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Characterization of alternative oxidase (AOX) gene expression in response to methyl salicylate and methyl jasmonate pre-treatment and low temperature in tomatoes ☆

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

[a]*Produce Quality and Safety Laboratory, Plant Sciences Institute, Beltsville Agricultural Research Center, US Department of Agriculture, Beltsville, MD 20705-2350, USA*
[b]*Biological Resources Engineering Department, Animal Sciences and Agriculture Engineering, Bldg 142, University of Maryland, College Park, MD 20742-2315, USA*
[c]*HortResearch, Mt Albert Research Centre, Private Bag 92169, Auckland, New Zealand*

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Chilling injury;
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RNA processing

Summary

Methyl salicylate (MeSA) vapor increased resistance against chilling injury (CI) in freshly harvested pink tomatoes. The expression patterns of alternative oxidase (AOX) before and during the chilling period demonstrated that pre-treatment of tomato fruit with MeSA vapor increased the transcript levels of AOX. We used 4 EST tomato clones of AOX from the public database that belong to two distinctly related families, 1 and 2 defined in plants. Three clones were designated as LeAOX1a, 1b and 1c and the fourth clone as LeAOX2. Using RT-PCR, 1a and 1b genes were found to be expressed in leaf, root and fruit tissues, but 1c was expressed preferentially in roots. RNA transcript from LeAOX1a of AOX subfamily 1 was present in much greater abundance than 1b or 1c. The presence of longer AOX transcripts detected by RNA gel blot analysis in cold-stored tomato fruit was confirmed to be the un-spliced pre-mRNA transcripts of LeAOX1a and LeAOX1b genes. Intron splicing of LeAOX1c gene was also affected by cold storage when it was detected in roots. This alternative splicing event in AOX pre-mRNAs molecules occurred, preferentially at low temperature, regardless of mRNA abundance. Transcript levels of several key genes

Abbreviations: AOX, alternative oxidase; CI, chilling injury; F, fruit; L, leaf; MeJA, methyl jasmonate; MeSA, methyl salicylate; R, root; wk, week

☆The nucleotide sequence data used were retrieved from TIGR tomato gene index Nucleotide Sequence databases.

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

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involved in RNA processing (splicing factors: 9G8-SR and SF2-SR, fibrillarin and DEAD box RNA helicase) were also affected by changes in storage temperature. The aberrant splicing event in AOX pre-mRNA and its possible association with the change in expression of genes involved in RNA processing in tomato fruit having chilling disorder was discussed.

Introduction

Chilling tolerance is a multigenic complex trait (Tokuhisa and Browse, 1999). Various mechanisms correlated with the acquisition of chilling tolerance have been identified including the 18:1 desaturase gene for the production of polyunsaturated membrane lipid (Miquel et al., 1993), the catalase and alternative oxidase (AOX) genes in the oxidative stress defense mechanism (Kerdnaimongkol and Woodson, 1999; Abe et al., 2002), and cold-regulated (COR) genes such as dehydrins (Thomashow, 1998). Most of these genes are functionally related to the reduction of reactive oxygen species (ROS).

In particular, it was suggested that AOX maintains the flow of mitochondrial electrons and avoids production of ROS (Purvis, 1997; Maxwell et al., 1999; Møller, 2001) which was thought to be the main factor resulted in chilling injury (CI; Purvis et al., 1995). Methyl salicylate (MeSA) and methyl jasmonate (MeJA) induced CaAOX1 gene expression is correlated with resistance to CI in peppers (Fung et al., 2004). One unexplained phenomenon of CaAOX1 expression was the detection of two CaAOX transcripts (1.5 and 3.5 kb) at low temperature. A longer transcript was also detected in rice and suggested to be the unprocessed transcript of AOX (Ito et al., 1997). Family 1 AOX genes typically occur as multigene families in plants (Ito et al., 1997). For example, closely related LeAOX1a and 1b gene transcripts are expressed in chilled tomatoes (Holtzapffel et al., 2003). By using a yeast expression system, the 1b protein was shown to be functional and had altered regulatory properties in comparison to 1a. The 1b protein was suggested to be a less regulated form of AOX that remains active under stress conditions (Holtzapffel et al., 2003).

Recent studies point to the presence of temperature-related splicing inhibition for genes originating from plastid organelles. For example, an increase in temperature fully inhibits splicing of the intron for chloroplast NAD(P)H dehydrogenase and ndhB genes (Karcher and Bock, 2002). In addition, pre-mRNA levels of cytochrome-*c* oxidase subunit 2 (cox2), which is involved in electron transport and evolved from mitochondria, are increased by cold (Kurihara-Yonemoto and Handa, 2001). Also, the uncoupling protein (UCP), which is exported to mitochondria and nuclear encoded, does not express functional UCPs in cold environments due to defective pre-mRNA processing which results in multiple abnormal forms of UCP transcripts (Watanabe and Hirai, 2002). These examples suggest that impairment of the RNA processing mechanism affects gene expression post-transcriptionally and contributes to the loss of chloroplast or mitochondrial function under temperature stress conditions.

RNA splicing is a highly regulated event and most of the splicing mechanism that takes place within the spliceosome protein complex has been established using yeast and metazoans, which share high conservation in many respects with plants (Kramer, 1996; Brown and Simpson, 1998; Lorkovic et al., 2000; Reddy, 2001). Some of the components included fibrillarin (Ochs et al., 1985), SR proteins (a family of proteins with arginine/serine-rich domains) and DEAD/H proteins (RNA-dependent ATPases or ATP-dependent RNA helicases) (Fuller-Pace, 1994; Staley and Guthrie, 1998).

