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Stimulated auxin levels enhance plum fruit ripening, but limit shelf-life characteristics

Islam El-Sharkawy[a,b], Sherif Sherif[b,c], Tawfiq Qubbaj[a], Alan J Sullivan[c], Subramanian Jayasankar[a,*]

[a] University of Guelph, Department of Plant Agriculture, 4890 Victoria Av. N., P.O. Box 7000, Vineland Station, ON L0R 2E0, Canada
[b] Faculty of Agriculture, Damanhour University, Damanhour, Egypt
[c] University of Guelph, Department of Plant Agriculture, Guelph, ON N1G2W1, Canada

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ABSTRACT

Ripening is a highly coordinated, irreversible event involving a series of physiological and biochemical changes, leading to the development of a soft fruit. One of the limiting factors that influence the economic value of fruit is the relatively short ripening period and limited shelf-life. In climacteric fruit, ethylene is a key regulator of ripening; however, recent research has shown that auxin also plays an important role during the event. To understand the contributions of ethylene, auxin and their interaction in ripening, two plum cultivars with widely varying fruit ripening behaviors were compared. The early cultivar, EG, exhibited a brief ripening process in association with rapid decline in firmness. The late cultivar, V9, displayed slow ripening behavior accompanying by notable extension in fruit firmness, resulting in prolonged shelf-life along with preserved fruit quality traits. Auxin has been suggested to play an indirect role in promoting fruit ripening via stimulating the transcription of several ethylene components, resulting in ethylene-induced fruit ripening and softening. This study shows further that there is a direct involvement of auxin in advancing ripening events independent of ethylene action through stimulating the transcription of several genes that encode cell-wall metabolism-related proteins critical for determining the fruit softening rate and potential shelf-life. These results support the hypothesis that the autonomous role played by auxin is as important as that of ethylene in determining not only fruit ripening behavior, but also in mediating other fruit quality traits including shelf-life.

1. Introduction

Fruit ripening is a genetically programmed event that is characterized by a number of biochemical, physiological and structural alterations. These include production of aromatic compounds, nutrients, pigmentation, and softening of flesh to an edible texture (Singh and Khan, 2010). These processes have direct impacts not only in general fruit quality traits, but also in shelf-life, consumer acceptability, and postharvest disease incidence (Giovannoni, 2004). The fundamental importance of these processes has prompted considerable research into how they are governed. Research into fruit developmental processes has been greatly aided by analyzing the outcomes of both naturally occurring and induced genetic diversity (Kelly and Bradford, 1986; Lau et al., 2008). One outcome of the research has been the identification of phytohormones as master regulators of the many processes involved (McAtee et al., 2013; Kumar et al., 2014). While the role of ethylene in triggering and regulating the ripening of climacteric fruit has been clearly demonstrated, little is known about the contribution of other hormones (Giovannoni, 2004; Bouzayen et al., 2010). The characterization of mutant plants in some species like tomato, whose fruit are unable to ripen even when treated with ethylene has helped to identify the developmental factors that act upstream of ethylene and control the ripening process. Given its almost ubiquitous importance, it was not unexpected that auxin would be shown to play a prominent role (Friml, 2003). Several studies showed the involvement of auxin in mediating the fruit ripening process and other fruit quality traits in many crop species (Vendrell, 1985; Cohen, 1996; Sagar et al., 2013). Collectively, data from hormone application and quantification have shown that auxin is an important component in the regulation of the onset and coordination of ripening

Abbreviations: DAB, Days after bloom; EG, Early Golden; V9, V98041 plum genotype.

* Corresponding author.
E-mail address: jsubrama@uoguelph.ca (S. Jayasankar).

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processes (Miller et al., 1987; Gillaspy et al., 1993; El-Sharkawy et al., 2008, 2009, 2010, 2014). For instance, Trainotti et al. (2007) highlighted the contribution of ethylene, auxin, and more importantly their interaction in maintaining peach fruit ripening. Further, recent studies have shown the impact of auxin in accelerating the onset of ripening-associated ethylene production in plum and peach, acting at least partially by triggering the expression of several ethylene synthesis and response components (El-Sharkawy et al., 2008, 2009, 2010; Tatsuki et al., 2013).

Several fruit parameters such as firmness, size, weight, TSS, and acidity are used to specify the progression of ripening. However, the significant postharvest losses of fresh fruits due to excessive softening have justified considerable research into the mechanism of fruit softening (Brummell and Harpster, 2001; Li et al., 2010). In plums, a typical climacteric fruit, cultivars considerably vary in their ripening behavior. Some cultivars show a brief ripening pattern associated with rapid decline in firmness, limiting their storage and shelf-life; however, others exhibit notable extension in both processes resulting in relatively firm fruit with prolonged shelf-life. Fruit softening during the ripening process results in part from numerous modifications of the cell-wall architecture, leading to a reduction in intercellular adhesion, depolymerization and solubilization of pectins, depolymerization of hemicelluloses, and loss of pectic galactose side chains (Brummell and Harpster, 2001; Rose et al., 2003; Fry, 2004). These modifications in cell-walls involve the coordinated and interdependent action of many of cell-wall modifying enzymes and proteins (Rose et al., 2003). Thus, investigating the developmental process and signal mechanisms involved in cell-wall protein regulation and fruit softening remain an important area of research (Li et al., 2010). Generally, the decline in fruit firmness due to softening in many fleshy fruits, including plum, is accompanied by elevated expression of numerous cell metabolism enzymes, including polygalacturonase, endo-1,4-β-mannanase, pectin methylesterase, pectate lyase, endo-1,4-β-glucanase, and β-galactosidase (Pressey and Avants, 1973; Carpita and Gibeaut, 1993; Smith and Gross, 2000; Brummell et al., 2004; Hayama et al., 2006; Khan and Singh, 2007) in which all these enzymes have the capacity to reduce the apparent molecular size of pectic polymers by cleaving the backbone or side chain residues (Ranwala et al., 1992; De Veau et al., 1993; Hadfield et al., 1998). Based on enzyme activity and genetic studies, other classes of proteins have been suggested to participate in ripening-related cell-wall disassembly. For instance, it has shown that down-regulation of genes encoding the *N*-glycan processing enzymes α-mannosidase and β-D-*N*-acetylhexosaminidase significantly increased fruit shelf-life (Meli et al., 2010). These enzymes act through breaking the glycosidic bonds between carbohydrates, or between carbohydrates and non-carbohydrate structural molecules (Brummell and Harpster, 2001). Further, phospholipase D-α is a key enzyme involved in membrane deterioration that occurs during fruit ripening and senescence. This enzyme catalyzes the hydrolysis of membrane phospholipids, which maintain cell viability and homeostasis into phosphatidic acid (Dawidowicz, 1987; Exton, 1997). Finally, expansins are cell wall-localized proteins facilitating cell-wall loosening. They are involved in many aspects of cell-wall modification during development through disruption of non-covalent bonds between matrix glycans and cellulose microfibrils (Rose et al., 1997; Rose and Bennett, 1999; Brummell and Harpster, 2001).

In climacteric fruits, although ethylene is the key regulator of the ripening process, not all of the ripening-related events are dependent on ethylene action as some other processes are controlled by other hormonal or developmental factors. For instance, the suppression of ethylene in many fruit species had impact on delay and reduce fruit softening, but was never be able to prevent the occurrence, indicating that the fruit firmness events are not exclusively regulated by ethylene (Murray et al., 1993; Flores et al., 2001; Nishiyama et al., 2007). Apparently, both ethylene-dependent and -independent pathways coexist to coordinate climacteric fruit ripening process (Lelièvre et al., 1997; Alexander and Grierson, 2002).

