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Full Length Research Paper

Effect of para-chlorophenoxyacetic acid on acid invertase gene expression and sucrose metabolism in tomato (*Solanum lycopersicum*) fruit

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Tomato cv. Liaoyuanduoli (*Solanum lycopersicum*) plants were cultivated in a greenhouse to allow sampling of the second fruit in the first cluster and comparison with tomato fruit that developed following para-chlorophenoxyacetic acid (PCPA) treatment. Sugar content, activities of sugar related enzymes and the effects of PCPA treatment on gene expression of soluble acid invertase (AI) during tomato fruit development were studied. Enhanced activity of AI and increased gene expression of soluble AI in pectinic tissues, pericarp and dissepiments were observed during fruit development, resulting in an increase in fructose and glucose levels. Following PCPA treatment, the activity of AI was amplified and gene expression of soluble AI was accelerated during the mature period of fruit so that fructose and glucose were increased. Upon ripening and following PCPA treatment, the activity of AI and gene expression of soluble AI in pectinic tissues were more pronounced than those in pericarp and dissepiments, but the concentrations of fructose and glucose in pectinic tissues were lower than in pericarp and dissepiments. PCPA appeared to affect vacuolar acid invertase activity by regulating corresponding gene expression, resulting in hexose accumulation in ripening tomato fruit.

Key words: Para-chlorophenoxyacetic acid (PCPA), tomato, acid invertase, sucrose metabolism.

INTRODUCTION

Sugar content is an important factor in determining tomato fruit quality and taste (Wang et al., 1993; Yelle et al., 1991). Most sugars in the fruit are derived from leaf photosynthates and are delivered to the fruits in the phloem. In tomato plants, sucrose represents the major transport form of photoassimilates and is translocated through the phloem from sources to the developing fruit (Walker and Ho, 1977; Ruan and Patrick, 1995; Patrick, 1997). However, fructose and glucose are the predominant sugar in the ripening tomato fruit rather than sucrose (Davies, 1966; Davies and Kempton, 1975; Cui et al., 2006a). Therefore, regulation of sugar accumulation occurs by regulating sucrose metabolism in tomato fruit (Yelle et al., 1991; Cui et al., 2006b). Sucrose is hydrolyzed by acid invertase (AI) into fructose and glucose for entry into sink tissue metabolism (Klann et al., 1993). Thus, AI is a key enzyme in plant growth and development (Zanor et al., 2009; Naegele et al., 2010).

Acid invertase exists in two states; soluble and insoluble, which are encoded by two different gene families (Le Clere et al., 2008, 2010). Different AIs play different roles in various developing stages and tissues in plants. Soluble acid invertase activity has been shown to be closely correlated with growth and cell expression in a number of systems (Sturm and Tang, 1999; Heyer et al., 2004; Sergeeva et al., 2006; Barratt et al., 2009). In cells of most fruit tissues, the mature vacuole is composed of about 80 to 90% volume of the plant cell and represents

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Abbreviations: PCPA, Para-chlorophenoxyacetic acid; **AI,** acid invertase; **IAA,** indole acetic acid; **PVPP,** polyvinylpolypyrrolidone; **SPS,** sucrose phosphate synthase; **SS,** sucrose synthase; **GA3,** gibberillic acid; **HPLC,** high performance liquid chromatography; **EDTA,** ethylenediaminetetraacetic acid; **DTT,** dithiothreitol; **HEPES,** 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid.

the major organelle for storage of soluble sugars. About 90% of the AI in the plant cell is soluble and accumulates primarily in the vacuole, where it regulates sucrose metabolism and hexose levels. Hence, the levels of gene expression and enzyme activity of soluble acid invertase directly affect sugar accumulation in tomato fruit, in turn influencing fruit quality. Qi et al. (2005) reported that, AI activity at the mature stage in tomato fruit was followed by fructose and glucose accumulation, and that the hexose content of pericarp and dissepiments was higher than that in pectinic tissues. Jiang et al. (2007) reported that, soluble sugar content and AI activity were increased by jasmonic acid treatment.It was also found that, exogenous indole acetic acid (IAA) alone stimulated *Ivr2* (gene of soluble acid invertase in maize) transcript accumulation in non-gravistimulated maize pulvini (Long et al., 2002). However, the effects of other exogenous hormones, such as auxins, on acid invertase gene expression and activity have not yet been reported.

