Middle-East Journal of Scientific Research 7 (6): 984-989, 2011
ISSN 1990-9233

Effects of Exogenous Auxin on Activity of Fructokinase and Gene Expression in Tomato Fruits

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Abstract: Sugar contents, related enzymes activities of sucrose metabolism, activity and gene expression of fructokinase(FRK) were studied during tomato fruit development by PCPA and 2,4-D treatment. The results showed that activity and gene expression of fructokinase were ascended firstly then descended during fruit development, but thr activity of fructokinase was the lowest during mature period of fruit. *FRK1* gene expression was higher than that of *FRK2* gene. The contents of fructose and glucose in fruit reached a peak point at the stage of mature. The activity of fructokinase was decreased, meanwhile the activity of acid invertase was increased and gene expression of fructokinase was accelerated during mature period of fruit after PCPA and 2,4-D treatment so that the contents of fructose and glucose were increased.

Key words: *Solanum lycopersicum* · Exogenous auxin · Fructokinase · Gene expression

INTRODUCTION

Sugar content is an important factor in determining tomato fruit quality. Fructose is the sweetest among the soluble sugar [1, 2]. Thus fructose accumulation is one of the key factor for forming the fruit quality. Fructose accumulation in tomato fruit is not only from sucrose transport and sucrose metabolism, but also from fructose metabolism. That means fructose accumulation is more derived from fast rate of sucrose transportation to fruits and metabolism and the rate of fructose metabolism is slowly. Therefore, those were more important to study the condition of fructose metabolism and regulation. The present studies showed that the key enzyme attending fructose metabolism was fructokinase (FRK, EC2.7.1.4). Fructose attended the next step of metabolism must be phosphorylation through fructokinase [3]. Moreover, gene expression, enzyme activities and regulation of FRK in tomato fruits can help to clear the sugar accumulation and sugar regulation.

Exogenous auxin was used in tomato produces so that the increasing production was obtained [4]. But how exogenous auxin to affect the fructose accumulation, FRK activity and regulating the expression of *FRK* were still not clear to affect fructose metabolism, tomato growth and development, fruit quality. In this study, we examined PCPA (para-chlorophenoxyacetic acid) and 2,4-D (2,4-dichlorophenoxy acetic acid) effects on sugar content, the related-enzyme activities of sucrose metabolism, activity of FRK and gene expression of *FRK*. Relationship between FRK and sugar accumulation in tomato fruit was discussed and was responded following exogenous auxin treatment.

MATERIALS AND METHODS

Plant Meterials: Liaoyuanduoli tomato (*Solanum lycopersicum*) seeds were sown on 28th April, 2009 and seedlings (41d seedlings) were transplanted to a solar greenhouse with array pitch of 50cm and row spacing 35cm. 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 and 2.4-D respectively with control in distilled water. All treatment and control plants were tagged at anthesis.

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Tissue Sampling: Tissues of fruits were harvested at 15, 25, 35, 45d and at the mature stage (55-60d) after anthesis. After harvest, fruit samples were frozen in liquid nitrogen and stored at -80□ for later analysis of sugar concentration and enzyme activity. Three replicate samples were collected. Fruit tissues were also harvested at different stages of fruit development, frozen in liquid nitrogen and stored at -80□ for later analysis of gene expression.

Sugar Determinations: Approximately 2g fresh weight of frozen tissue was extracted with 80% ethanol. Soluble sugar concentration was determined using an Angelient HPLC system. An amino column (Dikma) and a model 1100 refractive index monitor were used. The mobile phase was 80% acetonitrile and ultrapure water (80:20), the flow rate was 1.0mL.min^{-1} and the temperature of the column was 35□. Sucrose, glucose and fructose were identified by their retention times and quantified according to known standards. Angelient software was used for data analysis.

Enzyme Extraction and Assays: Approximate 1g 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) [5], with slight modification. The buffer contained 50mM Hepes-NaOH (pH7.5), 1mM EDTA, 10mM $MgCl_2$, 2.5mM DTT, 10mM ascorbic acid and 5% (w/v) PVPP. Homogenates were centrifuged at 12000g for 20min at 4□ and the pellets were discarded. Ammonia sulphate was gradually added to the supernatant to 80% saturation and the solution was again centrifuged at 12000g for 30min at 4□. The supernatant was discarded and the pellet was dissolved in 2-5mL of extraction buffer, then dialyzed against a tenfold volume of extraction buffer (without PVPP) for 20h. All steps were carried out at 0-4□.