DEAD/H-box RNA helicase genes is a superfamily with up to 53 members in *Arabidopsis* (Boudet et al., 2001). It functions by unwinding double-stranded RNA in an energy-dependent manner and disrupts RNA–protein interaction (Luking et al., 1998). DEAD/H-box RNA helicases are essential in all aspects of RNA metabolism at the level of expression and at the post-transcriptional level for pre-messenger RNA splicing, translation, and nucleocytoplasmic trafficking (Pause and Sonenberg, 1993; Aubourg et al., 1999; Graveley, 2000) and was recently shown to be required for chilling tolerance in *Arabidopsis* (Gong et al., 2002). The serine-arginine SR family proteins have multiple functions in pre-mRNA splicing reactions for removal of constitutively spliced introns and regulate alternative splicing via variation of its physiological concentration (Valcarcel and Green, 1996; Graveley, 2000). In *Arabidopsis*, various SR isoforms are themselves regulated by alternative splicing in a temperature-dependent manner (Lopato et al., 1996; Lazar and Goodman, 2002). Fibrillarin, on the other hand, is a single copy gene in *Arabidopsis* and is involved in post-transcriptional processing

and modification of ribosomal RNA (Schimmang et al., 1989; Pih et al., 2000). A point mutant of the fibrillarin gene was found to be temperature-sensitive and lethal in yeast (Schimmang et al., 1989; Tollervey et al., 1993). In fact, other evidence also supports the importance of pre-mRNA splicing factors under stress tolerance conditions. For example, lithium toxicity in yeast is due to the inhibition of RNA processing enzymes (Dichtl et al., 1997), whereas expression of SR-like splicing proteins in yeast and *Arabidopsis* plants increases their LiCl and NaCl tolerance (Forment et al., 2002). By transcriptome profiling, it was indicated in *Arabidopsis* that mRNA processing are one of the regulatory networks involved in cold acclimation (Fowler and Thomashow, 2002).

Here, we investigate the relationship of induced AOX gene expression and chilling tolerance by characterizing three closely related (Family 1) and one distinctly related (Family 2) LeAOX genes in chilled tomatoes. We identify the AOX gene members that are responsive to cold storage and induction by the plant growth regulators MeJA and MeSA. RT-PCR, using gene-specific primers spanning intron–exon boundaries, was used to determine if altered splicing occurred among the three LeAOX Family 1 genes at low temperature. Furthermore, we characterized the expression patterns of several genes involved in RNA metabolism under cold storage conditions.

Methods and materials

Plant materials and post-harvest treatments

Tomato fruit (*Lycopersicon esculentum* L. cv ''Beefsteak'') were harvested at the pink maturity stage from a local farm in Virginia during the summer season of 2003 with a mean daytime temperature of 24 °C. Fruit were transported at 20 °C and no exogenous ethylene was applied. Fruit were selected, washed and blotted dry (day 0). A total of 300 fruit were divided into three lots. Each lot was placed in a 200-L airtight container, together with 22.4 μL/L MeJA, 13.2 μL/L MeSA or water (control) spotted onto filter paper so that the final vapor concentration reached 10^{-4} M (if completely vaporized) and incubated for 1 day in darkness at 20 °C. After treatment (day 1), fruit were kept in the packing carton box and stored in darkness at 0 °C for 21 days. After the cold treatment, the fruit were transferred to 25 °C and the development of CI, decay, and ripening characteristics were measured. At each sampling time point, equatorial slices were taken from each fruit, diced, frozen in liquid nitrogen within 2 min and stored at −80 °C until used.

Evaluation of chilling and ripening characteristics

Chilling symptoms were evaluated 2 days after cold storage. Tomato CI was ranked according to an index and composite color measurement according to Ding et al. (2002) with slight modification. CI index rank was recorded based on the overall appearance of fruit marketability, which takes into account symptoms including the degree of shrivel (skin shrinkage), pitting, decay and fruit color. Each of these symptoms was also evaluated independently.

Preparation of gene specific and family specific probes

The four LeAOX clones corresponding to EST sequences TC99848, AI781103, TC111033, TC109296 were obtained from Clemson University. These clones were fully sequenced and the 3′ untranslated regions (UTRs) were compared. Gene-specific primer pairs of the four LeAOX clones (represented by 2 families) [LeAOX1 (MST1 and MST2), LeAOX1a (Uni 1-1 and LeAOX1arev), LeAOX1b (Uni 1-1 and LeAOX1brev), LeAOX1c (Uni 1-1 and LeAOX1crev), LeAOX2 (RvcmLeAOX2fd and RvcmLeAOX2rev primers) (Table 1)] were designed. Gene-specific primer pairs of genes involved in RNA processing (Le9G8-SR, LeSF2-SR1, LeFibrillarin and LeDEAD-box RNA helicase using their respective -1 and -3 primers (Table 1) were designed based on sequence information from the TIGR Tomato *L. esculentum* database [splicing factor 9G8-like SR protein (TC125078), putative pre-mRNA splicing factor SF2 (SR1 protein) (TC117073), Fibrillarin (TC118045) and DEAD box RNA helicase-like protein (TC116803)]. Complementary DNA was prepared with SuperScript™ reverse transcriptase (Invitrogen™) using odtRACE1 primer and total RNA of tomato fruit as a template (freshly harvested time point). PCR conditions were as outlined below. PCR products were gel-purified and sequenced to confirm their identity before being used as probes for RNA gel blot analyses.