Para-chlorophenoxyacetic acid (PCPA) is one of the plant growth regulators which regulates fertile fruit, significantly enlarges fruit and increases concentration of sugar. In this study, we examined PCPA effects on AI activity and sucrose metabolism to determine its potential contribution as a regulator of tomato fruit quality. We assayed the sugar content, the AI activity and the gene expression of soluble acid invertase during tomato fruit development following PCPA treatment of tomato flowers at anthesis.

MATERIALS AND METHODS

Plant materials

Liaoyuanduoli tomato (*Solanum lycopersicum*) seeds were sown on 19 January, 2008 and seedlings (41days seedlings) were transplanted to a solar greenhouse with array pitch of 50 cm and row spacing 35 cm. The plants were pruned to one branch and growing points were removed above the first cluster fruits. Other management was the same as general practice.

The second flowers of the first cluster were dipped in PCPA with control in distilled water. All treatment and control plants were tagged at anthesis.

Tissue sampling

Pericarp, dissepiments and pectinic tissues of fruits were harvested at 15, 25, 35, 45 days and at the mature stage (55 to 60 days) after anthesis. After harvest, representative portions from whole fruits were frozen in liquid nitrogen and stored at -80°C for later analysis of sugar concentration and enzyme activity. Three replicate samples were collected. Pericarp, dissepiments and pectinic tissues were also harvested at different stages of fruit development, frozen in liquid nitrogen, and stored at -80°C for the analysis of gene expression.

Sugar determinations

Approximately, 2 g fresh weight of frozen tissue was extracted with 80% ethanol. Soluble sugar concentration was determined using a Waters 600E High Performance Liquid Chromatography (HPLC) system. An amino column (Dikma) and a model 2410 refractive index monitor were used. The mobile phase was 75% acetonitrile and ultrapure water (75:25), the flow rate was 1.0 ml min^{-1} and the temperature of the column was 30°C. Sucrose, glucose and fructose were identified by their retention times and quantified according to known standards. Waters millennium software was used for data analysis.

Enzyme extraction and assays

Approximately, 1 g fresh weight of frozen tomato tissue was ground in buffer of three times sample volume in a chilled mortar according to the methods of Wang and Zhang (2000), with slight modification. The buffer contained 50 mM HEPES-NaOH (pH7.5), 1 mM EDTA, 10 mM $MgCl_2$, 2.5 mM DTT, 10 mM ascorbic acid and 5% (w/v) polyvinylpolypyrrolidone (PVPP). Homogenates were centrifuged at 12000 g for 20 min at 4°C and the pellets were discarded. Ammonium sulphate was gradually added to the supernatant to 80% saturation and the solution was again centrifuged at 12000 g for 30 min at 4°C. The supernatant was discarded and the pellet was dissolved in 2 to 5 ml of extraction buffer, then dialyzed against a ten-fold volume of extraction buffer (without PVPP) for 20 h. All steps were carried out at 0 to 4°C.

The activities of enzymes related to sucrose metabolism were assayed in desalted extracts as described by Qi et al. (2005), with minor modifications. Uridine diphosphoglucose (UDPG) and fructose-6-phosphate were purchased from Sigma (St. Louis, MO, USA).

RNA extraction and northern blot analysis

Total RNA was isolated from frozen tomato tissue and northern blot analysis was performed as described by Jiang et al. (2005). The sequences of the primers used were as follows: forward primer, 5'-AACTCCGCCTCTCGTTACACA; reverse primer, 5'-TAGGATGGTAGCGGACCC TG. Probe labelling was performed with DIG high prime DNA labelling and detection starter kit II (Roche).