The activities of enzymes related to sucrose metabolism were assayed in desalted extracts as described by Yu (1985) [6], with minor modifications. UDPG and Fru-6-P were purchased from Sigma (St.Louis, MO, USA).

Fruictokinase extraction was according to the methods of Schaffer and Petreikov [7]. The activity of fructokinase was assayed as described by Huber and Akazawa [8].

Total RNA Extraction and Real-Time PCR Analysis: Total RNA was extracted using the RNAprep pure plant total RNA extraction kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The possible DNA contamination was eliminated by adding RNase-free DNase I. The integrity of the RNA was detected by 0.8% agarose gel electrophoresis.

The RNA samples were reversely transcribed into cDNAs. In brief, 5μL RNA mixed with 5μL dNTP(2.5mM) and 1μL oligo $d(T)_{15}$ were incubated for 5min at 65□, then cooled immediately and plused 5μL M-MLV 5□×Reaction buffer, 1μL cloned ribonuclease inhibitor, 1μL M-MLV reverse transcriptase and RNase-free ddH_2O to final volume 25μL, mixed gently and incubated at 37□ for 1h, ended the reaction at 70□ for 15min, the product cDNAs were stored at -20□.

The quantitative real time PCR analysis was performed as described by (Jain *et al.*, 2006) [9]. In brief, the cDNA samples were used as template and mixed with 200nM of each primer and SYBR Green PCR Real Master Mix (Tiangen, Beijing, China) for real-time PCR analysis, using ABI 7500 Real Time PCR System and Software 7500 v2.0.3 (Applied Biosystems) according to the manufacturer's instructions. The temperature procedure was: 95°C for 3min; 40 cycles of 95°C for 30s, 57°C for 30s and 68°C for 1min. The fluorescence signal was collected during the elongation at 68°C of every cycle. Each pair of primers designed by using Primer Express 3.0 software (Applied Biosystems) was checked by the BLAST program in tomato genomic sequence available in SGN and NCBI database to ensure that the primers amplify a unique and desired cDNA segment. The primer sequences were listed in Table 1. The specificity of the reactions was verified by melting curve analysis. The relative mRNA levels for each of the FRK genes in RNA isolated from various samples were quantified with respect to the internal standard actins. The real-time PCR was conducted with three replications.

Table 1: Primer sequences used for analysis of fructokinase genes expression in tomato fruit by real-time fluorescent quantitative PCR

Gene	Accession number	Primers sequences
FRK1	U64817	F 5'-CTCCGTTACATATCTGATCCTT -3'
FRK2	U64818	R 5'-GACAGCATTGAAGTCACCTT-3'
Actin	Q96483	F 5'-TTGTTGGTGCCCTTCTAACCA -3'
		R 5'-ACGATGTTTCTATGCTCCTCCCT -3'
		F 5'-TGTCCCTATTTACGAGGGTTATGC-3'
		R 5'-AGTTAAATCACGACCAGCAAGAT-3'

RESULTS

Effect of Exogenous Auxin on Sugar Content in Tomato Fruits: The content of fructose was gradually increased following the tomato fruits development. No significantly difference was observed between treatment and control groups before 35 days after anthesis. The fructose contents of PCPA and 2,4-D treatment groups were significantly higher than control group from 45d after anthesis to ripening stage. The content of glucose was fluctuated within a certain range following the tomato fruits development, namely that, the glucose content of PCPA and 2,4-D treatment groups was slightly lower than that of control group before 35d after anthesis. Meanwhile, the glucose content was higher in treated plants than that of control group 45d after anthesis. When in the ripening fruits, the glucose content of 2,4-D treatment group was higher than those of both PCPA treatment group and control group. The content of sucrose was low in the whole stage of tomato fruit development. No significant difference between treatment and control groups. But the sucrose content of PCPA treatment group was slightly increased 45d after anthesis (Fig. 1).

