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Full Length Research Paper

Regulation effects of exogenous gibberellin acid (GA_3) on the formation of tomato (*Solanum Lycoperscium*) ovary locule and *fasciated* transcription

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To fully understand the regulation effects of gibberellin on tomato (*Solanum Lycoperscium*) ovary locule formation and the *fasciated* transcription, two varieties: multi-locule 'MLK1' and few- locule 'FL1' which were highly different in locule number and *fasciated* transcriptional levels, were used in this study. By spraying GA_3 and PAC (paclobutrazol; an inhibitor of gibberellin biosynthesis) with different concentrations, we found that GA_3 increased the locule number, especially, in 'MLK1'. At the same time, we chose the suitable treatment concentrations of GA_3 and PAC (GA_3, 80 mg·L^{-1}, PAC, 80 mg·L^{-1}) which were applied on the 'MLK1' and 'FL1' to analyze the *fasciated* transcriptional levels and we found that GA_3 repressed the *fasciated* transcriptional level while PAC increased it. The results implied that the GA_3 regulated the ovary locule formation through controlling the *fasciated* transcription levels.

Key words: Tomato, *fasciated*, GA_3, locule number.

INTRODUCTION

The locule number of tomato (*S. Lycoperscium*) is closely related to malformation (Li, 1993). The more the ovary locule number and the larger fruit, the more is the incidence of fruit malformation. The development of ovary locules is affected by environmental and nutritional conditions, which induce exogenous substances (Asahira et al., 1982; Li et al., 1997; Xu et al., 1997; Li et al., 2007; Zhang et al., 1998; Chen et al., 2006), but the most important factor is their genes (Younis et al., 1988; Lippman et al., 2001). There are six QTLs associated with fruit size: *fw*1.1, *fw*1.2, *fw*2.1, *fw*2.2, *fw*3.1 and *fw*11.3 (Frary et al., 2000; Nesbitt et al., 2001; Lippman et al., 2001; Cong et al., 2002; Liu et al., 2003; Tanksley 2008). But only *fw*2.2 (*locule-number*) and *fw*11.3 (*fasciated*) have been identified to cause the change in fruit size through a change in the number of carpels in the flower (Foolad, 2007). These two loci have superior sexual function from each other (Lippman et al., 2001), either one can improve the locule number.

Tanksley (2008) cloned the *fw*11.3 first and later confirmed the function by the transgene way, which is associated with tomato locule formation (Foolad, 2007; Cong et al., 2008). Relevant research shows that the abundance of *fasciated* mRNA accumulation is higher in high-locule number tomatoes than low-locule number tomatoes (Cong et al., 2008). In the meantime, applying GA_3 at the bud differentiation stage increase markedly the locules number (Li et al., 1997). But the relationship between the *fasciated* and GA_3 in tomato locule formation have been unknown. In this paper, through spraying GA_3 and PAC, the effect of GA_3 on the locule formation was discussed

MATERIALS AND METHODS

Plant material

Two stable inbred lines of tomatoes, multi-locule (*S. Lycoperscium*) 'MLK1' and few-locule (*S. Lycoperscium*) 'FL1' were established at Shenyang Agricultural University. The locule number of 'MLK1' is

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Table 1. Real-time RT-PCR primers used to amplify gene-specific regions.

Category	Accession number	Primer sequences (5'-3')
Fasciated	EU557674	Sense: ATA TTA GCC ATC GTG AAG C Antisence: TCG CTA TTT GTT GCC CTC C
Actin	Q96483	Sense: TGT CCC TAT TTA CGA GGG TTA TGC Antisence: AGT TAA ATC ACG ACC AGC AAG AT

14 ± 1(Figure 1E), with oblate and light-red fruit and more sepals and petals (Figure 1A and C). The 'FL1' locule number is 2 or 3 (Figure 1F), with elongated round and scarlet fruit and less sepals and petals (Figure 1B and D). Except for the differences in locule number, sepals and petals, and other agronomic traits are similar to these two variants (Figure 1). Tomato plants were grown in greenhouse conditions under temperatures ranging between 18 and 25°C.