RESULTS

Effect of PCPA on the sugar content of different tomato fruit parts during development

Pericarp and dissepiments

In para-chlorophenoxyacetic acid (PCPA) treated plants, the fructose and glucose contents in pericarp and dissepiments were slightly higher than those in similar tissues from untreated control tomato fruits 15 days after anthesis. However, no difference was observed between treatment and control groups from 25 to 45 days after anthesis. Fructose and glucose contents were significantly increased in ripening fruits in both the treatment and control groups, but the levels of sugars were much higher in the treatment group than in the control group (Figure 1).

Fruit sucrose content following PCPA treatment was slightly lower in treated plants than in control.

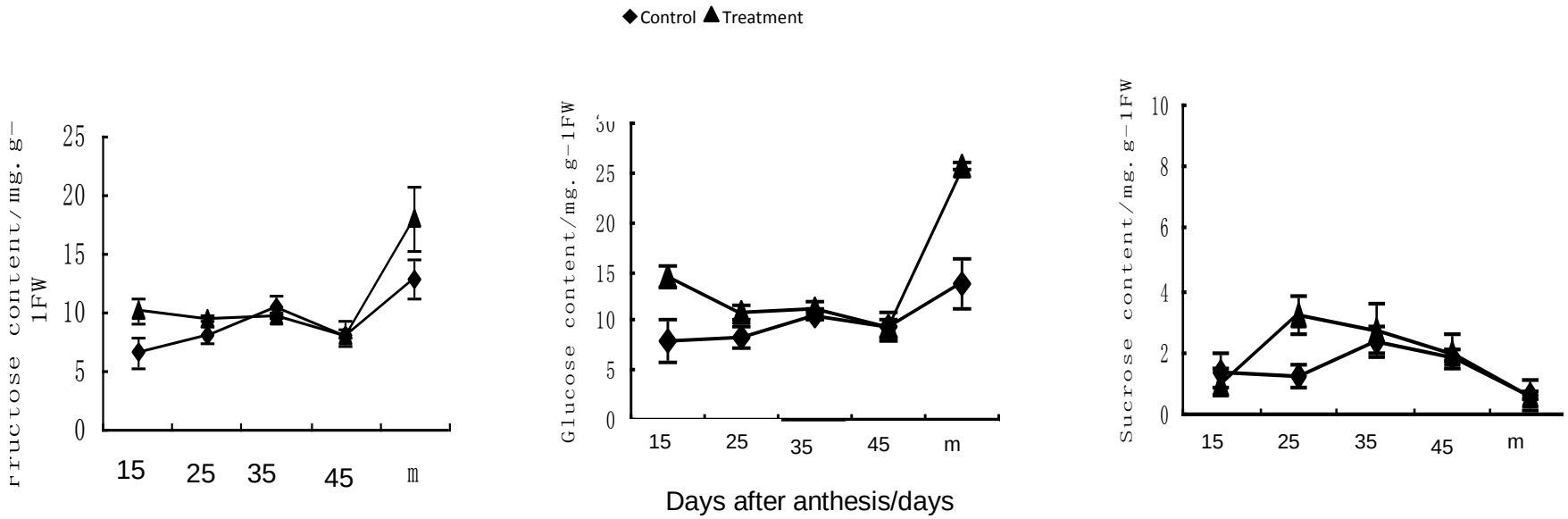

Figure 1. Effects of PCPA on sugar contents in pericarp and dissepiment during tomato fruits development. CK, Control; PCPA, PCPA treatment; m, ripening tomato fruit.

An increase was observed in both treatment and control groups until 35 days after anthesis and during this time, sucrose content of fruit from the treated plants was slightly higher than that in control fruits. No difference was observed between fruits from PCPA treated and control groups during fruit ripening.

Overall, an increasing trend in fructose and glucose contents was observed during fruit development. Fructose and glucose concentrations were increased by PCPA treatment in ripening tomato fruit, while an increasing trend in sucrose content was observed at 35 days after anthesis, followed by a decline in sucrose content in ripening fruit.

Pectinic tissues

An increase in fructose and glucose contents was observed in pectinic tissues during tomato fruit development. No difference in fructose content was observed between the PCPA treated and control groups, while glucose content was slightly higher in the treated group.