Several researchers have investigated the role of ethylene in mediating cell-wall metabolism-related gene expression and enzyme activity. However, little is known about the contribution of other ethylene-independent factors that can play an important role as that of ethylene in synchronizing fruit softening process. In this study, the role of auxin and its joined effect with ethylene in coordinating fruit ripening of two plum cultivars that vary in their ripening and shelf-life characteristics was studied. El-Sharkawy et al. (2014) previously showed that the diversity in fruit ripening behaviors between the early- (EG) and late-ripening (V9) cultivars (i.e., the same cultivars studied in the present work) was associated with the levels of auxin and ethylene that occurred during fruit maturation and ripening. In this study, the importance of auxin in accelerating ripening through stimulating autocatalytic ethylene production by activation of the transcription of several ethylene signaling components is assessed along with the potential autonomous role of auxin in advancing ripening events by triggering the transcription of several cell-wall disassembly-related genes that contribute to fruit softening independently of ethylene action.

2. Materials and methods

2.1. Plant materials and postharvest treatments

Japanese plum (*Prunus salicina* L.) cultivars Early Golden (EG) and V98041 (V9) were harvested at commercial maturity in 2011 and 2012 from the experimental farm at the Vineland Research and Innovation Center (Vineland Station, ON, Canada). These two cultivars were chosen due to the diversity of their fruit ripening behavior (early and late, respectively) and shelf-life characteristics (short and extended, respectively). Uniform sized fruit from both cultivars were collected from early maturation until a post-climacteric stage of fruit ripening; 50–82 days after bloom (DAB) for EG and 50-128 DAB for V9. Tissue from nine fruit exhibiting similar ethylene production and firmness at every stage were collected and frozen for further analysis.

To evaluate the effect of hormones in fruit ripening, mature fruit of EG (76 DAB) and V9 (108 DAB) were harvested before autocatalytic ethylene production had risen, surface sterilized, and subjected to various treatments, including: 1-naphthalene acetic acid (NAA), propylene (C_3H_6), the ethylene-inhibitor 1-methylcyclopropene (1-MCP), and 1-MCP followed by dipping in NAA as described previously (El-Sharkawy et al., 2014). Water-dipped fruit were used as controls. After assessing ethylene production and flesh firmness at different intervals post-treatment, climacteric V9 fruit from each treatment were collected (9 fruits/replicated treatment, three independent biological replicates). Fruit were sampled ~6 d and ~15 d after treatment for propylene and NAA treatment, respectively. In case of treatments with no alteration in ethylene emission as MCP and MCP/NAA treated fruit, samples were collected ~24 d after treatment. All samples were frozen and stored for further analysis.

2.2. Fruit shelf-life assessment and other quality traits determination

To determine shelf-life characteristics, fruit of similar size from both cultivars were harvested at commercial maturity (76 and 108 DAB for EG and V9, respectively), surface sterilized, stored at 4 °C for 30 d, and then were transferred to 20 °C. Physical and chemical properties of fruit, such as flesh firmness, weight loss, total soluble

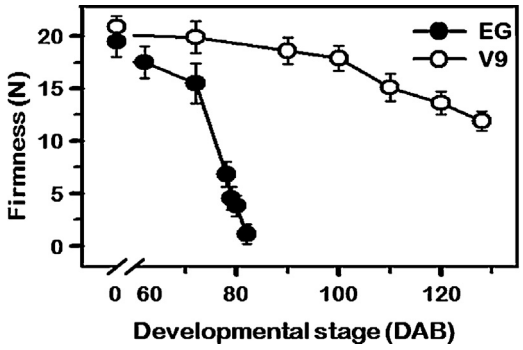

Fig. 1. Changes in firmness during plum fruit ripening for the early- (open circle) and late-ripening (filled circle) cultivars EG and V9, respectively. Values represent the mean ± SD, as derived from 9 fruit. The *x*-axis represents developmental stage, indicated by number of days after bloom (DAB). The *y*-axis refers to fruit firmness (N).

solids (TSS), titratable acidity (TA), and sugar/acid ratio were evaluated, using 20 fruits/replicate sample (three independent biological replicates). Firmness of intact fruit (N) was determined on the opposite sides of the fruit after peel removal using digital penetrometer equipped with an 8 mm diameter probe (FHT200, Extech Instruments, USA). Loss of fruit weight was calculated as percentage of the initial fruit weight at harvest. TSS (%) of the plum juice was measured with a digital refractometer (PR-32 α Palette; Atago, Japan). For titratable acidity evaluation, a 2 mL of fruit juice was titrated with 0.1 M NaOH to pH 8.2, expressed as percentage of malic acid (%). All fruit samples were frozen in liquid N_2 and stored at −80 °C for further analysis. Ethylene production was assessed using gas chromatography in nine independent biological replicates (3 fruits/replicate).

2.3. Nucleic acid extraction and qPCR assays

Total RNA extraction, DNase treatment, cDNA synthesis and qPCR reactions were performed as described previously (El-Sharkawy et al., 2014). Gene-specific primers were designed using Primer Express (v3.0, Applied Biosystems, Carlsbad, CA, USA) (Table S1). Three technical replicates from three biological replicates for each reaction were analyzed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The expression of several genes related to ethylene and auxin signaling, including ethylene-biosynthesis (*ACS* and *ACO*), perception (ethylene-receptors, *ETR*-like), signal transduction elements (ethylene response factor, *ERF*), and auxin-receptors (*ABP1*, *GLP*-like, and *TIR1*-like) transcripts (El-Sharkawy et al., 2007, 2008, 2009, 2010, 2012, 2014) were assessed during ripening of V9 fruit untreated or treated with hormones. Further, different groups of cell-wall related genes that have been shown to be actively associated with cell-wall disassembly were identified using Phytozome v9.1: Peach Genome website, including five genes encoding α-mannosidase (*α-Man*), three β-D-*N*-acetylhexosaminidase (*β-Hex*), three genes encoding phospholipase D-α (*PLD*), one gene of endo-polygalacturonase (*PG*), pectin methylesterase (*PME*), pectate lyase (*PL*), and expansin (*Exp*) (Wang, 1999; Laxalt et al., 2001; Hayama et al., 2006; Meli et al., 2010). Transcript abundance was quantified using standard curves for both target and reference (*PslAct*) genes, which were generated from serial dilutions of PCR products from corresponding cDNAs. The expression level of β-actin (*PslAct*) among different treatments used in this study was assessed using absolute quantitative real-time PCR (qPCR). The qPCR was performed based on the standard curve generated from recombinant plasmids. No significant differences in *PslAct* expression were detected between different treatments and tissue samples (Fig. S1). Thus, it is concluded that *PslAct* could be used as a reliable internal reference gene for qPCR.

3. Results

3.1. Changes in firmness during plum fruit ripening

To assess the shelf-life longevity, the changes in firmness were determined during ripening of early EG and late V9 plum fruit. Flesh firmness of both EG and V9 fruit at harvest was approximately 20 N. Thereafter, EG fruit firmness declined linearly and reached the extreme softness at post-climacteric phase, while V9 fruit exhibited gradual reduction in fruit firmness and the fruit generally remained firm until the end of ripening period (Fig. 1).