RNA gel blot analysis

Total RNA was extracted from *L. esculentum* L. fruit tissues (Verwoerd et al., 1989). Electrophor-

Table 1. Oligonucleotide primers used to carry out family specific and gene-specific PCR

Primer name	Oligo sequence
Le9G8-SR-1	5′-CTAGGGTTTTACTCTCTCGCGTTCT-3′
Le9G8-SR-3	5′-TAAGGAACTTCATCTCTTCCACGAT-3′
LeSF2-SR1-1	5′-ACGCAACCTGTCGTCGTTGTATATC-3′
LeSF2-SR1-3	5′-GAGAATTGATTGCGAAACAGAGAGT-3′
LeFibrillarin-1	5′-GTAAGGTTGTGGTCGAGCCTCATAG-3′
LeFibrillarin-3	5′-CAATACAGTTTGCCTTGATTGAGAT-3′
LeDEAD box RNA helicase-1	5′-ATCTTGGCGCCTACCAGAGAACTTG-3′
LeDEAD box RNA helicase-3	5′-GGCGAAAACCTGATAAAGTTCTGTC-3′
LeAOX1arev	5′-TAAGTAAACAAAAAGATGATATAA-3′
LeAOX1brev	5′-CATCTATATTTCATATTTTGGAGC-3′
LeAOX1crev	5′-TAGTAAATTGTGATCTTTTTCACT-3′
RvcmLeAOX2fd	5′-CAACCTAAATGGTACGAGAGGTTGT-3′
RvcmLeAOX2rev	5′-GAATTCGGCACGAGGTGAGCAATAA-3′
New1aFd2	5′-TCATCCTACTCTACCTCTTATCGAT-3′
RvcmLeAOX1bfd	5′-TTGATCGATATAGAAAAGGGACT-3′
RvcmLeAOX1brev	5′-TTGGAGATATTGTTTTCACTTCAA-3′
HT110	5′-CATAACTGCAGTGGAGGTGTTGTTGGAGCAT-3′
HT111rev	5′-CACAAATGCTCCAACAACACCTCCA-3′
MST1	5′-ACWGTRGCWGCWGTVCCYGGRATGGT-3′
MST2	5′-GGTTKACATCWCGRTGRTGWGCCTC-3′
odtRACE1*	5′-GACTCGAGTCGACATCGA-$(T)_{17}$-3′

*Parentheses indicate the number of nucleotide repeats used in the odtRACE1 primer.

esed RNA was transferred to Hybond N^+ (Amersham BioSciences™) membrane using 20 × SSC, according to manufacturer's instructions. RNA gel blot hybridization was performed according to Virca et al. (1990). Probes were labelled with [α-^{32}P]-dCTP by DNA random prime labelling RTS system (Invitrogen™). Plasmid DNAs representing each gene fragment were applied individually in 10 ng aliquots to dot blots and hybridized together with each corresponding RNA gel blot. After an overnight hybridization at 65 °C, unbound probe was removed by washing the membrane in 0.2 × SSC at 65 °C. The membranes were sealed in plastic bags and exposed to X-ray film. The membranes were then stripped and rehybridized with an 18S ribosomal DNA probe from apple (*Malus domestica*) (S. Pechous, personal communication) as a loading control. For other blots, ethidium bromide staining of rRNA was used as RNA loading control.

Analysis of intron splicing using RT-PCR

Oligonucleotides were designed so that each pair flanked an intron located 5′ of the stop codon for LeAOX1a (uni1-1, New1aFd2 primer pairs), LeAOX1b (RvcmLeAOX1bfd, RvcmLeAOX1brev primer pairs) and LeAOX1c (uni 1-1, LeAOX1crev primer pairs) (Table 1). As a control to detect gDNA in RT preparations, primers (HT110 and HT111rev) were obtained from the promotor region of tomato β-galactosidase gene TBG2 (D. Smith, unpublished data). Total RNAs were extracted from tomato fruit (F) (cv. 'Beefsteak') harvested from a local farm and tomato leaf (L) and roots (R) harvested from 8 weeks old plants (cv. 'Brandywine') grown in a greenhouse. 'Beefsteak' fruit and intact 'Brandywine' tomato plants were stored at 0 °C for 2 days before the corresponding cold treated tissues were harvested. Genomic DNA extracted from leaf tissue of both Beefsteak and Brandywine was used as a size control for intron-containing pre-mRNA. RNA samples were treated with DNaseI (Invitrogen™) to remove contaminating genomic DNA. RT-PCR was performed using Platinum-Taq (Invitrogen™) following manufacturer's instructions. The cycling conditions were 94 °C, 2 min initial denaturation; 94 °C, 30 s (denaturation), 60 °C, 30 s (annealing), 72 °C, 30 s (extension) for 30 cycles; and a final extension at 72 °C for 5 min. PCR product was visualized by electrophoresis using 3.5% NuSieve 3:1 agarose gels (Cambrex) and staining with GelStar staining solution (Cambrex). Electrophoresis results were recorded by using a Bio-Rad Fluor-S™ MultiImager. At least three independent runs were performed.

Results

MeSA reduced chilling injury of pink tomatoes

Overnight exposure to MeSA or MeJA was effective at increasing resistance to CI in tomatoes at the mature green maturity stage (Ding et al., 2002) and in peppers (Fung et al., 2004). We elected to study the response of tomatoes at the pink maturity stage to cold storage based on this stage's potential usefulness in the fresh cut produce market. Pink tomato fruit were treated overnight with MeJA or MeSA at a concentration of 10^{-4} M before cold storage at 0 °C. CI index was recorded based on overall fruit appearance by taking into account the degree of shrivel, pitting, decay and fruit color. Significant differences in CI index were found among treated and untreated pink tomato fruit only after 3 weeks of storage and only with MeSA treatment (Fig. 1). For MeJA treated fruit, no differences were observed from untreated fruit except for less decay. For MeSA treated fruit, CI index was significantly lower in terms of the degree of shrivel and decay. Red color development was inhibited in both untreated and MeJA treated fruit and was characterized by the development of pale orange and yellow pigmentation. In summary, MeSA (but not MeJA) efficiently delayed CI in tomatoes treated at the pink maturity stage.