An increasing trend in sucrose content was maintained until 35 days after anthesis and sucrose content was slightly higher in the treated group. A slight decline in sucrose content was then observed. The sucrose content was the lowest in ripening fruit (Figure 2).

Effect of PCPA on the enzyme activities related to sucrose metabolism in different fruit parts during development

Pericarp and dissepiments

As shown in Figure 3, acid invertase (AI) activity in pericarp and dissepiments was low prior to 45 days after anthesis except for a spike at 25 days after anthesis and no difference was observed between PCPA treated and control groups. A sharp increase i7n AI activity was observed in ripening fruit and AI activity became higher in the PCPA treated group.

The activity of sucrose synthase in pericarp and dissepiments in the PCPA treated group was lower than that of the control group from 15 to 35 days after anthesis. After 35 days, only a low activity of sucrose synthase (SS) was observed in both PCPA treatment groups and control groups and no difference was observed.

Low levels of sucrose phosphate synthase (SPS) activity were noted during tomato fruit development. A slight decline in SPS activity was observed following PCPA treatment during early stages of fruit development and a slight increase

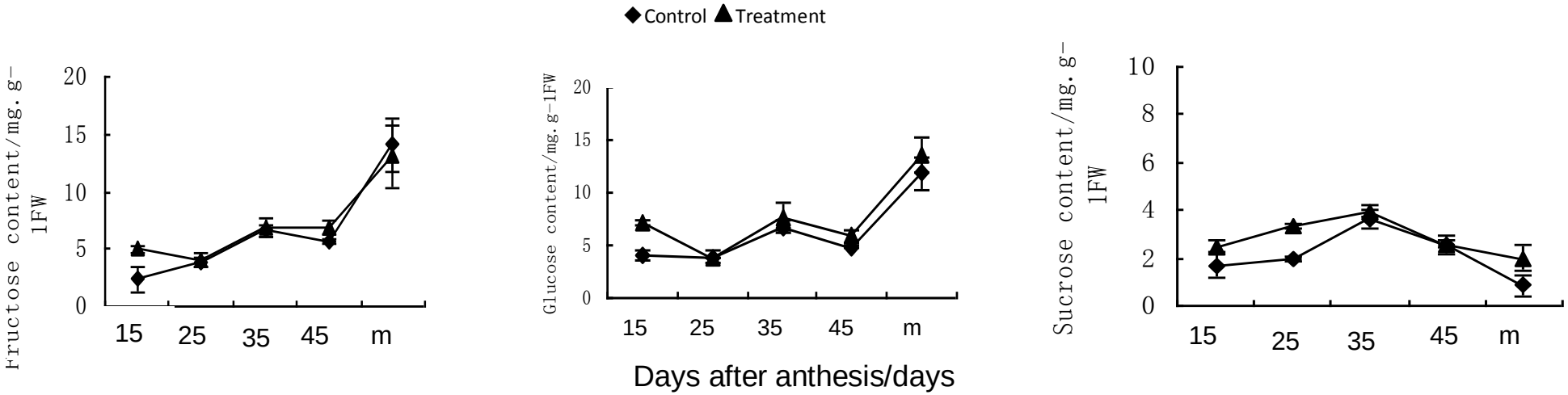

Figure 2. Effects of PCPA on sugar contents in pectinic during tomato fruits development. CK, Control; PCPA, PCPA treatment; m, ripening tomato fruit.

in SPS activity was observed during fruit ripening in the control group.

Pectinic tissues

In pectinic tissues of tomato fruit, similar trends in the activity of acid invertase (AI) were noted in both pericarp and dissepiments, in both the PCPA treated and control groups. A low level of AI activity was seen during initial stages and middle stages of fruit development. A sharp increase in AI activity was noted at the mature stage of fruit and AI activity in the PCPA treated group was markedly stronger than that in the control group (Figure 4).

A declining trend in sucrose synthase (SS) activity was observed in pectinic tissues during tomato fruit development. The SS activity level in fruits of the control group was higher than that in fruits of the PCPA treated group during initial stages of fruit development. In contrast, a low level of SS activity was noted in ripening fruit, with no significant differences between treated and control groups. The SPS activity in pectinic tissues was low throughout tomato fruit development. The peak activity of SPS was only 8 μmol sucrose g^{-1}FW h^{-1}. SPS activity showed no significant change in response to PCPA treatment.