3.1.1. Assessment of fruit shelf-life quality

Several fruit quality and physiological variables were evaluated to determine the shelf-life characteristics of both plum cultivars (Table 1). All fruit exhibited very low levels of ethylene production during cold storage, but ethylene increased sharply when fruit were transferred to 20 °C. In V9 fruit, cold storage reduced the fruit softening process rate; however, considerable loss in EG fruit

Table 1
The differences in fruit firmness, weight loss, TSS, acidity and TSS/acidity between early-ripening (EG) and late-ripening (V9) plum cultivars at harvest and during shelf-life at 20 °C after storage for 30 d at 4 °C.

Fruit characteristics	Cultivar	Shelf-life at 20 °C				
		Harvest	0[a]	4[a]	8[a]	12[a]
Fruit firmness (N)	EG	19.5 ± 1.9	6.9 ± 1.2**	4.5 ± 1.1**	2.1 ± 0.8**	0.8 ± 0.4**
	V9	18.9 ± 1.3	17.9 ± 1.2^{NS}	15.1 ± 1.5**	13.6 ± 0.9**	9.9 ± 1.2**
Weight loss (%)	EG	0	8.8 ± 1.3**	13.0 ± 1.5**	14.1 ± 1.6**	18.2 ± 1.4**
	V9	0	3.1 ± 0.6**	3.4 ± 0.7**	4.9 ± 0.9**	5.3 ± 1.1**
TSS (%)	EG	13.0 ± 0.16	15.6 ± 0.19**	19.2 ± 0.08**	17.1 ± 0.15**	16.5 ± 0.1**
	V9	9.5 ± 0.31	11.4 ± 0.12**	13.1 ± 0.14**	14.4 ± 0.13**	16.6 ± 0.19**
Acidity (%)	EG	1.32 ± 0.11	1.07 ± 0.12*	0.92 ± 0.12**	0.8 ± 0.15**	0.62 ± 0.16**
	V9	1.69 ± 0.08	1.40 ± 0.13*	1.31 ± 0.14**	1.24 ± 0.13**	1.16 ± 0.18**
TSS/acidity	EG	9.8 ± 0.24	14.6 ± 0.19**	20.9 ± 0.25**	21.4.0 ± 0.3**	26.6 ± 0.27**
	V9	5.6 ± 0.19	8.1 ± 0.17**	10.0 ± 0.19**	11.6 ± 0.18**	14.3 ± 0.13**

Shelf-life fruit characteristics of the EG and V9 plum cultivars. The measurements are the means (±SD) of 20 fruits. Measurements were taken in two growing seasons. Statistically significant differences from the fresh harvested fruit are indicated by (*) and (**) for the probability levels ($P < 0.05$) and ($P < 0.01$), respectively. NS, non-significant ($P > 0.05$).

[a] Number of days at 20 °C after removing from cold storage (30 d at 4 °C).

firmness occurred. All fruit showed continuous decline in firmness during subsequent storage at 20 °C, but the rate of decline was slower in V9 than in EG. During the 12 d storage period, EG fruit firmness decreased from 6.9 N to 0.8 N and a number of fruit started rotting by the end; however in V9, firmness was reduced only from 17.9 N to 9.9 N. Further, the amount of weight loss increased during cold storage and shelf-life of both plum cultivars. TSS increased as the fruit approached maturity and throughout the storage period, but in two different manners. In EG, TSS were initially increased to a peak after 4 d of storage and declined afterward; however, those of V9 continued accumulating throughout storage duration. TA of both plum cultivars was relatively high at harvest and declined progressively with the advance in storage duration. In general, V9 displayed higher TA content than in EG and its degradation during storage was much slower. When the sugar/acid ratio was evaluated, a significant increase of the ratio from the initial levels at harvest was observed during cold storage and subsequent shelf-life of both plum cultivars; however, the rate of the increase was significantly faster during the shelf-life storage at 20 °C.

3.2. Expression of cell-wall associated genes at harvest and during shelf-life

To understand the regulation of fruit softening during shelf-life, the transcription levels of a number of genes that have been shown to alter cell-wall disassembly in fleshy fruit was assessed during shelf-life storage (Fig. 2). In the course of storage at 20 °C, EG fruit exhibited a dramatic increase in all studied genes along with the progression of fruit shelf-life, excluding *α-Man4, β-Hex2, PME, PL*, and *Exp* that were expressed at nearly constant levels with significant increase in *α-Man4* and *Exp* by the end of storage period. In V9, the expression patterns of all transcripts had the same trend as in EG, but in much lower levels. Clear differences between the two cultivars in the accumulation profile of *α-Man5, β-Hex2, β-Hex3, PLD2, PLD3* and *PG* mRNAs were detected. The transcripts of *α-Man5*, *β-Hex3, PLD2*, and *PG* were expressed at basal constant levels. The *β-Hex2* transcription decreased along with the progression of storage period, whereas that of *PLD3* peaked at 6 d storage and declined thereafter. However, transcripts of *PME, PL*, and *Exp* were almost undetectable.

3.3. Dissecting the role of auxin-induced ethylene production in climacteric ripening

In view of the natural variations between early EG and late V9 plum cultivars in terms of auxin accumulation, ethylene production, ripening behavior, and fruit shelf-life characteristics (El-Sharkawy et al., 2014 and this study), the question was raised whether the prolonged shelf-life in V9 fruit is partially due to inadequate quantities of auxin necessary to coordinate different aspects of ripening. In order to determine the regulatory mechanism of auxin-induced ethylene production and ripening, the ethylene production and fruit firmness were assessed during the ripening of EG and V9 fruit exposed to different treatments that alter auxin- or ethylene-responses, including NAA, propylene, and 1-MCP. Fruit kept in air were used as control (Fig. 3A and B). Auxin stimulated the autocatalytic ethylene production of both plum cultivars; however, only EG fruit displayed acceleration in the ripening process. Ethylene production in the auxin-treated EG fruit peaked at a high level (6.9 ± 0.54 pmol kg^{-1} s^{-1}, ~44% more than control) and shortly; ~3 d after treatment (~2 d earlier than control). Similarly, an activated climacteric ethylene peak of 2.8 ± 0.52 pmol kg^{-1} s^{-1} (~4-fold more than control) was observed