Hormone treatment

Treatment one

When plants of tomato grew to two leaves just before flower bud differentiation began, GA_3 and PAC were sprayed with different concentrations on the whole-plant until run-off. GA_3 (5% Methanol and 0.08% Tween 20), 5, 20, 50, 80, 100, 150 and 200 $mg \cdot L^{-1}$ respectively, PAC (9% Methanol and 0.8% Tween 80), 20, 40, 60, 80, 100, 150 and 200 $mg \cdot L^{-1}$ respectively, and application of distilled water as control. Two weeks later, the plants were transplanted. Thereafter, the stereo microscope (Olympus, SZX16) was used to investigate the ovary locule number of the first three flowers of the first truss.

Treatment two

Suitable treatment concentrations (greater influence on locule number, less on plants growing) of GA_3 (80 $mg \cdot L^{-1}$) and PAC (80 $mg \cdot L^{-1}$) was chosen, sprayed on the whole-plant until run-off. Plants decapitation was performed by severing about 3 mm of the apical shoot with the first and the second true leaves at 1, 3 and 5 days after treatment. During sample collection, the tissue was snap frozen in liquid nitrogen and stored at -80°C until RNA isolation. Absolute amounts of mRNA in samples were quantified using three biological replicates, with each replicate containing 5 plants.

Quantitative RT-PCR

RNA was isolated with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Samples of 1 µg of RNA were used for cDNA synthesis with the Superscript reverse transcriptase kit (Invitrogen), subsequently diluted 5 times, and stored at -20°C until further use. Quantitative RT-PCR was performed using gene-specific primers (Table 1) in a total volume of 20 µL with 2 µL of the cDNA , 4 µL (2 µM) gene-specific primers, 5 µL sterile ultrapure water and 9 µL power SYBR Green PCR Master Mix (TaKaRa) solution on a 7500 real-time PCR machine (Applied Biosystems) according to the manufacturer's instructions.

Cycle threshold (CT) values were obtained with the accompanying software. Obtained values were normalized against the actin that was used as an internal standard. The mean expression level of the *fasciated* was calculated from three biological repeats and obtained from three independent experiments. *Fasciated* / actin (the untreatment sample from day 0) ratios were then averaged and presented as a ratio of a control treatment with the value set to 1. The primers were annealed at 58°C and run 40 cycles, after normalization using the actin cDNA level and averaging over three replicates.

Statistical analysis

The data presented correspond to experiments processed using Origin 7.5 (Microcal Software Inc., Northampton, MA, USA). Analysis of variance was performed using one-way ANOVA with SPSS 11.0 Software (SPSS Inc., Chicago, IL, USA).

RESULTS

The effects of GA_3 on locule number of 'MLK1' and 'FL1' tomatoes

Accompanied to the increase of the concentration of GA_3, the locule number had also been improved in 'MLK1', but there were no significant differences from 80 to 200 $mg \cdot L^{-1}$ respectively, and was the same of the first three flowers. In 'FL1', the locule number resulted in an increasing trend but there was also no significant difference. The higher the concentration the more it improved the plants growth (with longer internode) and as such, we chose the 80 $mg \cdot L^{-1}$ as a suitable one to the next treatment (Tables 2 and 3).

The effects of PAC on the locule number of 'MLK1' and 'FL1' tomatoes

Increasing the concentrations of PAC, the locules number were dropped off as compared to control in 'MLK1', but with no significance difference from 60 to 200 $mg \cdot L^{-1}$ and the effects declined gradually from the first to the third flower. In 'FL1', the variation trends of the locule number followed a similar behavior to that of GA_3 treatments (Tables 4 and 5). The locules number showed a decreasing trend, but there were no significant difference. Considering the influence on the locule number and normal growth of plants, we chose 80 $mg \cdot L^{-1}$ as a suitable concentration for the next treatment (Tables 4 and 5).

Table 2. Effects of different GA_3 concentration treatments on 'MLK1' locule number.