Effect of PCPA on *AI* gene expression in different parts during tomato fruit development

No obvious effect was observed on sucrose synthase (SS) and sucrose phosphate synthase (SPS) activity by para-chlorophenoxyacetic acid (PCPA) treatment in the mature stages of tomato fruit development. In contrast, acid invertase (AI) activity was affected by PCPA during ripening. Thus, AI appeared to be the main enzyme responsible for effects on the quality of ripening tomato fruit and its activity was directly affected by PCPA. Therefore, we examined *AI* mRNA expression in different parts of the tomato fruit at the initial, middle and mature stages of fruit development. Soluble *AI* mRNA was expressed in ripening fruits. The expression of *AI* gene in pectinic tissues was stronger than that in pericarp and dissepiments. An increase in acid invertase mRNA level was noted in fruit tissues following PCPA treatment (Figure 5).

DISCUSSION

The quality of tomato fruit is determined by sugar accumulation (Sturm, 1999; Kortstee et al., 2007). Previous investigations have indicated that, sucrose is the major transport form of photo-assimilates, while fructose and glucose are the predominant sugars in ripening tomato fruit (Davies, 1966; Davies and Kempton, 1975; Cui et al., 2006a). Therefore, the main factor for

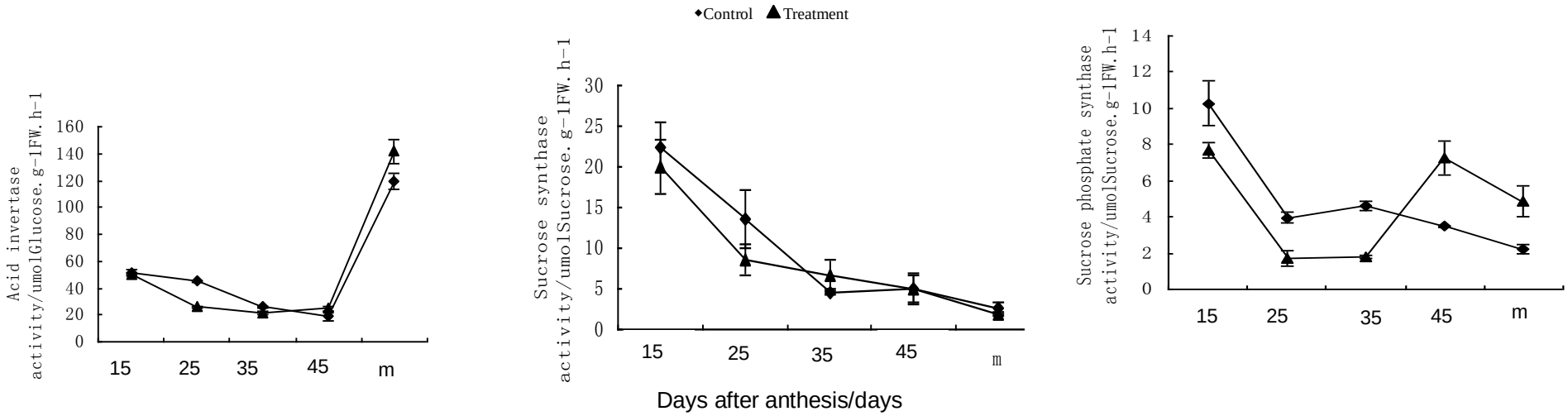

Figure 3. Effects of PCPA on the activity of sucrose related enzyme in pericarp and dissepiment during tomato fruits development. CK, Control; PCPA, PCPA treatment; m, ripening tomato fruit