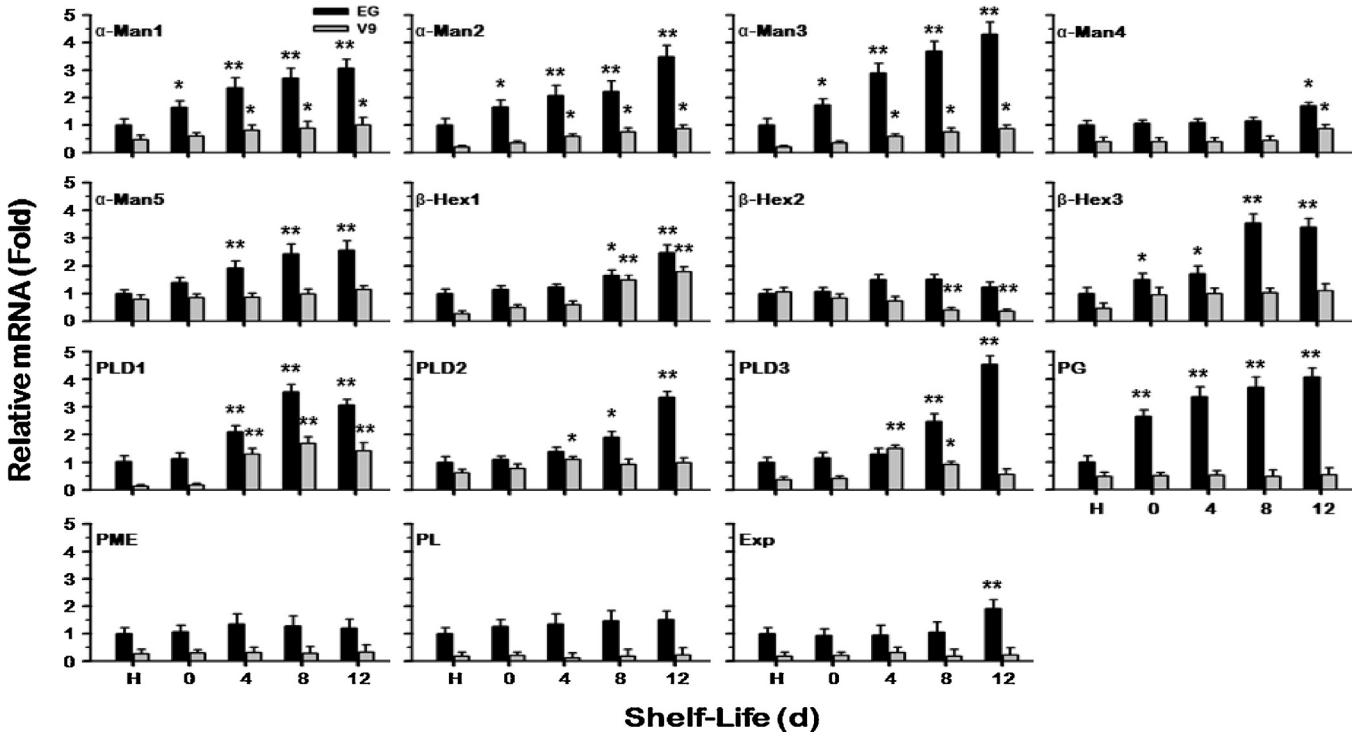

Fig. 2. Steady-state transcript levels of several plum cell-wall disassembly genes, including α-mannosidase (*α-Man*), β-D-*N*-acetylhexosaminidase (*β-Hex*), phospholipase D-α (*PLD*), endo-polygalacturonase (*PG*), pectin methylesterase (*PME*), pectate lyase (*PL*), and expansin (*Exp*). Transcripts were assessed by qPCR in EG and V9 fruit at harvest (H) and at different intervals during shelf-life at 20 °C after storage for 30 d at 4 °C. Results represent data from three biological and three technical replicates. Standard curves were used to calculate the number of target gene molecules per sample. These were then normalized relative to *PslAct* expression. Error bars represent standard deviation. The *y*-axis refers to the fold change in the target gene levels relative to its levels at harvest. The *x*-axis in each figure represents the number of days during storage at 20 °C. Statistically significant differences from EG and V9 fruit at harvest (H) are indicated by (*) and (**) for the probability levels ($P < 0.05$) and ($P < 0.01$), respectively.

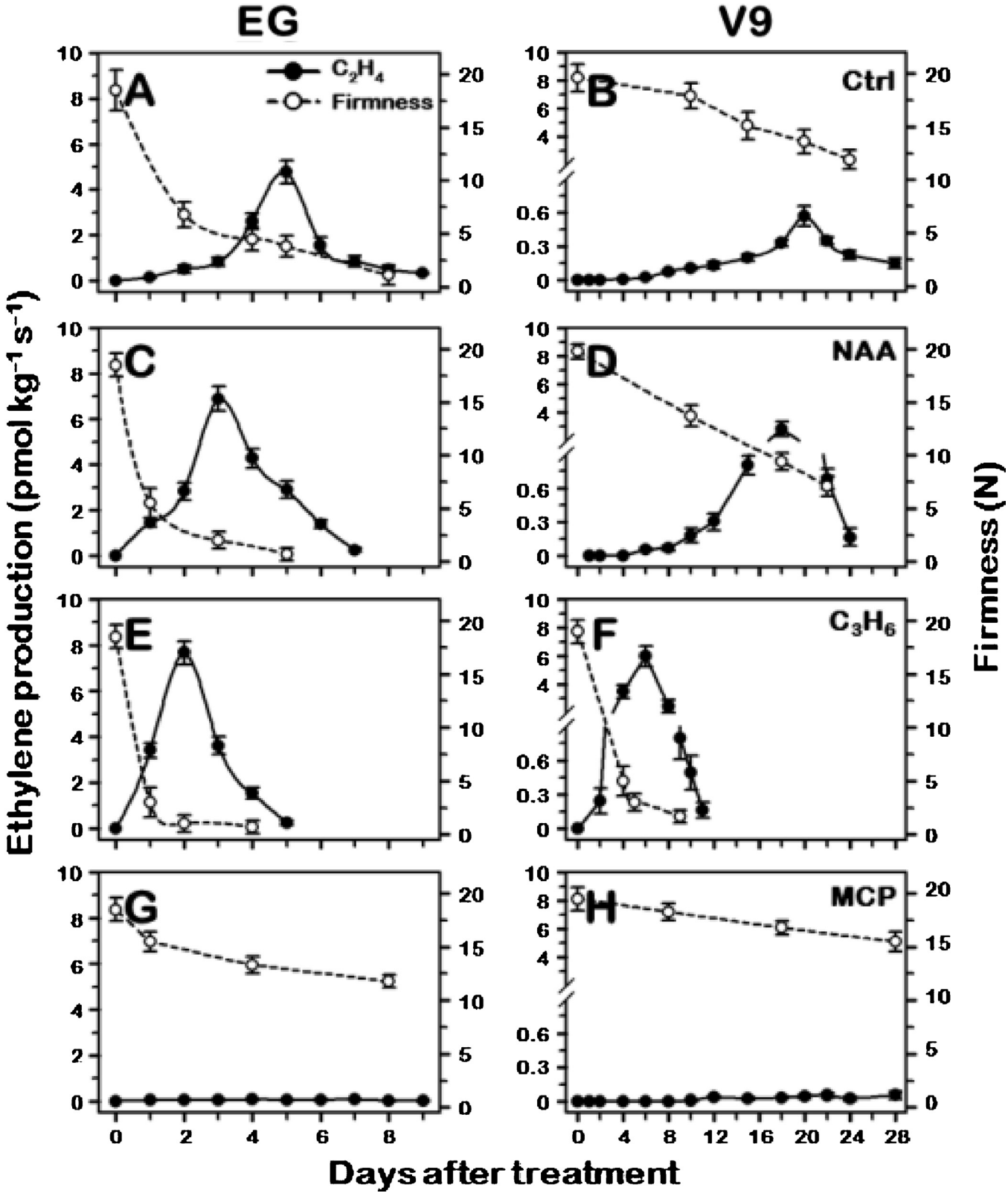

Fig. 3. Changes in ethylene production (filled circle) and fruit firmness (open circle) in control EG and V9 fruit (A and B) and in fruit treated with NAA (C and D), propylene (E and F), or 1-MCP (G and H). Fruit were stored at 20 °C after respective treatments. Values represent the mean ± SD, as derived from 9 fruit. The *x*-axis represents the number of days after treatment. The *y*-axis in left- and right-side refers to ethylene levels and fruit firmness, respectively.

in auxin-treated V9 fruit and this peak occurred ~18 d after treatment (~2 d earlier than control) (Fig. 3C and D). Fruit from both cultivars treated with propylene were characterized by rapid and brief ripening profiles in association with increased ethylene levels. Maximal levels of ethylene were observed at ~2 d (~3 d earlier than control) and ~6 d (~14 d earlier than control) post-treatment with a correspondingly increased ethylene production of 7.7 ± 0.51 (~61% more than control) and 6 ± 0.72 pmol kg^{-1} s^{-1}