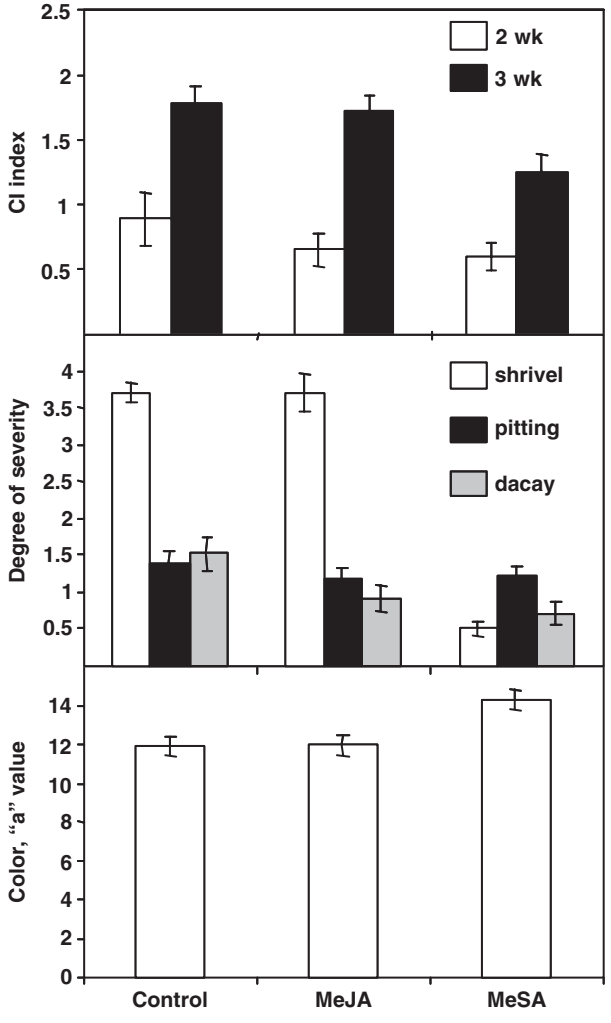
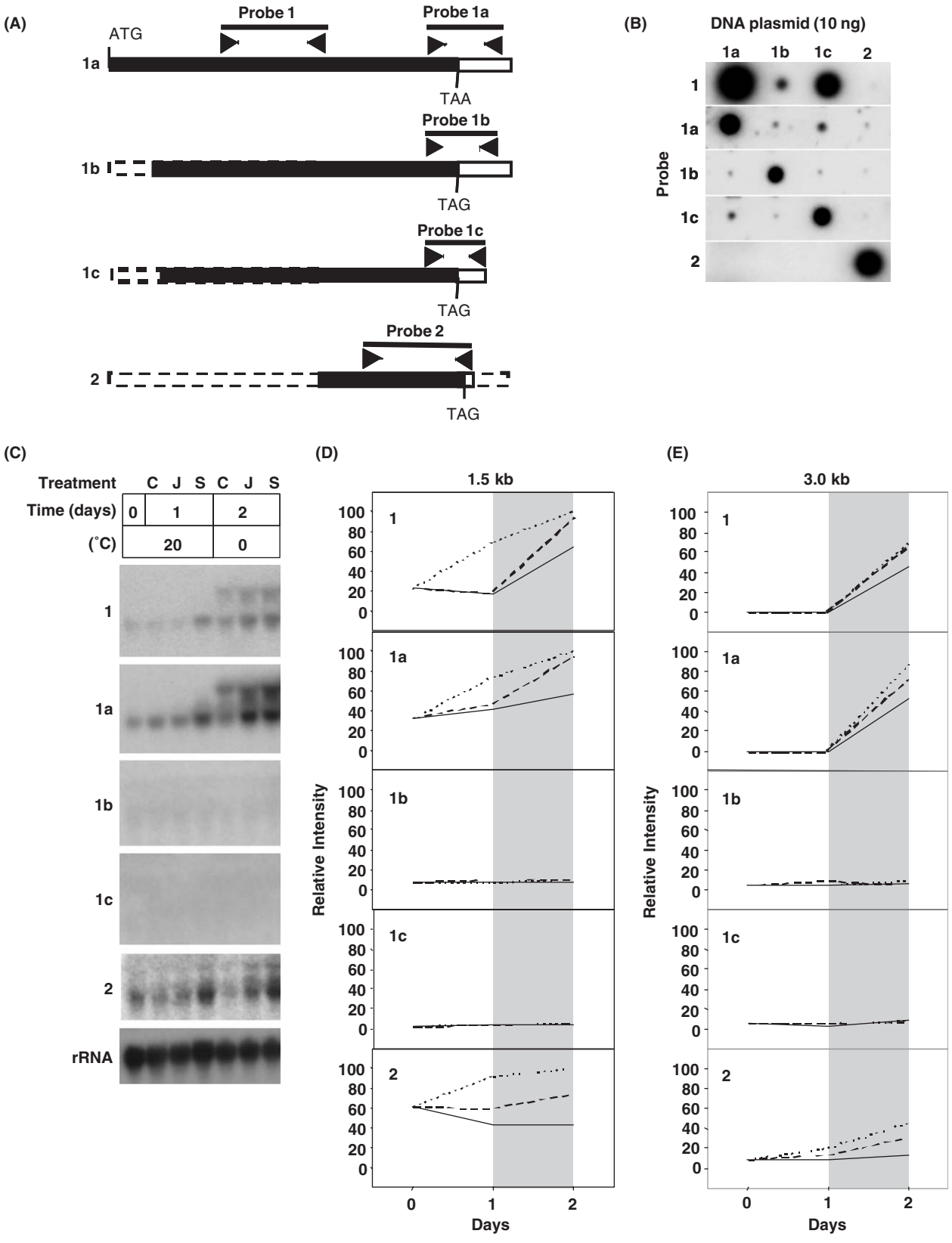


Figure 1. Effect of MeSA and MeJA on chilling injury of tomatoes stored at 0 °C. Fruits at pink maturity stage were harvested from a local farm and treated at 20 °C overnight with 10^{-4} M of MeSA, MeSA or with air as a control. Fruit were then stored at 0 °C for 2–3 weeks. After cold treatment, fruits were rewarmed at 25 °C for another 3 days. Chilling injury evaluation was performed at day 2 upon rewarming based on the general marketable appearance of fruit (CI index, top panel). Individual symptoms were evaluated for fruit stored in 0 °C for 3 weeks (degree of shrivel, pitting or decay in middle panel; color development in bottom panel). Error bars represent SE with $n = 24$.