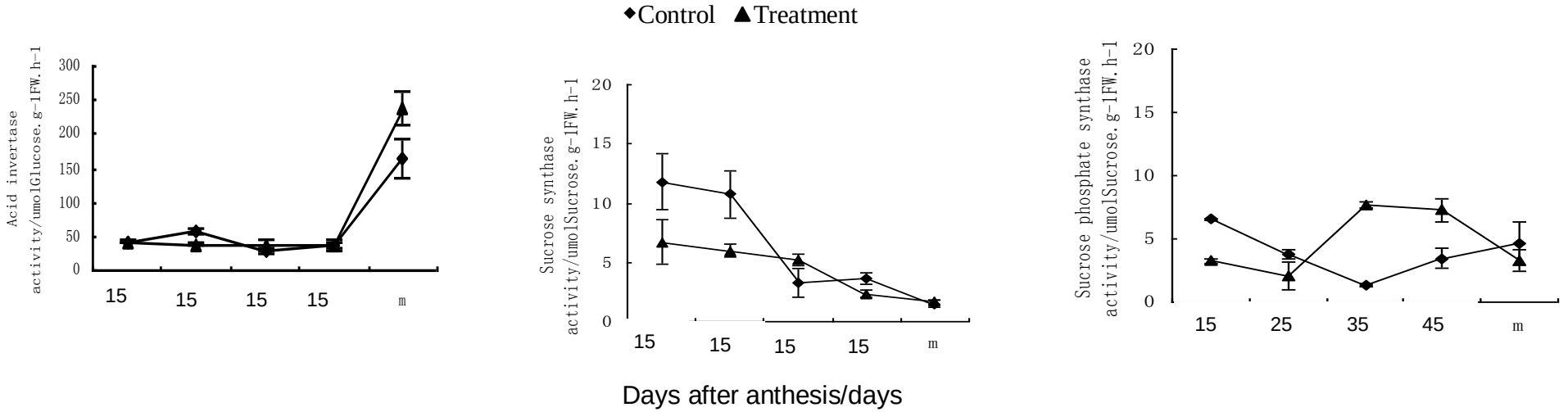

Figure 4. Effects of PCPA on the activity of sucrose related enzyme in pectinic during tomato fruits development. CK, Control; PCPA, PCPA treatment; m, ripening tomato fruit.

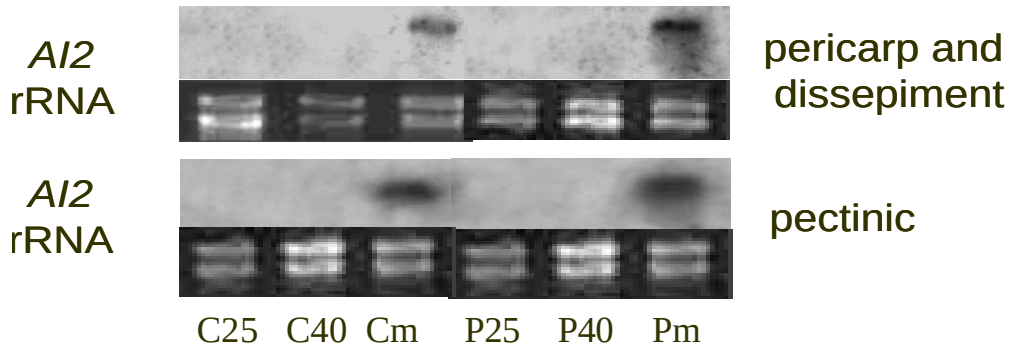

Figure 5. Effects of PCPA on mRNA expression of soluble acid invertase in tomato fruits. C25, Control, 25 days after anthesis; C40, control, 40 days after anthesis; Cm, control, mature stage; P25, PCPA treatment, 25 days after anthesis; P40, PCPA treatment, 40 days after anthesis; Pm, PCPA treatment, mature stage; AI_2, gene of soluble acid invertase (*TIV1*).

Table 1. Correlation coefficients of soluble sugar content and related enzyme activities in the pericarp and dissepiments of tomato fruit during ripening stage.