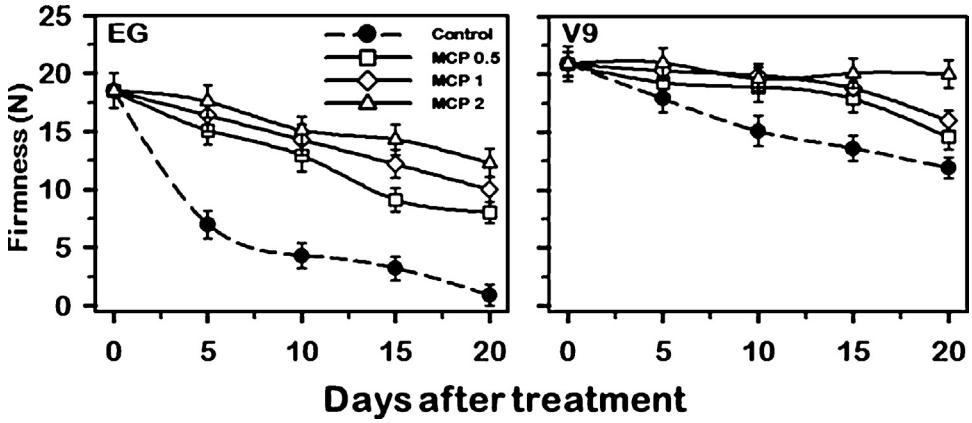

Fig. 4. Effect of 1-MCP concentration on the firmness of EG and V9 fruit during ripening. Fruit were treated with three different concentrations of 1-MCP (0.5, 1, and 2 $\mu L\,L^{-1}$) for 24 h at 20 °C. Then fruit were stored on air and assessed for firmness at different intervals during storage. Values represent the mean ± SD, as derived from 9 fruit. The *x*-axis represents the number of days after treatment. The *y*-axis refers to fruit firmness (N).

(~10-fold more than control) for EG and V9, respectively (Fig. 3E and F). As expected, all fruit treated with the ethylene response inhibitor; 1-MCP, were unable to ripen autonomously and their ethylene production remained low (Fig. 3G and H).

Further, the assessment of firmness in treated fruit indicated that the alteration in flesh firmness correlated well with the changes of ethylene production due to treatments (Fig. 3). Relative to control, auxin treatment triggered the loss of firmness in both plum cultivars, but EG fruit softened more quickly and were ~8-fold softer than V9 auxin-treated fruit at fully ripe stage. Likewise, propylene treatment caused a sharp decline in fruit firmness with no significant difference between the two plum cultivars. Although 1-MCP treatment considerably reduced the rate of fruit softening in both plum cultivars, significant loss of firmness still occurred during postharvest storage.

In order to determine the exact effect of ethylene on firmness, fruit from both plum cultivars were treated with three different concentrations of the ethylene inhibitor; 1-MCP (0.5, 1, and 2 $\mu L\,L^{-1}$). Ethylene production and fruit firmness were assessed in the treated fruit during 20 d of postharvest storage. All treated fruit produced no ethylene and did not ripen due to the treatment (data not shown). Similarly, the treatment caused significant inhibition in fruit softening of both plum cultivars, but the fruit remained able to lose firmness. However, the profile of 1-MCP-reduced fruit softening was dependent on the dosage used, since the higher concentration of 1-MCP resulted in lower rate of fruit softening. However, in V9 fruit treated with 2 $\mu L\,L^{-1}$ of 1-MCP, no apparent changes in fruit softening were observed (Fig. 4).

3.4. Hormone application alters the expression of ethylene- and auxin-related genes

In order to strengthen understanding of the independent and mutual contributions of auxin and ethylene in coordinating climacteric ripening, the accumulation of several ethylene and auxin signaling components were assessed during ripening of V9 fruit untreated or treated with hormones (Figs. 5 and 6). EG fruit were not used for this study due to the major effect of endogenous

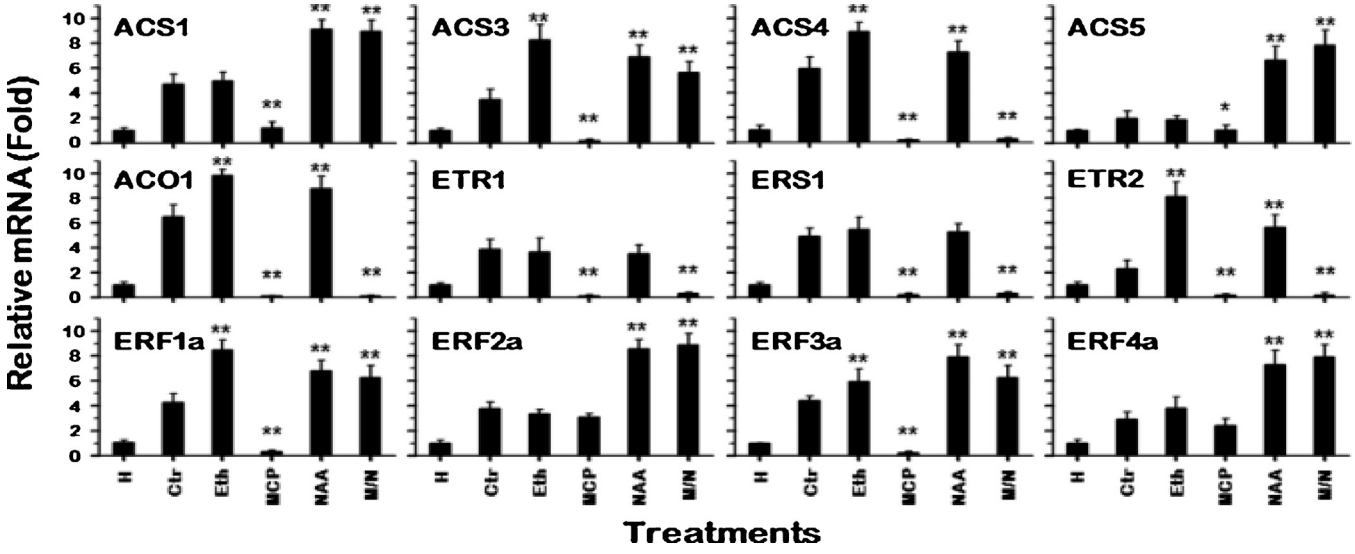

Fig. 5. Steady-state transcript levels of several plum genes involved in ethylene signaling, including four 1-aminocyclopropane-1-carboxylate synthase (*ACS*), one 1-aminocyclopropane-1-carboxylate oxidase (*ACO*), three ethylene-receptors (*ETR1/ETR2*-like), and four ethylene response factor (*ERF*). Transcripts were assessed by qPCR in mature non-climacteric V9 fruit at harvest (H) and climacteric fruit ripened in air (Ctr), propylene-treated climacteric fruit (Eth), MCP-treated fruit (MCP), auxin-treated climacteric fruit (NAA), and fruit treated with MCP and NAA (M/N). All fruit were stored at 20 °C until sampling. Results represent data from three biological and three technical replicates. The *y*-axis refers to the fold change in the target gene levels relative to its levels at harvest (H). Statistically significant differences between control climacteric fruit (Ctr) and climacteric fruit from different treatments are indicated by (*) and (**) for the probability levels ($P < 0.05$) and ($P < 0.01$), respectively. The *x*-axis in each figure represents the different treatments.