GA3 treatment concentrations (mg•L-1)	The first flower of the first truss	The second flower of the first truss	The third flower of the first truss
0	13.67 ± 0.58^{a}	13.33 ± 0.58^{a}	14.00 ± 0.00^{a}
5	15.00 ± 0.00^{ab}	15.00 ± 1.00^{ab}	14.33 ± 0.58^{a}
20	15.67 ± 1.53^{ab}	14.67 ± 0.58^{ab}	15.33 ± 0.58^{a}
50	15.67 ± 1.15^{ab}	16.33 ± 1.53^{ab}	19.00 ± 1.00^{b}
80	18.00 ± 1.00^{bc}	16.67 ± 1.15^{b}	19.33 ± 0.58^{bc}
100	18.67 ± 0.58^{bc}	16.67 ± 1.15^{b}	21.00 ± 1.00^{bc}
150	20.33 ± 3.21^{c}	16.67 ± 1.53^{b}	21.67 ± 0.58^{c}
200	21.00 ± 1.00^{c}	17.00 ± 1.00^{b}	21.67 ±1.53^{c}

Data are means of fruits locule number ± SD (n = 20), a, b, c denote significant differences at $P = 0.05$.

Table 2. Effects of different GA_3 concentration treatments on 'FL1' locule number.

GA_3 treatment concentration ($mg \cdot L^{-1}$)	The first flower of the first truss	The second flower of the first truss	The third flower of the first truss
0	2.33 ± 0.5^{8a}	2.33 ± 0.58^{a}	2.33 ± 0.58^{a}
5	2.33 ± 0.58^{a}	2.67 ± 0.58^{a}	3.00 ± 1.00^{a}
20	2.67 ± 1.15^{a}	2.33 ± 0.00^{a}	2.67 ± 0.58^{a}
50	2.67 ± 0.58^{a}	2.67 ± 0.58^{a}	2.67 ± 0.58^{a}
80	2.33 ± 0.00^{a}	2.33 ± 0.58^{a}	2.67 ± 0.58^{a}
100	2.67 ± 0.58^{a}	2.33 ± 0.58^{a}	2.67 ± 0.58^{a}
150	2.33 ± 0.58^{a}	2.67 ± 0.58^{a}	2.33 ± 0.58a
200	2.33 ± 0.58^{a}	2.67 ± 0.58^{a}	2.67 ± 0.58^{a}

Data are means of fruits locule number ± SD (n = 20), a, b, c denote significant differences at $P = 0.05$.

Table 4. Effects of different PAC concentration treatments on 'MLK1' locule number.

PAC treatment concentration ($mg \cdot L^{-1}$)	The first flower of the first truss	The second flower of the first truss	The third flower of the first truss
0	13.75 ± 0.50^{a}	13.25 ± 0.50^{a}	13.75 ± 0.50^{a}
20	13.25 ± 0.50^{a}	13.25 ± 0.96^{a}	12.00 ± 1.15^{ab}
40	12.75 ± 0.50^{ab}	11.00 ± 1.15^{ab}	12.50 ± 0.58^{ab}
60	11.75 ± 0.50^{bc}	11.25 ± 0.96^{ab}	12.75 ± 0.50^{ab}
80	11.75 ± 0.50^{bc}	11.25 ± 0.96^{ab}	12.50 ± 0.58^{ab}
100	11.75 ± 0.50^{bc}	11.25 ± 0.96^{ab}	12.00 ± 0.82^{ab}
150	11.75 ± 0.50^{bc}	10.25 ± 1.26^{b}	12.25 ± 1.50^{ab}
200	11.00 ± 0.82^{c}	10.50 ± 1.00^{b}	11.50 ± 0.58^{b}

Data are means of fruits locule number ± SD (n=20), a, b, c denote significant differences at $P = 0.05$.

The comparison of fasciated mRNA level between 'MLK1' and 'FL1'

At the beginning of the flower bud differentiation, we chose the apical shoot, the first and the second leaves to compare the *fasciated* mRNA levels between 'MLK1' and 'FL1'. It showed that there were enormous differences between the two varieties. In leaves, 'FL1' *fasciated* mRNA levels were 53 times higher than in 'MLK1' (Figure 2A). In apical shoot, it was about 3.8 times higher (Figure 2B).