Treatment	Soluble sugar	AI	SS	SPS
CK	Fructose	0.643931	-0.674075	-0.698708
	Glucose	0.738554	-0.730690	-0.616503
	Sucrose	-0.875510*	0.182776	0.157975
PCPA	Fructose	0.982136**	-0.410066	0.000893
	Glucose	0.991470**	-0.284090	0.124854
	Sucrose	-0.790622	0.102473	-0.696040

*P < 0.05; **P < 0.01 (*t* test).

increasing fruit quality is regulation of sucrose content. In this study, an increasing trend in fructose and glucose contents was observed in pericarp, dissepiments and pectinic tissues during development of the Liaoyuanduoli tomato. The concentrations of fructose and glucose reached the highest levels at the mature fruit stage. The contents of fructose and glucose were notably increased in pericarp, dissepiments and pectinic tissues of mature fruits following para-chlorophenoxyacetic acid (PCPA) treatment.

Research into sucrose metabolism related enzymes is an important part of the investigation of fruit sugar accumulation. The metabolism of sucrose transported into tomato fruit and the changes in enzyme activities are the major aspects that will regulate the tomato fruit development and these bear a close relationship to final fruit quality (Moriguchi et al., 1991). The combined actions of acid invertase (AI) and sucrose synthase (SS) were the main factors affecting sugar accumulation in tomato fruit (Qi et al., 2006).

In the present study, an increase in acid invertase activity was observed in pericarp, dissepiments (Table 1) and pectinic tissues (Table 2) during the development of Liaoyuanduoli tomato fruit. During the early stage of tomato fruit development, when the activity of AI was low, sucrose accumulated. Then, along with the increasing activity of AI, sucrose concentration became progress-sively low. In the ripening fruit, a sharp increase in AI activity was followed by a steady decline in sucrose concentration concomitant with an increase in the concentrations of fructose and glucose. These results suggested that, the sucrose accumulation had a reverse relationship with the AI activity.

No obvious changes in the activity of acid invertase were observed in response to PCPA treatment in immature fruit. However, the activity of AI in pectinic tissues (Table 2) was higher compared to pericarp and dissepiments (Table 1) in ripening fruit. AI activity in all parts of the fruit was increased by PCPA treatment at the mature stage and this was followed by a sharp increase in the concentrations of fructose and glucose. Thus, hexose concentration was effectively increased by PCPA treatment during ripening stage in Liaoyuanduoli fruit.

The significant positive correlation found between hexose concentration and AI following PCPA treatment in different parts of the fruit further confirmed that, PCPA may affect the accumulation of fructose and glucose in ripening fruit.

Table 2. Correlation coefficients of soluble sugar content in the pectinic tissues of tomato fruit and related enzymes activities during ripening stage.

Treatment	Soluble sugar	AI	SS	SPS
CK	Fructose	0.870593*	-0.801036	0.139856
	Glucose	0.865006*	-0.751333	0.078541
	Sucrose	-0.798430	0.131282	-0.777929
PCPA	Fructose	0.982019**	-0.809567	0.028327
	Glucose	0.973112**	-0.602049	0.055396
	Sucrose	-0.619689	0.512563	-0.394663

*P < 0.05;**P < 0.01 (*t* test).

Acid invertase (AI) exists in two states which are soluble (in vacuole) and insoluble (cell wall-bound invertase). In recent years, many researches on the molecular level have reported that, the two types of AI were encoded by different gene family. And soluble AI was mainly in vacuole, regulating hexose concentration and affecting sucrose content in vacuole (Fridman and Zamir, 2003).

Acid invertase plays an important role in the process of plant growth and development (Zanor et al., 2009; Naegele et al., 2010). The gene expression of AI was quite complicated, because there are several types of invertase isoenzyme in every type of plant and are all encoded by different invertase genes. The expression patterns of diverse invertase genes were different in distinct species and organs at different times and plant growth regulator. Ohyama et al. (1995) reported that, invertase activity was inhibited by antisense expression of an invertase gene, which resulted in sucrose accumulation in tomato fruit. Klann et al. (1996) reported that, sucrose concentration in the fruit of an *AI* antisense gene translation mutant was 5 times that of control, but the concentrations of fructose and glucose were only half of the control, suggesting that the enzyme activity was affected by the change of enzyme at gene level and that sucrose metabolism was further affected.