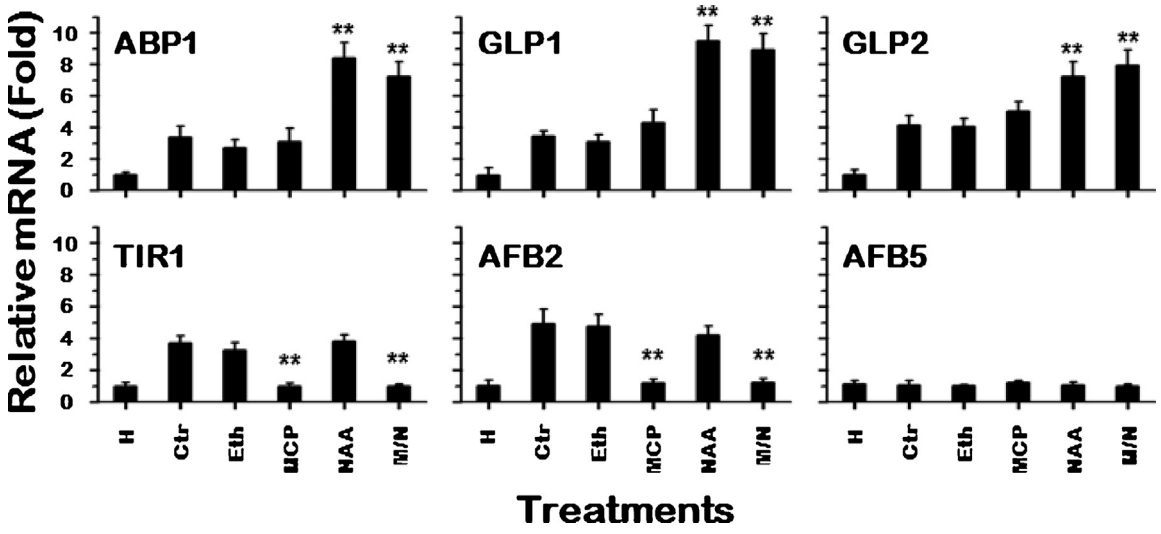

Fig. 6. Steady-state transcript levels of several plum genes putatively encode auxin-receptors, including one auxin-binding protein1 (*ABP1*), two germin-like protein (*GLP*), and three F-box auxin-receptors (*TIR1*-like). Other details as in Fig. 5.

high levels of auxin content and ethylene production (El-Sharkawy et al., 2014). Among all studied transcripts, only *AFB5* did not show any alteration during fruit ripening or due to treatments, indicating that this gene is not involved in the regulation of plum fruit ripening. Analysis of expression data classified the diverse transcripts based on their responses to different treatments into four main groups. *ETR1*, *ERS1*, *TIR1*, and *AFB2* belong to group-1 that appear to be ripening-induced genes, but insensitive to both hormones. Although the transcription levels of group-1 members were down-regulated by MCP, propylene treatment did not cause any obvious change in their transcription levels, compared to air-ripening control fruit (Ctr). Group-2 includes all mRNAs that greatly accumulated in an auxin-dependent manner, with no visible response to any of ethylene-related treatments (*ACS1*, *ACS5*, *ERF2a*, *ERF4a*, *ABP1*, *GLP1*, and *GLP2*). The third group contains all transcripts upregulated in both auxin- and ethylene-dependent manners, including *ACS3*, *ERF1a*, and *ERF3a*. Although the expression profile of *ACS4*, *ACO1*, and *ETR2* seems to be ethylene/auxin-dependent, the dual treatments of MCP/NAA, generated this fourth group that holds only transcripts accumulated in an ethylene-dependent manner.

3.5. Cell-wall associated genes differentially responded to hormone application

To establish the regulatory mechanism(s) of fruit softening during ripening, the expression of the different cell-wall associated genes was quantified in the firm V9 fruit exposed to hormones treatments; however, air-ripened climacteric fruit (Ctr) were used as control (Fig. 7). The expression of the diverse cell-wall metabolism genes was differentially altered in response to hormones application. Analysis of expression data divided the

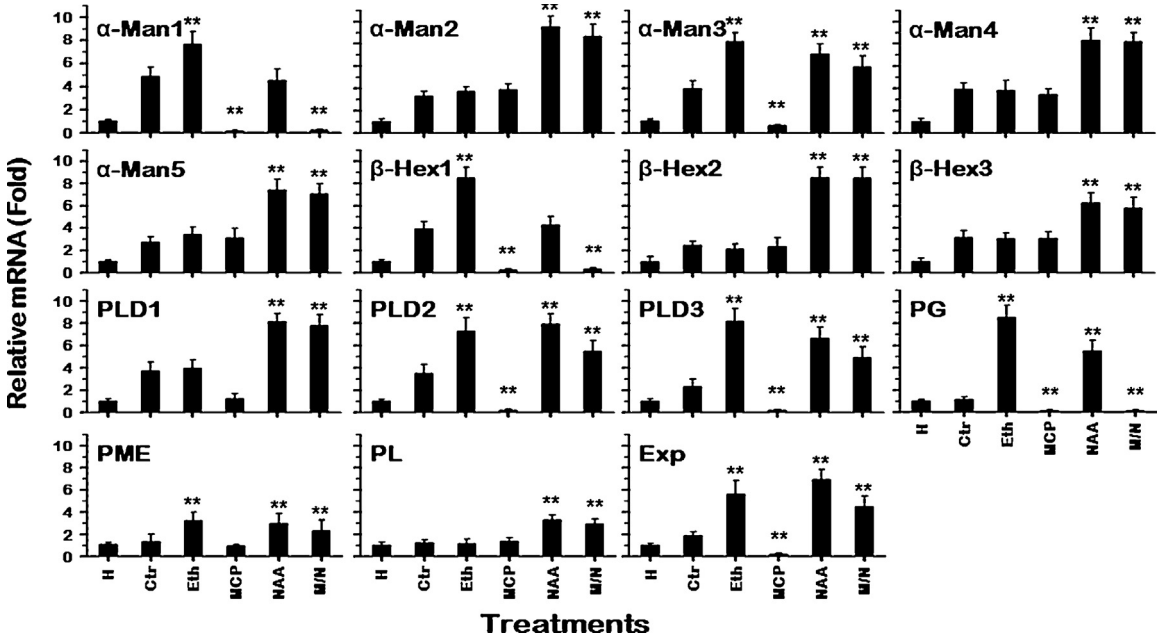

Fig. 7. Steady-state transcript levels of several plum cell-wall disassembly genes, including five genes encoding α-mannosidase (*α-Man*), three β-D-*N*-acetylhexosaminidase (*β-Hex*), three genes encoding phospholipase D-α (*PLD*), one gene of endo-polygalacturonase (*PG*), pectin methylesterase (*PME*), pectate lyase (*PL*), and expansin (*Exp*). Other details as in Fig. 5.

various transcripts based on their responses to different treatments into three main groups. Group-1 comprises the ethylene-induced transcripts with no sensitivity to auxin presence (*α-Man1*, *β-Hex1*, and *PG*). The second group contains all transcripts up-regulated in an auxin-dependent manner and this includes large number of the studied genes (*α-Man2*, *α-Man4*, *α-Man5*, *β-Hex2*, *β-Hex3*, *PLD1*, and *PL*). Finally, the last group represents the genes greatly abundant in response to both ethylene and auxin treatment, including *α-Man3*, *PLD2*, *PLD3*, *PME*, and *Exp*.