Effect of Exogenous Auxin on the Enzyme Activities Related to Sucrose Metabolism in Tomato Fruits: As shown in Fig. 2, AI(acid invertase) activity was low prior to 45d after anthesis in tomato fruits and no difference was observed between PCPA and 2,4-D treated and control groups. A sharp increase in AI activity was observed in ripening fruit and AI activity became higher in the PCPA and 2,4-D treated groups. The trend of NI(neutral invertase) activity was similar as AI except that the activity of 2,4-D treated group was slightly higher than PCPA treated and control groups in the ripening fruits, but the NI activity was obviously lower than AI during the tomato fruit development.

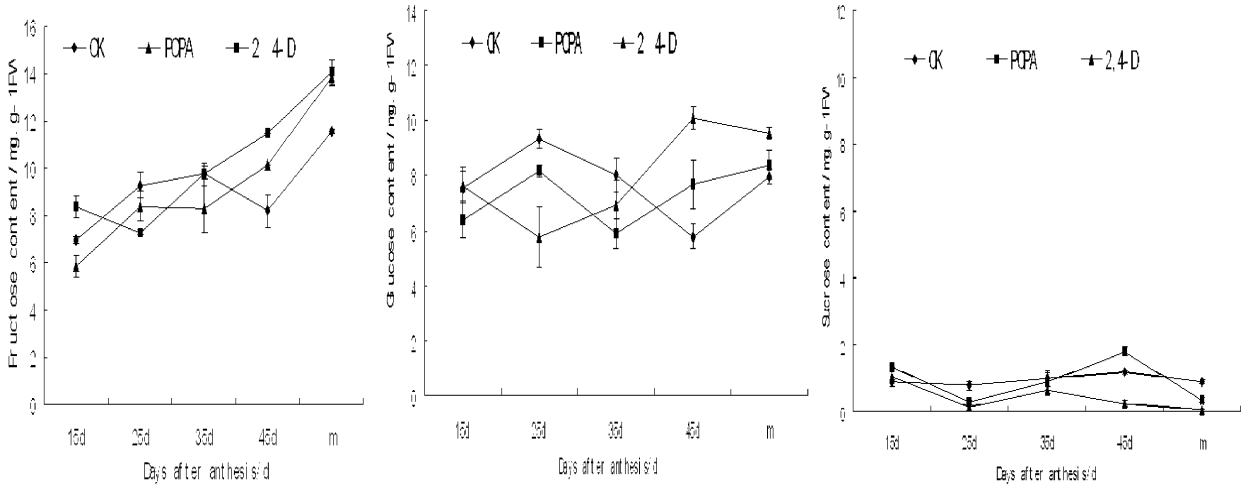

Fig. 1: Effects of PCPA and 2,4-D on sugar contents in tomato fruits

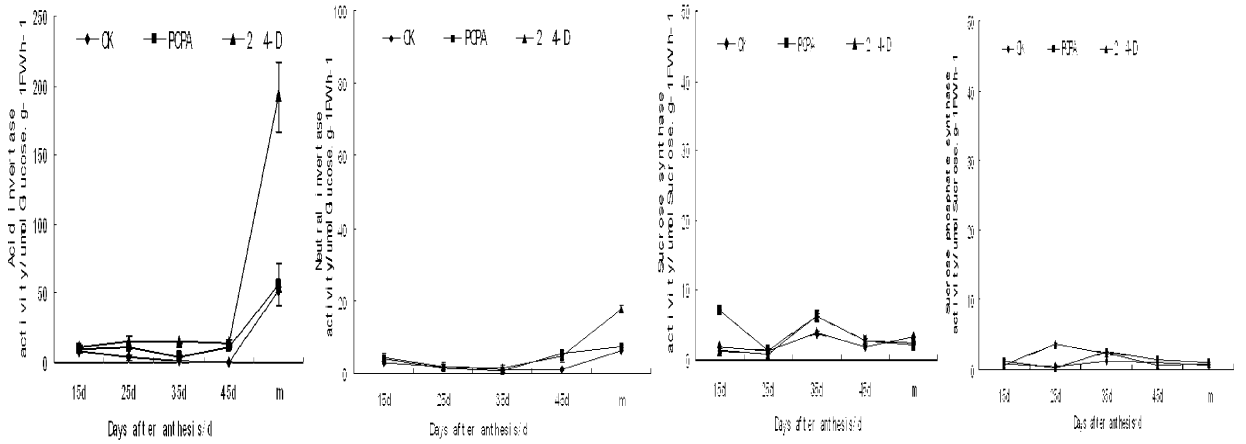

Fig. 2: Effects of PCPA and 2,4-D on activities of sucrose metabolism related enzymes in tomato fruits