MeSA and MeJA treatment of fruit leads to an induction of LeAOX1 (1a) transcript

We examined the transcript levels of four LeAOX genes in tomatoes treated with MeSA or MeJA vapors. Total RNA was extracted from tomatoes freshly harvested from a local farm (day 0). Gene specific probes (probes 1a, 1b, 1c and 2) were designed to detect the divergent 3′ regions of each of the three LeAOX Family 1 and LeAOX Family 2 genes (Fig. 2A). A family specific probe (probe 1) that hybridized to all three LeAOX Family 1 genes was also used in order to determine the total LeAOX transcript profile in comparison with gene specific transcript profile of each LeAOX member. In general, all family and gene-specific probes show no cross-hybridization other than to their respective EST plasmid clones except for 1a and 1c probes, where slight cross-hybridization was observed even under high stringency washing condition (Fig. 2B).

RNA gel blot analysis indicated that LeAOX1a mRNA abundance increased during the 24 h exposure to exogenous MeSA and also during the first day at 0 °C in both MeJA- or MeSA-treated fruit (Figs. 2C and D). In addition to the 1.6 kb LeAOX1a transcript, a 3 kb transcript was detected when fruits were transferred to 0 °C (Figs. 2C and E). Both 1.6 and 3 kb LeAOX1a transcript levels remained high in treated and untreated fruit throughout the first 5 days of 0 °C storage (data not shown). The expression profile of LeAOX Family 1 transcripts in fruit


(A)
Probe 1
Probe 1a
ATG
1a
TAA
Probe 1b
1b
TAG
Probe 1c
1c
TAG
Probe 2
2
TAG
(B)
DNA plasmid (10 ng)
1a 1b 1c 2
Probe
1
1a
1b
1c
2
(C)
Treatment C J S C J S
Time (days) 0 1 2
(°C) 20 0
1
1a
1b
1c
2
rRNA
(D)
1.5 kb
Relative Intensity
Days
(E)
3.0 kb
Relative Intensity
Days

appeared to be determined almost entirely by LeAOX1a expression (Fig. 2C). The transcript levels of the LeAOX Family 2 gene appeared to be increased by MeSA and MeJA during days 1 and 2 (Fig. 2C), and thereafter remained unchanged during cold storage (data not shown). A 3 kb transcript was also detected for the LeAOX Family 2 gene during 0 °C storage (Fig. 2E). Expression of LeAOX1b and 1c was not detected in fruit by RNA gel blot analysis (Figs. 2C–E).

Inhibition of intron splicing at 0 °C in both high and low abundant LeAOX Family 1 genes

In addition to the expected 1.6 kb AOX transcript, a second 3 kb transcript was detected in cold stored tomatoes with probes LeAOX1 and 1a (Figs. 2C–E). The following experiment was conducted to identify the source of the 3 kb transcripts. Based on AOX genomic sequences from *Arabidopsis*, AOX Family 1 genes typically consist of three introns. An "intron-3" region of LeAOX Family 1 genes was identified and confirmed by comparing sequence information between gDNA and cDNA PCR fragments covering the corresponding region. In tomato, intron-3 was localized to a site, just 5′ of the stop codon for 1a, 1b and 1c genes (Fig. 3A). Gene-specific primer pairs flanking each intron-3 region of LeAOX1a, 1b and 1c were selected. The expected PCR product size was determined when cDNA versus gDNA were used as templates (Fig. 3A). The transcript sizes of the three LeAOX Family 1 genes were evaluated in fruit, leaf and root tissues at both warm (>20 °C) and cold conditions (0 °C). PCR products from gDNA indicated that all three LeAOX Family 1 genes are present in tomato cultivars 'Beefsteak' and 'Brandywine' (Fig. 3B, lanes 2 and 9, respectively). PCR fragments derived from gDNA were used as size controls to indicate the presence of intron-containing transcripts for RT-PCR analysis (Fig. 3B, lanes 3–8). The LeAOX1a, 1b and 1c genes were expressed in both cultivars, and similar sized fragments were amplified (Fig. 3B). LeAOX1a and 1b were expressed in fruit, leaves and roots but LeAOX1c expression was not detected in fruit. RT-PCR results confirmed the presence of intron-containing LeAOX1a, 1b and 1c transcripts when expressed at low temperature (Fig. 3B, lanes 4, 6, 8). The identity of PCR fragments from the normal spliced cDNA and the aberrant intron-containing cDNA of each LeAOX gene was confirmed by gel purification of the corresponding bands and direct sequencing of the PCR products (data not shown). Intron-containing LeAOX1a transcripts were absent in fruit stored at warm temperature (Fig. 3, lane 3) and roots harvested from greenhouse-grown tomato plants (Fig. 3, lane 7). Interestingly, intron-containing LeAOX1b transcripts were detected in leaf, but not fruit or root tissues under warm conditions (Fig. 3, lane 5). As a control for contamination of gDNA in cDNA samples, a pair of primers designed from the promoter region of β-galactosidase gene TBG2 was used. No signal was detected in RT products when this promoter primer pair was used for PCR (Fig. 3B, lower panel). Also, RNA samples prepared with no RT step resulted in no PCR products using the LeAOX1a, 1b and 1c primers (data not shown). Based on the results presented above, we conclude that the 3 kb band detected by RNA gel blot analysis was unspliced AOX pre-mRNA. Furthermore, this inhibition in intron splicing seems