The effect of GA_3 and PAC on the transcriptional levels of *fasciated*

In apical shoot of 'MLK1', GA_3 induced abundance of

Table 5. Effects of different PAC concentration treatments on 'FL1' locule number.

PAC treatment concentration ($mg \cdot L^{-1}$)	The first flower of the first truss	The second flower of the first truss	The third flower of the first truss
0	2.67 ± 0.58[a]	2.33 ± 0.58[a]	2.33 ± 0.58[a]
20	3.00 ± 1.00[a]	2.67 ± 0.58[a]	2.33 ± 0.58[a]
40	3.00 ± 0.00[a]	2.67 ± 0.58[a]	2.67 ± 0.58[a]
60	2.67 ± 0.58[a]	2.67 ± 0.58[a]	2.33 ± 0.58[a]
80	2.33 ± 0.58[a]	2.00 ± 0.00[a]	2.33 ± 0.58[a]
100	2.00 ± 0.00[a]	2.33 ± 0.58[a]	2.00 ± 0.00[a]
150	2.33 ± 0.57[a]	2.67 ± 0.58[a]	2.67 ± 0.58[a]
200	2.33 ± 0.57[a]	2.67 ± 0.58[a]	2.67 ± 0.58[a]

Data are means of fruits locule number ± SD (n = 20), a, b, c denote significant differences at $P = 0.05$.

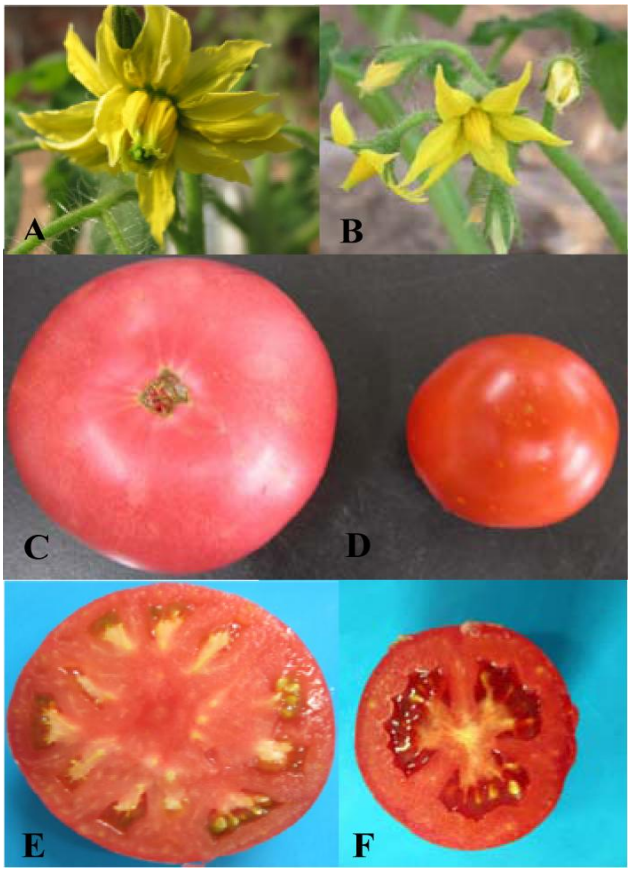

Figure 1. 'MLK1' and 'FL1' flowers and fruit; Panel A, C, E = 'MLK1'. Panel B, D, F= 'FL1'; Panel A, B = open flowers. Panel C, D = mature fruit. Panel E, F= fruit crosscut.

increasing of *fasciated* transcripts on day 1 in camparison to the unsprayed and then reduced at a level lower than the control on days 3 and 5 (Figure 3A). PAC improved the *fasciated* mRNA levels at a higher level than the control until day 3 and then, lower than the unsprayed on day 5 (Figure 3A). In 'FL1' apical shoot, application of GA_3 produced the same result to 'MLK1' (Figure 3C). But the PAC treatment increased the *fasciated* mRNA level higher than the control observed only on the 5th day (Figure 3C).