4. Discussion

Fruit ripening involves well-orchestrated coordination of several regulatory steps that bring about subtle changes to the physiological traits of fruit ripening. With the progression of ripening, complex carbohydrates are converted to simple sugars, the acidity declines with the accumulation of sugar, the aroma compounds accumulate, the color changes due to anthocyanin accumulation, and cell-wall dynamics change leading to either dehiscence or softening (Klee and Giovannoni, 2011). The above ripening-related parameters involve initiation of multiple genetic and biochemical pathways. However, the molecular hierarchy of their regulators remains to be ascertained. Although, the ripening-related changes have been observed in the context of response to various hormones, ethylene remains the typical ripening promoting hormone due to its predominant role in climacteric fruit ripening (Lelièvre et al., 1997; Giovannoni, 2004). Results in this study show an important linkage in auxin and ethylene levels during ripening of early EG and late V9 plum fruit. The evaluation of auxin content and ethylene production in plum fruit (i.e., the same cultivars studied in the present work) revealed that the elevated auxin levels during EG fruit ripening are associated with high climacteric ethylene production and brief ripening process (El-Sharkawy et al., 2014). In contrast, low auxin levels in V9 are associated with suppressed climacteric ethylene profile and an extended ripening process. During storage EG displayed more rapid fruit senescence than V9, as determined by TSS accumulation, acidity degradation, weight loss, and, most importantly, fruit softening that markedly limited the fruit storability and shelf-life. Consequently, EG fruit exhibited short shelf-life duration; however, V9 fruit showed extended shelf-life along with preserved fruit quality characteristics.

EG fruit displayed rapid fruit softening during shelf-life, a pattern that occurred late and at a much slower rate in V9. Interestingly, the accumulation profile of several genes involved in cell-wall disassembly was well correlated with the fruit softening pattern of both plum cultivars during shelf-life storage. In EG, their transcription steadily increased along with the progression in storage duration; however in V9, they were either almost undetectable, or constantly low, or showed minimal increase. Ethylene and its biosynthetic components have been shown to be involved, at least in part, in the regulation of fruit softening and maintenance of shelf-life in several fleshy fruits (Xiong et al., 2005; Hayama et al., 2006; Khan and Singh, 2007; Nishiyama et al., 2007; Lopez-Gomez et al., 2009). However, both ethylene-dependent and ethylene-independent pathways were demonstrated to be involved in the regulation of cell-wall related genes and fruit softening by Pech et al. (2008) and in this study. The results herein demonstrated that auxin could be a significant part of the network of mechanisms that control the capacity of the fruit to ripen and soften. In both cultivars, treatment with auxin elicited an enhanced climacteric ethylene and accelerated the degradation in fruit firmness comparable to that observed in fruit treated with propylene. The changes in ripening profile due to hormone application suggested that the difference in ripening pattern between the two plum cultivars could be partially due to the variation in auxin content. This notion is supported by the observations of the concomitant increase of auxin and ethylene during EG and V9 ripening (El-Sharkawy et al., 2014), and the stimulatory accumulation of ethylene elements in several fleshy fruits by auxin and vice versa (Gillaspy et al., 1993; Agustí et al., 1999; Jones et al., 2002; Bregoli et al., 2007; Trainotti et al., 2007; Tatsuki et al., 2013). Importantly, auxin further showed an ability to regulate several cell-wall modifying genes critical in controlling fruit softening. It was suggested that auxin can indirectly accelerate fruit softening via stimulating the transcription of several ethylene elements, which consequently increases the ethylene production, resulting in ethylene-induced fruit softening events (Tatsuki et al., 2013). Results in this study added more strength to this mechanism by demonstrating the direct involvement of auxin in advancing the ripening process independent of ethylene action. Apparently, auxin acts upstream of ethylene to not only control the initiation of autocatalytic ethylene and the transition of a fruit from the end of growth into the onset of ripening, but also to start other ripening-related events such as fruit softening. Thereby, the high auxin levels in EG (El-Sharkawy et al., 2014) should accelerate fruit ripening due to the accumulation of auxin-, ethylene-, and many other ripening-related genes in an auxin-dependent, ethylene-independent manner. Once autocatalytic ethylene production is initiated, the ripening process will progress in both auxin- and ethylene-dependent manners, as the transcripts of some ethylene components and cell-wall disassembly elements are still present in considerable levels in ethylene deprived tissues such as MCP- and MCP/NAA-treated fruit. Therefore, the finding that many ripening-related genes are regulated exclusively by auxin is the best alternative that explains the significant accumulation of such transcripts during ripening in an ethylene-independent manner (El-Sharkawy et al., 2008, 2009, 2014; Ziliotto et al., 2008; Kumar et al., 2014). Under these circumstances, the scarcity of auxin in V9 cultivar might influence the capacity of the fruit to progress in ripening, including softening, resulting in the differentiation in ripening behavior and shelf-life characteristics thereafter.

5. Conclusion

Auxin influences multiple steps in the initiation and progression of plum fruit ripening and softening. The notion is supported by the autonomous regulation of several ethylene components and cell-wall disassembly transcripts by auxin. These findings add information to the growing body of knowledge concerning one of the ethylene-independent mechanisms that mediate climacteric fruit ripening.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.postharvbio.2015.09.012.

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Supplemental information

Table S1. The oligonucleotide primers.

Name	Oligonucleotide sequence
1-PslAct(FQ)[a]	**5'**-CTGGACCTTGCTGGTCGT-**3'**
2-PslAct(RQ)[a]	**5'**-ATTTCCCGCTCAGCAGTG-**3'**
Cell-wall-related genes	
3-PslMan1(FQ)	**5'**-TGACGTGGAAAGTGGAAGG-**3'**
4-PslMan1(RQ)	**5'**-GAGCAACATCCACCACCAG-**3'**
5-PslMan2(FQ)	**5'**-CTCCTCTTCCAACCCCAAA-**3'**
6-PslMan2(RQ)	**5'**-GGCCGATGGTGAAGAAGTT-**3'**
7-PslMan3(FQ)	**5'-**TGATTCAAATGGGCGTGAT-**3'**
8-PslMan3(RQ)	**5'-**ATTTCCCGCAATAGGTTGG-**3'**
9-PslMan4(FQ)	**5'**-GGTGAAGCGCTTGATGAAA-**3'**
10-PslMan4(RQ)	**5'**-CCAGCCCCTACCTGGTTTA-**3'**
11-PslMan5(FQ)	**5'**-GCCTCTGGGGCATATGTTT-**3'**
12-PslMan5(RQ)	**5'**-GACCGCGCATGACAGTTAG-**3'**
13-PslHex1(FQ)	**5'**-CCATTGAACCCCAAGACCT-**3'**
14-PslHex1(RQ)	**5'**-CAGCTCCAGCGTGGTAAAA-**3'**
15-PslHex2(FQ)	**5'-**GGGAGCGCTACACAGTTGA-**3'**
16-PslHex2(RQ)	**5'-**TGCATGACCAGGGACATCT-**3'**
17-PslHex3(FQ)	**5'**-CTCAACCCATTGTTTGTGAT-**3'**
18-PslHex3(RQ)	**5'**-GGTATGGTGCTTTGTTGTT-**3'**
19-PslPLD1(FQ)	**5'**-GCTGCATTTGGATTTCCTG-**3'**
20-PslPLD1(RQ)	**5'**-GCATCCTGAATGCTTCGAT-**3'**
21-PslPLD2(FQ)	**5'-**GGATCTGCCAACATCAACC-**3'**
22-PslPLD2(RQ)	**5'-**TCCCTGACCGACAGATGAT-**3'**
23-PslPLD3(FQ)	**5'**-ATCCTTGGATCTGCCAACA-**3'**
24-PslPLD3(RQ)	**5'**-AATGTTCAGGCTGGAATGC-**3'**
25-PslPG(FQ)	**5'**-CTTGTGCCTCGATGAATCCC-**3'**
26-PslPG(RQ)	**5'**-TTGCAAGGCCCACTAAACAC-**3'**
27-PslPME(FQ)	**5'**-TAGCGGCAGAGGGTTCATAG-**3'**
28-PslPME(RQ)	**5'**-CTTAGTGCCACTGCTTGGTG-**3'**
29-PslPL(FQ)	**5'**-CAAGCCCACAGGAAATGCTT-**3'**
30-PslPL(RQ)	**5'**-GCATCACCATCAGCCATTGT-**3'**
31-PslExp(FQ)	**5'**-ATCAAGTGTGCAAACGACCC-**3'**
32-PslExp(RQ)	**5'**-GTTTGGAGGGCAGAAGTTGG-**3'**
Ethylene-related genes	
33-PslACS1(FQ)[b]	**5'**-ACCGCGTTCACGTTGTTT-**3'**
34-PslACS1(RQ)[b]	**5'**-TGCCACAACCATGTCGTC-**3'**
35-PslACS3(FQ)[b]	**5'**-GTGCAACTGCAGCCAATG-**3'**
36-PslACS3(RQ)[b]	**5'**-GGTTGGCACAAGCAAAGC-**3'**
37-PslACS4(FQ)[b]	**5'-**GCGTCGATGCTCTTGGAT-**3'**
38-PslACS4(RQ)[b]	**5'-**AAAACCCCGTGCCTCTTC-**3'**
39-PslACS5(FQ)[b]	**5'**-GATCTTGGATGGCGAACG-**3'**
40-PslACS5(RQ)[b]	**5'**-AGCCTCCAAGGCTGCTCT-**3'**
41-PslACO1(FQ)	**5'**-GAGCACAGAGTGATTGCCCA-**3'**
42-PslACO1(RQ)	**5'**-GCATCACTGCCAGGGTTGTA-**3'**
43-PslETR1(FQ)[a]	**5'**-CAACCGCGCTTCAAAAAT-**3'**
44-PslETR1(RQ)[a]	**5'**-CCTGGCATGTGCTTTCCT-**3'**
45-PslERS1(FQ)[a]	**5'**-CCAAATGGCACCTCAACC-**3'**
46-PslERS1(RQ)[a]	**5'**-GGTCTGTGCGCAATGAGA-**3'**
47-PslETR2(FQ)	**5'**-ACCGCTGGAATAGTGCCCTCTGA-**3'**
48-PslETR2(RQ)	**5'**-TTCGGGTTGCTTCGGATAATGG-**3'**