Figure 2. Expression of AOX gene transcript. (A) Schematic representation of the three closely related AOX Family 1 clones (LeAOX1a, LeAOX1b, LeAOX1c) and a divergent Family 2 (LeAOX2) clone obtained from TIGR Tomato database. The open reading frame of each clone (designated 1a, 1b, 1c and 2) is in black filled box. Dashed box indicated regions with unknown sequence information. The 3′UTR is indicated in white box. Relative locations of family and gene specific probes (horizontal bars) or primers (arrowheads) are indicated. (B) DNA dot blot analysis. DNA plasmid of each AOX clones were applied individually in 10 ng aliquots in each set of dots. Each row displays the hybridization of all four plasmid DNAs with the individual AOX family or gene-specific probes. (C) AOX Family 1 and 2 transcripts in response to MeSA 'S', MeJA 'J' in chilled tomato fruits were compared with that of untreated control fruit 'C'. Tomato fruits were harvested from a local farm (field temperature was 20 °C, day 0) and treated as described in Fig. 1. RNA gel blot analysis shows expression of Family 1 AOX transcripts by using probe 1. Expression profiles of gene 1a, 1b, 1c and 2 transcripts were showed by using the 3′end of the gene as probes 1a, 1b, 1c and 2, respectively. Fruits from selected timepoint of experiments were sampled and 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. Relative AOX transcript levels, 1.5 and 3.0 kb, were quantified by densitometry and normalized to hybridization signals with an 18S ribosomal probe for each timepoint. Graphical representation of relative amount of 1.5 kb (D) and 3.0 kb (E) transcripts were plotted against time (days). Sampling time point during the treatment at air control (solid line), MeJA (long dashed line) and MeSA (dotted line) are shown. Gray area indicated storage at 0 °C. Expression levels were expressed as a ratio to the timepoint with the highest intensity for each gene, which is set to 100. Highest intensity timepoint for gene 1b and 1c is set to that of 1a.

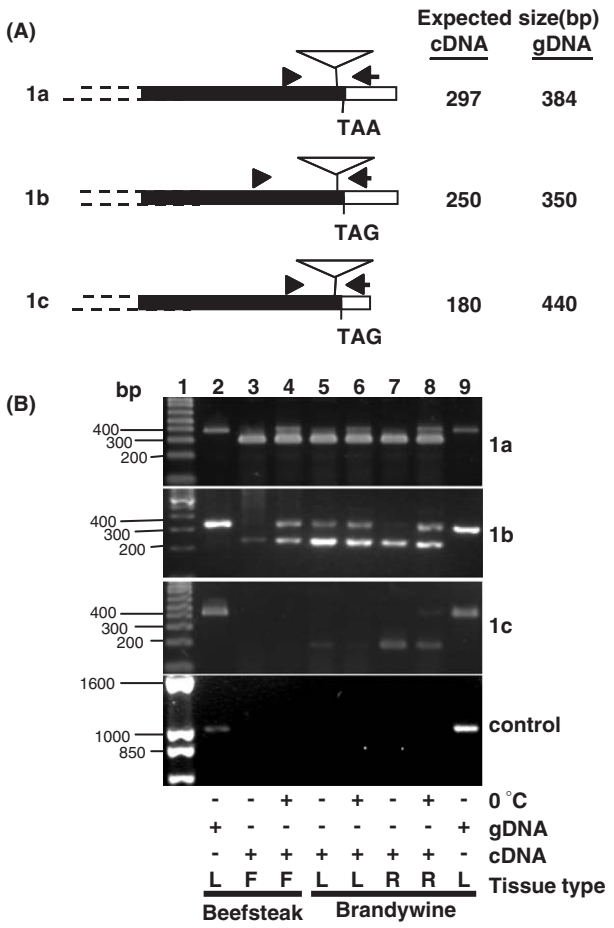


Figure 3. Analysis of intron splicing of AOX pre-mRNA in response to low temperature. (A) Position of intron located 5′ of the stop codon for 1a, 1b and 1c genes are indicated with open triangle. Locations of primers flanking each intron are indicated as arrowhead in the coding sequence and arrow in the 3′UTR. Expected sizes (bp) of AOX transcript and its pre-mRNA are estimated by PCR using gene specific primer pairs and its respective EST clone plasmid or genomic DNA as template. (B) Total RNA were extracted from tomato Beefsteak cultivar fruit (F) harvested from local farm, tomato Brandywine cultivar leaf (L) and roots (R) harvested from 8 weeks old plant grown in greenhouse. Beefsteak fruit and Brandywine tomato plant were stored in 0 °C for 2 days before the corresponding cold treated samples were harvested. The figure shows bands amplified in PCR by the indicated primer pairs. Genomic DNA extracted from leaf tissue of both Beefsteak and Brandywine tomato cultivars were used as size control for the presence of pre-mRNA (lane 2 and 9). Primer pair designed from promoter region of tomato beta-galactosidase gene is used as positive control for presence of gDNA contamination in cDNA samples.

to happen equally to both the highly abundant LeAOX1a and the rarer transcripts LeAOX1b and 1c under low temperature conditions.