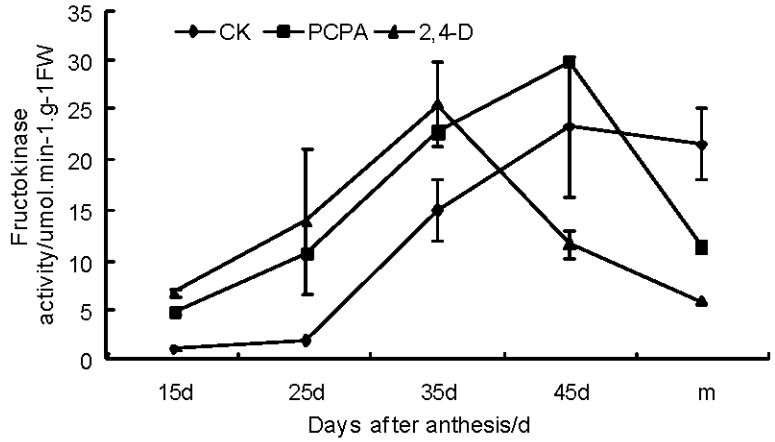

Fig. 3: Effects of PCPA and 2,4-D on activity of fructokinase in tomato fruits

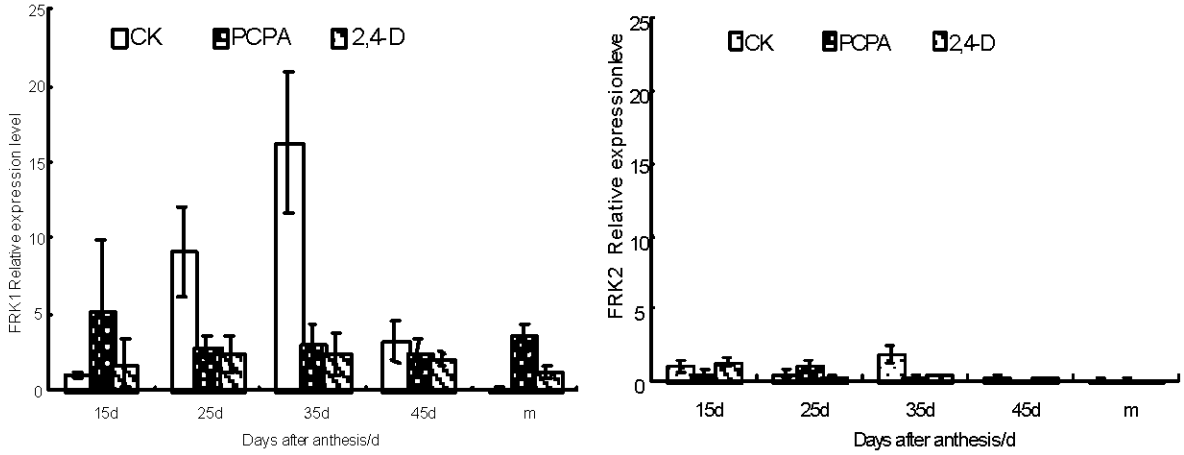

Fig. 4: Effects of PCPA and 2,4-D treaments on fructokinase gene expression during the tomato fruit development

The activity of sucrose synthase(SS) was low at the whole stage of tomato fruit development and no difference was observed except for a slightly high activity of SS was observed of PCPA treated group 15d after anthesis. Low levels of SPS(sucrose phosphate synthase) activity were noted during tomato fruit development and no difference was observed between treated and control groups.