In 'MLK1' leaf, GA_3 declined the *fasciated* transcripts at an extremely lower level (Figure 3B). PAC increased the *fasciated* mRNA level especially, on day 3 (Figure 3B). In 'FL1' leaf, the inhibiting effects on *fasciated* transcripts of GA_3 was notable particularly, on the 3rd and 5thday (Figure 3D). PAC increased the level from the 1st day, thereafter, the differences between the treatments and control gradually decreased. On the 5th day, the effects of PAC on *fasciated* transcripts were weakened (Figure 3D).

DISCUSSION

The effect of GA_3 on the locule number

The regulation effects of GA_3 on the locule number were different in 'MLK1' and 'FL1'. In 'FL1', neither GA_3 nor PAC could change the locule number markedly. In contrast, GA_3 increased the locule number and PAC declined the locule number in 'MLK1'. Our results showed that the locule number of the 'FL1' varieties (locule numbers 2 or 3) had not been easily regulated by exogenous GA_3 treatment, but could regulate the formation of ovary locule in 'MLK1' (locule number 14 ± 1). Sprayed GA_3 on tomato of 'Qiang Li Xv Guang' (the locule number is 7) could also increase the locule number (Li et al., 1997). Sawhney and Greyson (1971) in their study reported that GA_3 can improve the differentiation and development of floral organs and also increase the locule number. These results were similar to ours, but we found that in low locule number tomato, such as 'FL1', the effects of GA_3 on locule number was small.

The relationship between the locule formation and *fasciated* transcription

Tanksley (2008) isolated the *fasciated* locus and used a positional cloning approach, then confirmed its function

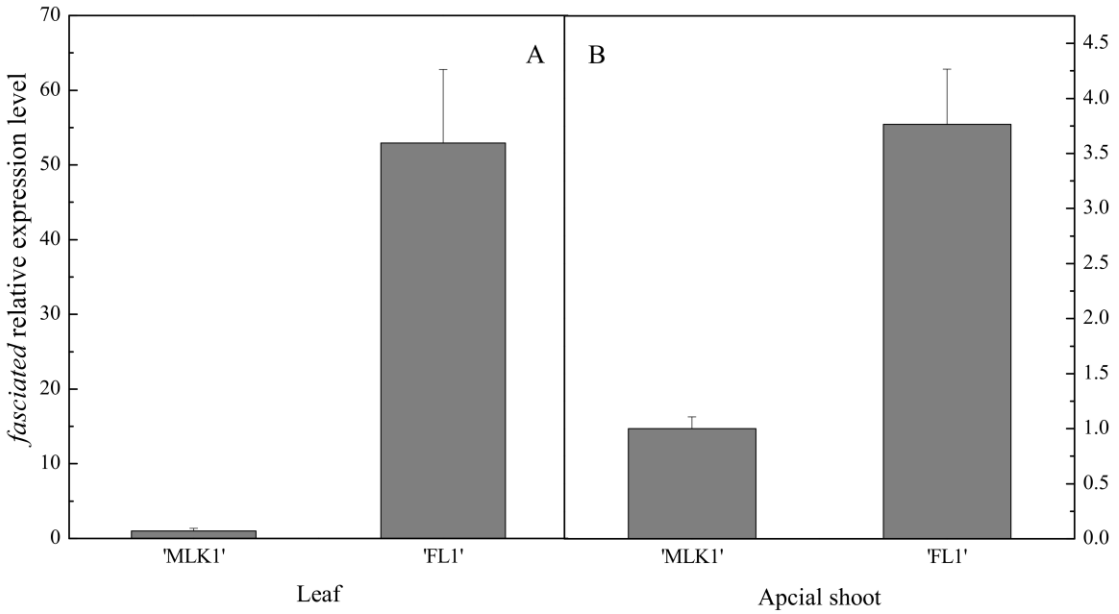

Figure 2. Expression analysis of the fasciated in leaf and apical shoot of 'MLK1' and 'FL1' using Real-time RT-PCRL; Panel A = leaf. Panel B = apical shoot.