Differential expression of genes involved in RNA processing during chilling-temperature storage

We described in the introduction the importance of highly regulated pre-mRNA splicing mechanism under stress conditions. The results above showed intron splicing of LeAOX Family 1 members was specifically inhibited at low temperature. To further investigate possible sources of altered intron splicing events in cold stored tomatoes, the transcript levels of four genes encoding RNA processing enzymes in tomatoes treated with MeSA or MeJA vapors was examined. The transcript levels of splicing factors, 9G8-SR and SF2-SR1, were high on day 0 compared with those of fibrillarin and DEAD box RNA helicase (Figs. 4A and B). Interestingly, in addition to the expected 1.2 kb fragment, a 3.4 kb band was detected for the 9G8-SR gene (see Discussion for details). Overnight incubation at 20 °C with MeJA or MeSA vapors, resulted in essentially no change in gene expression (Fig. 4). During the 0 °C storage period, transcript levels of 9G8-SR (3.4 kb) and SF2-SR1 decreased to low levels (Fig. 4). On the other hand, the transcript levels of 9G8-SR at 1.2 kb decreased at a much slower rate. Fibrillarin transcript levels increased slightly during 0 °C storage, while DEAD box RNA helicase transcripts remained at high levels during cold storage (Fig. 4). Within 7 h of rewarming to 25 °C, transcript levels of 9G8-SR (both 1.2 and 3.4 kb), SF2-SR1 and fibrillarin increased rapidly. DEAD box RNA helicase transcript levels remained at high levels after rewarming (Fig. 4). No significant differences were observed in the expression of RNA processing gene transcripts between MeJA or MeSA treatments and the controls. In summary, expression of the RNA processing and splicing genes SF2-SR1, 9G8-SR and fibrillarin was highly sensitive to temperature variation and the reduced expression of SF2-SR1 and 9G8-SR transcripts correlated with the appearance of unspliced LeAOX transcripts at low temperature.

Discussion

The major conclusion from the results described here is that CI index and degree of severity (shrivel and decay) correlates with the expression pattern of the LeAOX1a and LeAOX2 genes in MeSA treated fruit before and during the first day of cold storage. The three closely related LeAOX Family 1 genes are expressed at different levels in fruit and LeAOX1a is responsible for the vast majority of LeAOX Family 1

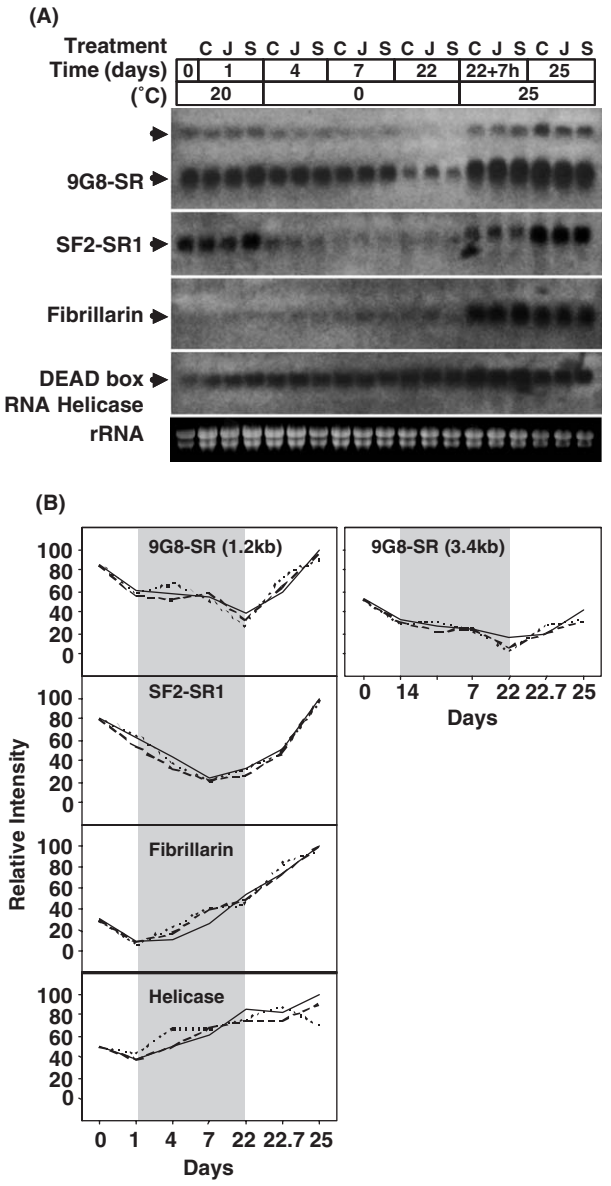


Figure 4. Expression of RNA processing gene transcripts for splicing factors (9G8-SR and SF2-SR1 proteins); fibrillarin and DEAD box RNA helicase in response to MeSA (S), MeJA (J) in chilled tomato fruits. Fruit from selected timepoint of experiments were sampled for RNA gel blot analysis (see Materials and methods for details). (A) RNA gel blot analysis was performed as described in Fig. 2. Relative transcript levels were quantified by densitometry and normalized to ethidium bromide signals of ribosomal RNA for each time point. Graphical representations of relative amount of each RNA processing gene transcripts were plotted against time (day) (B). Sampling time point during the treatment at air control (solid line), MeJA (long dashed line) and MeSA (dotted line) are shown. Gray area indicated storage at 0 °C. Expression levels were expressed as a ratio to the time point with the highest intensity of each gene, which is set to 100.

expression in fruit during cold storage and accumulation of its mRNA correlates with chilling resistance. Additionally, splicing of each of the closely related AOX genes was found to be altered in response to temperature changes. Inhibition of LeAOX intron splicing by low temperature was most notable in fruit and roots and was not dependent on transcript abundance. The precise mechanism of this aberrant transcription event was not ascertained, but expression levels of genes involved in pre-mRNA intron splicing and RNA processing are also dramatically altered in cold stored tomatoes.