Table S1. The oligonucleotide primers.

Name	Oligonucleotide sequence
49-PslERF1a(FQ)[c]	**5'-**GGAACAGACGCGGATATCACA-**3'**
50-PslERF1a(RQ)[c]	**5'-**GATCCGTCTCGCCCAGCAA-**3'**
51-PslERF2a(FQ)[c]	**5'**-TGCTGAAATCCTCTGCTCCAT-**3'**
52-PslERF2a(RQ)[c]	**5'**-GCTTCCCTGATCAGAGCTGAA-**3'**
53-PslERF3a(FQ)[c]	**5'**-TGCGACTCGTCGTCTTCA-**3'**
54-PslERF3a(RQ)[c]	**5'**-CCAACTCCCCCTCCTCAT-**3'**
55-PslERF4a(FQ)	**5'**-TCAGAGATCCCGGGAAGAAGA-**3'**
56-PslERF4a(RQ)	**5'**-CCTCCTCGGCAGTGTCAAAG-**3'**
Auxin-related genes	
57-PslABP1(FQ)[e]	**5'**-CACGAGAAGTCCCCTGGA-**3'**
58-PslABP1(RQ)[e]	**5'**-GGACCTGGTGAGCATCGT-**3'**
59-PslGLP1(FQ)[d]	**5'**-GCCCAATTTCCTGGTGTG-**3'**
60-PslGLP1(RQ)[d]	**5'**-CGGGGTGAGTGTGAAAGG-**3'**
61-PslGLP2(FQ)[d]	**5'**-CCCGGGTCTCCAAATTCT-**3'**
62-PslGLP2(RQ)[d]	**5'**-CCCCCAAGAACACCCTTC-**3'**
63-PslTIR1(FQ)[f]	**5'**-CTCTCGGGATGCAAGGAA-**3'**
64-PslTIR1(RQ)[f]	**5'**-TGATGTCAGCCCAGAGCA-**3'**
65-PslAFB2(FQ)[f]	**5'**-GACGCTGTCACCATGCAG-**3'**
66-PslAFB2(RQ)[f]	**5'**-CAGTCAAAAGGCCGGAGA-**3'**
67-PslAFB5(FQ)[f]	**5'**-TCTCCGCCGTTTGATCTCA-**3'**
68-PslAFB5(RQ)[f]	**5'**-CAGTTCCGGGCCTTGTTG-**3'**

The primer sequences marked with a, b, c, d, e, and f have been reported previously by El-Sharkawy et al., 2007, 2008, 2009, 2010, 2012, and 2014; respectively.

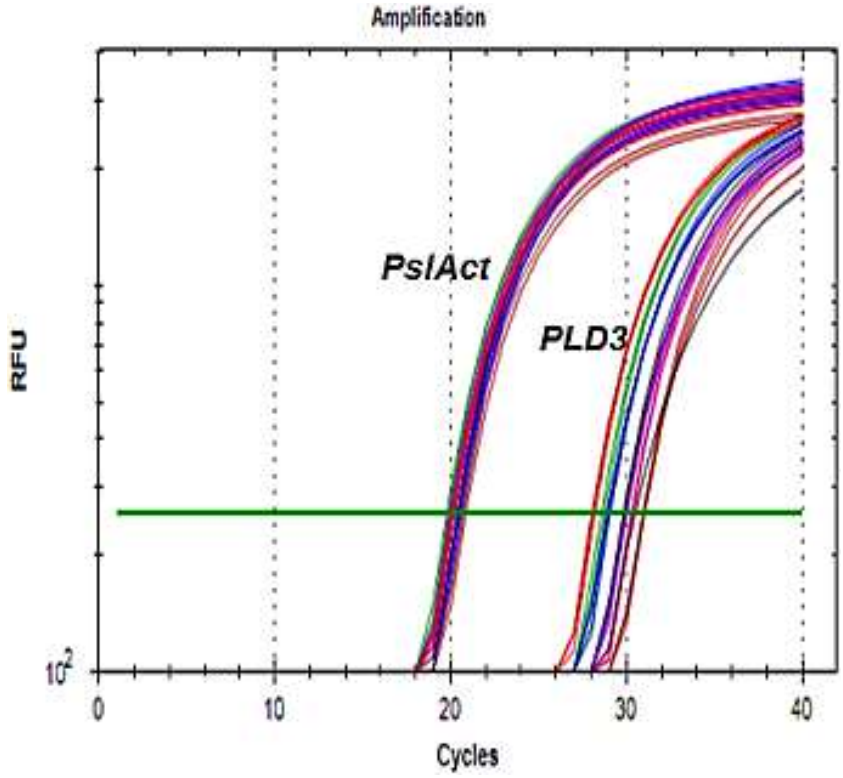

Fig. S1: The uniformity of *β-actin* expression in different samples. The qPCR amplification plot for *Actin* and *PLD3* transcripts in cDNAs generated from plum fruit. *X*-axis shows the number of PCR cycles and the *Y*-axis shows the relative fluorescent units (RFU). The amplification plot was generated for seven cDNA samples and three biological replicates for each sample.