Effect of Exogenous Auxin on the Activity of Fructokinase in Tomato Fruits: The activity of FRK was gradually increased from 15d to 35d after anthesis and the FRK activities were higher in treated plants than that of the control group. In 35d after anthesis, the FRK activity of 2,4-D treated group was reached the peak point and FRK activity was slightly low in PCPA treated plants, while the FRK activity was the lowest in control group. Then the FRK activity of 2,4-D treated group was significantly decreased and those in PCPA treated plants and control group were reached peak 45d after anthesis and then decreased at ripening fruits. The FRK activities were lower in both treated plants than that of control group in ripening fruits, among them, the 2,4-D treated group was the lowest (Fig. 3).

Effect of Exogenous Auxin on the *FRK* Gene Expression During Tomato Fruit Development: *FRK1* and *FRK2* mRNA expressions for having confirmed present in tomato were examined in this study as shown in Fig. 4. The expression of *FRK1* gene was stronger than that of *FRK2* gene during the whole stages of tomato fruit development. The expression of *FRK2* gene was difficult to examine in ripening fruits.

The *FRK1* gene expression was higher in treated groups than that of control group 15d after anthesis and *FRK1* gene expression was stronger than those of treated groups from 25d to 35d after anthesis. No difference was observed in treated and control groups 45d after anthesis and the levels of *FRK1* expression were slightly high in PCPA treated plants in mature stage. The levels of *FRK2*

gene expression were low during tomato fruit development and a little high level of *FRK2* expression in young and expanding stages of fruits. While, *FRK2* mRNA level was slightly high in 2,4-D treated group 15d after anthesis and its expression level in control group was slightly high 35d after anthesis.

DISCUSSION

The quality of tomato fruit is basically determined by sucrose metabolism and fructose accumulation [10]. The changes in sugar content are mainly regulated by activities of sucrose metabolism related enzymes and fructokinase during tomato fruit development [11, 12] and sugar contents are regulated through effect on the activities of sucrose metabolism related enzymes, fructokinase following exogenous auxin treatment [13, 14]. In this study, an increasing trend in fructose and glucose content was observed during development of tomato fruit. The concentrations of fructose and glucose reached the highest levels at the mature fruit stage. The contents of fructose and glucose were obviously increased in mature fruits following exogenous auxin treatment and the AI activity was increased at the mature stage. This showed the same result as the previous investigations [15]. Meanwhile, the FRK activity was decreased in mature tomato fruits following the exogenous auxin treatment. Thus, fructose and glucose accumulated in ripening tomato fruit by exogenous auxin treatment, was probably a reflection of the higher AI activity and the lower FRK activity. Sucrose was promptly broken down into fructose and glucose by the increased AI activity and fructose accumulation was ensured by the decreased FRK activity.

In this study, we found by the real-time PCR that *FRK1* and *FRK2* genes were expressed during the tomato fruit development, but expression levels were obviously different. *FRK1* expressed level was stronger than *FRK2* and *FRK1* was expressed in ripening tomato fruit, which indicated that *FRK1* gene was the key factor for forming the fruit quality (Fig. 4). Thus, we suppose that *FRK1* and *FRK2* genes might be encoded different isozymes of fructokinase and play different roles in fructose metabolism during different developmental stages of tomato fruit. However, expression levels of fructokinase gene were different from the activity of fructokinase (Fig. 3, 4), which showed that there might be exist posttranscriptional regulation from gene expression to activity of protein. In addition, expression of many genes in plants was activated or blocked by sugar as regulating signaling [16]. Fructose and glucose accumulated in ripening tomato fruit was improved by exogenous auxin treatment and expression levels of *FRK1* was increased, which was probably activated by increasing of sugar content. Meanwhile, the enzyme protein activity of fructokinase was feedback inhibition by increasing of sugar content, which suggested that *FRK1* gene might belong to sugar regulating gene [17, 18].

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 action mechanism of sugar for plant growth and development, regulation of sugar metabolism and gene expression are still unclear as nutrition and signaling molecule [19, 20]. Auxin is also signaling molecule [21] and its regulating mechanism, coordinated function with sugar signaling for sugar metabolism are still unclear and require further investigation, establishing basic for further showed the regulating mechanism of sugar metabolism.

ACKNOWLEDGEMENT

This study was supported by the Post Expert Foundation- Project of National Modern Agricultural System(Nycytx-35-gw23), Liaoning Provincial Education Department Scientific and Technical Research Foundation (2008623) and Postdoctoral Foundation of Shenyang Agricultural University (62658).

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