MeJA and MeSA treatments induced chilling resistance in mature green tomatoes (Ding et al., 2002) and in green bell peppers (Fung et al., 2004). However, MeJA is not as effective as MeSA in alleviating CI of pink maturity stage tomatoes. This result confirms previous work demonstrating that tomato fruit at different maturity stages respond differently to plant growth regulator treatment (Ding and Wang, 2003). Also, chilling tolerance of maize, cucumber, and rice seedling leaves and roots was shown to be differentially affected by salicylic acid (Kang and Saltveit, 2002). The delayed induction of LeAOX1a and LeAOX2 transcript levels in MeJA treated fruit may provide one explanation for the lower degree of shrivel found among chilling resistant MeSA treated tomato fruit but not in MeJA or control fruit.

Among the AOX gene family members, LeAOX1 transcripts accumulate to the highest levels during cold storage and LeAOX1a mRNA abundance is higher in comparison to that of the 1b and 1c genes in fruit throughout the course of treatment (Fig. 2). This observation is supported by the fact that 30 EST clones for LeAOX1a but only two for LeAOX1b were present in TIGR tomato EST database. This may also be one of the contributing reasons why LeAOX1a was the only isoform identified via amino acid sequencing (Holtzapffel et al., 2003). LeAOX1c is expressed most abundantly in roots, at a low level in leaves, but not in fruit tissues (Fig. 3B). This 1c expression profile is also supported by the fact that the only two EST clones of LeAOX1c from the TIGR tomato database were both derived from a root cDNA library. The expression profile of LeAOX1c suggests its possible functional importance in vegetative tissues. The biological functions of the AOX isoforms is still under debate. Enzymatically, LeAOX1a and 1b proteins were shown to be different in their regulatory properties (Holtzapffel et al., 2003). This suggests that closely related AOX isoforms may have subtly different biochemical characteristics. However, the expression of distinctly related isoforms may be regulated by varied

developmental and environmental cues (Considine et al., 2001).

Low temperature affected RNA splicing efficiency of AOX transcripts. By using intron-3 of AOX Family 1 for analysis, we confirmed partial inhibition of intron splicing in all LeAOX Family 1 genes at low temperature by RT-PCR analysis. Variation in AOX intron splicing efficiency appears not to correspond with transcript abundance (Fig. 3B). Under low temperature conditions, the LeAOX1a, 1b and 1c genes are predicted to encode a premature stop codon if intron-3 is unspliced. Furthermore, we anticipate that intron-1 and -2 splicing of the LeAOX genes may also be inhibited at low temperature. Most likely, at low temperature, this partial inhibition of splicing events results in production of aberrant transcript and non-functional LeAOX Family 1 proteins in cold stored tomatoes.

We identified, based on expression patterns, RNA processing genes that are responsive to changes in temperature and may possibly be related to LeAOX pre-mRNAs splicing. In *Arabidopsis*, splicing factor SR proteins, which control splicing of other genes, are themselves tightly controlled by phosphorylation (Tacke et al., 1997) and by alternative splicing in a temperature-dependent manner (Lazar and Goodman, 2002). This is consistent with our results in tomatoes where higher SF2-SR1 transcript levels are found in fruit both before and after cold storage.

A subset of SR family proteins (9G8) was shown to promote the nucleocytoplasmic export of mRNAs in mammalian cells (Huang and Steitz, 2001). We observed a gradual decrease in 9G8-SR transcript levels in cold stored tomatoes (Fig. 4). The tomato 9G8-SR gene probe detected two bands by RNA gel blot analysis. Interestingly, the longer of the two transcripts disappears during storage at 0 °C, and leads us to suggest that this splicing factor in tomato might itself be regulated by alternative splicing in a temperature-dependent manner, similar to *Arabidopsis* described above. Lazar and Goodman (2002) suggest that co-expression of SR1 transcripts is required for its function by the combinatorial actions of various isoforms. In summary, the dramatic change in transcript levels of fibrillarin, SF2-SR1 and 9G8-SR splicing factors suggests they are potentially important for adaptation to different temperature conditions. The splicing factor(s) that specifically influence the splicing efficiency of AOX genes are yet to be identified. It is unlikely that the higher rate of transcription detected for LeAOX1a on its own, could exceed its intron splicing capacity, resulting in insufficient splicing and accumulation of unprocessed transcript. This idea is supported by our data showing that intron splicing for gene 1b, which is much lower in transcript abundance, is also inhibited.

As the AOX transcript levels are correlated with chilling tolerance, there should be a mechanism to ensure the expression of an AOX transcript-specific factor responsible for splicing. This temperature sensitive splicing event occurs not only selectively to the AOX gene in rice (Ito et al., 1997) and peppers (Fung et al., 2004), but also to other subgroups of genes that coincidentally share mitochondria or plastid organelle evolutionary origins (see Introduction). The high occurrence of splicing inhibition for genes evolving from organelles indicates that there may be a discrete splicing mechanism that either specifically regulates a subset of genes as suggested by Clark et al. (2002) or there is a low temperature sensitive splicing mechanism.

Our study suggests that RNA processing steps may be important in the regulation of stress-related AOX expression at low temperature and are potentially related to chilling tolerance in tomatoes. Given the importance of the RNA processing genes (splicing factors 9G8-SR, SF2-SR1, fibrillarin and DEAD box RNA helicase) in the control of gene splicing or expression leading to functional protein, it seems likely that the dramatic change in transcript levels of these splicing factors represents a "master switch" in cold tolerance. For example, the Myb4 gene functions to increase the chilling and freezing tolerance of *Arabidopsis* (Vannini et al., 2004). A better knowledge of how splicing factors are differentially regulated and how these processes in RNA metabolism might be disrupted will help our understanding of CI in horticultural crops.

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