At present, one acid invertase gene in the vacuole (*TIV1*) and four *AI* genes binding to cell walls (*Lin5, Lin6*, *Lin7* and *Lin8*) have been isolated from tomato (Zanor et al., 2009). A few reports have shown *AI* gene regulation by endogenous and exogenous inductors (Godt and Roitsch, 1997; Sturm and Chrispeels, 1990; Wu et al., 1993; Roitsch et al., 1995; Linden et al., 1996; Serrani et al., 2008). Godt and Roitsch (1997) studied the effect of gibberillic acid (GA_3) and zeatin on the *TIV1* and *LIN6* and showed that, the mRNA level of *LIN6* was elevated by zeatin, but the *TIV1* mRNA level was not affected. In tomato cell culture, the *TIV1* and *LIN6* mRNA levels were not affected by gibberillic acid ($GA_{3)}$. Exogenous indole acetic acid (IAA) alone stimulated *Ivr2* (gene of soluble acid invertase in maize) transcript accumulation in non-gravistimulated maize pulvini (Long et al., 2002). However, little is known regarding effects of exogenous auxins on *AI* gene expression following affecting fruits quality in tomato.

In this study, we found that the vacuolar *AI* gene was expressed in ripening fruit of Liaoyuanduoli tomato. This result agreed with previous reports by Elliott et al. (1993) and Godt and Roitsch (1997), in which *TIV1* expression was undetectable in green fruit, suggesting a tissue, organ and developmental specificity of *TIV1* gene expression. The *TIV1* and *Lin6* genes were cloned and the effects of exogenous auxin on gene expression were tested in Liaoyuanduoli tomato. No *Lin6* expression was detected during fruit development (data not shown), but a strong expression of the *TIV1* gene was found in ripening fruit and this *TIV1* gene expression was increased by PCPA treatment. No *Lin6* expression was detected, perhaps mainly due to its low abundance which limited its detection.

The expression of *TIV1* gene, in pectinic tissues was stronger than in pericarp and dissepiments in ripening fruit and *TIV1* expression in pericarp and dissepiments was obviously improved by PCPA treatment.

Fructose and glucose accumulated in ripening fruit of Liaoyuanduoli tomato and the hexose accumulation in fruit tissues were increased by para-chlorophenoxyacetic acid (PCPA) treatment, which were probably a reflection of the higher AI activity. *TIV1* mRNA expression in pectinic tissues of ripening fruit was stronger than in pericarp and dissepiments and *TIV1* expression was also improved by PCPA treatment indicating that, PCPA may affect sucrose metabolism at the transcription level. The AI activity in pectinic tissues was stronger than in pericarp and dissepiments and *TIV1* expression in pectinic tissues was also higher than in pericarp and dissepiments. However, the concentrations of fructose and glucose in pectinic tissues were lower than in pericarp and dissepiments, because fructose and glucose may be consumed by respiratory metabolism or seed development, in which nutrition is used for seeds after-ripening. In the present study, AI activity and *TIV1* expression were increased at mature stage in tomato fruit both control and PCPA treatment groups. The reasons might be that, PCPA directly or indirectly affected the AI activity and *TIV1* expression. Para-chlorophenoxyacetic

acid is hardly metabolized because it is a synthetic growth regulator such that it is a long-acting agent. In addition, the trend of endogenous auxin was not changed after PCPA treatment during growth and development of tomato fruit and it reached peak at 15 days after anthesis. However, endogenous indole acetic acid (IAA) level was improved after PCPA treatment compared with control group (Yu, 2005). Thus, PCPA might be involved in the signalling pathway controlling acid invertase activity and *TIV1* expression. And that, different sugar signalling in green fruit might influence enzyme activity, or *TIV1* expression in red fruit (Bolouri et al., 2010).

The regulatory mechanism of sugar accumulation is quite complicated. It may be regulated not only by transcription and translation but also by posttranscriptional and posttranslational modifications. The essential function and mechanism of action of exogenous auxin and sugar signalling on sucrose metabolism are still unclear and require further investigation. However, the results from the present study suggest that, para-chlorophenoxyacetic acid directly or indirectly affects the acid invertase activity by regulation of gene expression and thus, affects hexose accumulation in ripening tomato fruit.

ACKNOWLEDGEMENTS

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