K. Ding, C.Y. Wang, K.C. Gross, D.L. Smith, Reduction of chilling injury and transcript accumulation of heat shock protein genes in tomatoes by methyl jasmonate and methyl salicylate, Plant Sci. 161 (2001) 1153–1159.
[38] S. Meir, S. Philosoph-Hadas, S. Lurie, S. Droby, M. Akerman, G. Zauberman, B. Shapiro, E. Cohen, Y. Fuchs, Reduction of chilling injury in stored avocado, grapefruit, and bell pepper by methyl jasmonate, Can. J. Bot. 74 (1996) 870–874.
[39] J.G. Buta, H.E. Moline, Methyl jasmonate extends shelf life and reduces microbial contamination of fresh-cut celery and peppers, J. Agric. Food Chem. 46 (1998) 1253–1256.
[40] D. Saisho, E. Nambara, S. Naito, N. Tsutsumi, A. Hirai, M. Nakazono, Characterisation of the gene family for alternative oxidase from *Arabidopsis thaliana*, Plant Mol. Biol. 35 (1997) 585–596.
[41] R.W.M. Fung, G. Langenkämper, R.C. Gardner, E. MacRae, Differential expression within an SPS gene family, Plant Sci. 164 (2003) 459–470.
[42] T.C. Verwoerd, B.M.M. Dekker, A. Hoekema, A small-scale procedure for the rapid isolation of plant RNAs, Nucl. Acids Res. 17 (1989) 2362.
[43] G.D. Virca, W. Northemann, B.R. Shiels, G. Widera, S. Broome, Simplified Northern blot hybridization using 5% sodium dodecyl sulfate, BioTechniques 8 (1990) 370–371.
[44] K.D. Livingstone, V.K. Lackney, J.R. Blauth, R. van Wijk, M.K. Jahn, Genome mapping in *Capsicum* and the evolution of genome structure in the *Solanaceae*, Genetics 152 (1999) 1183–1202.
[45] J. Whelan, M.K. Smith, M. Meijer, J.W. Yu, M.R. Badger, G.D. Price, D.A. Day, Cloning of an additional cDNA for the alternative oxidase in tobacco, Plant Physiol. 107 (1995) 1469–1470.
[46] A.L. Moore, A.L. Umbach, J.N. Siedow, Structure–function relationships of the alternative oxidase plant mitochondria: a model of active site, J. Bioenerg. Biomembr. 27 (1995) 367–377.
[47] J.N. Siedow, A.L. Umbach, Plant mitochondrial electron transfer and molecular biology, The Plant Cell 7 (1995) 821–831.
[48] M.J. Considine, D.O. Daley, J. Whelan, The expression of alternative oxidase and uncoupling protein during fruit ripening in mango, Plant Physiol. 126 (2001) 1619–1629.
[49] P.M. Finnegan, J. Whelan, A.H. Millar, Q. Zhang, M.K. Smith, J.T. Wiskich, D.A. Day, Differential expression of the multigene family encoding the soybean mitochondria alternative oxidase, Plant Physiol. 114 (1997) 455–466.
[50] T.C. McCabe, P.M. Finnegan, H. Millar, D.A. Day, J. Whelan, Differential expression of alternative oxidase genes in soybean cotyledons during postgerminative development, Plant Physiol. 118 (1998) 675–682.
[51] O. Karpova, E.V. Kuzmin, T. Elthon, K.J. Newton, Differential expression of alternative oxidase genes in maize mitochondrial mutants, The Plant Cell 14 (2002) 3271–3284.
[52] M.H.N. Hoefnagel, H.A. Miller, J.T. Wiskich, D.A. Day, Cytochrome and alternative respiratory pathways compete for electrons in the presence of pyruvate in soybean mitochondria, Arch. Biochem. Biophys. 318 (1995) 394–400.
[53] A.C. Purvis, Regulation and role of the alternative oxidase in chilling injury of green bell pepper (*Capsicum annuum* L.), Acta Hort. 553 (2001) 289–291.
[54] M.E. Alvarez, Salicylic acid in the machinery of hypersensitive cell death and disease resistance, Plant Mol. Biol. 44 (2000) 429–442.
[55] C.Y. Wang, J.E. Baker, Effects of two free radical scavengers and intermittent warming on chilling injury and polar lipid composition of cucumber and sweet pepper fruits, Plant Cell Physiol. 20 (1979) 243.
[56] L. Rizhsky, E. Hallak-Herr, F. van Breusegem, S. Rachmilevitch, J.E. Barr, S. Rodermel, D. Inzé, R. Mittler, Double antisense plants lacking ascorbate peroxidase and catalase are less sensitive to oxidative stress than single antisense plants lacking ascorbate peroxidase or catalase, Plant J. 32 (2002) 329–342.