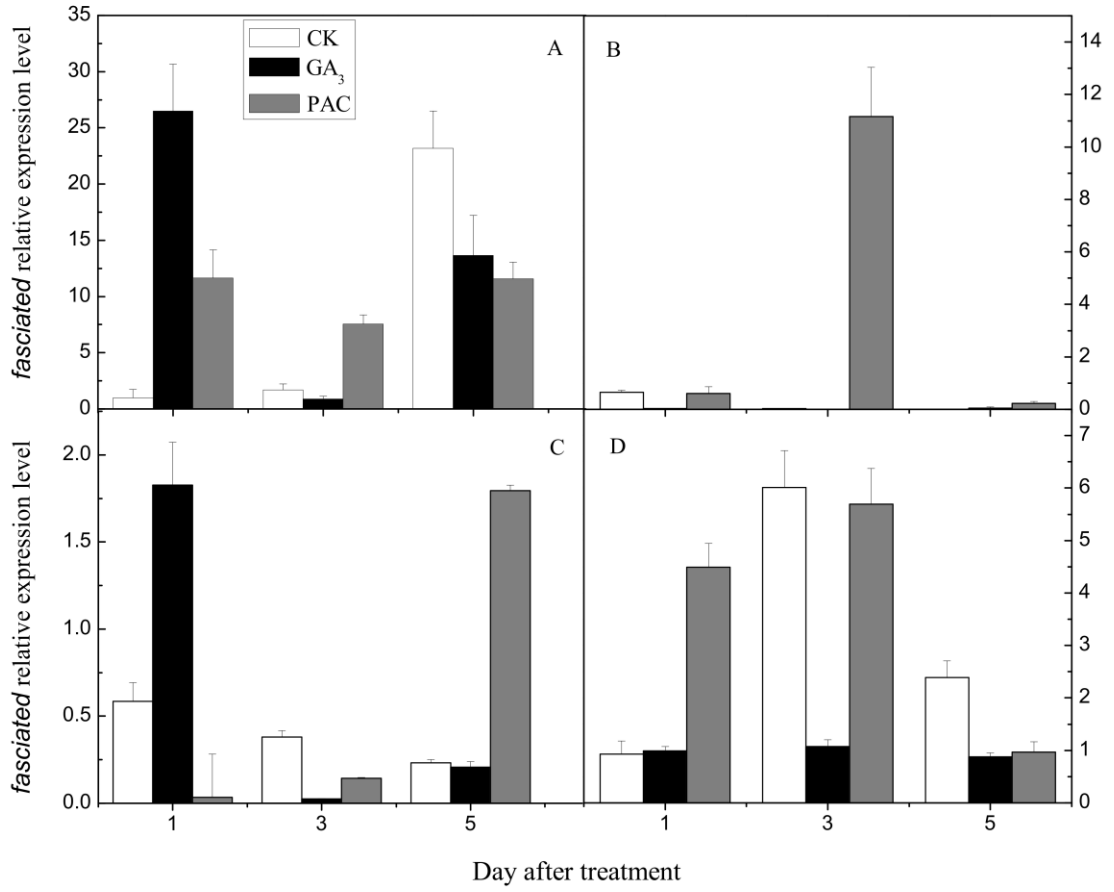

Figure 3. The effect of GA3 and PAC on fasciated transcriptional level. CK, GA3, PAC were sprayed with distilled water, GA3 and PAC separately; Panel A = Apical shoot of 'MLK1'. Panel B = leaf of 'MLK1'. Panel C = apical shoot of 'FL1'. Panel D = leaf of 'FL1'.

by the way of transgene, which is associated with fruit size through changing the number of carpels. To investigate whether the difference of carpel number in our plant material is caused by the transcriptional level of *fasciated,* we analyzed its mRNA level in leaf and apical shoot (we also conducted the *fasciated* cDNA sequence alignment and found that there were no difference between the two materials, though, the result was not shown). Transcription levels of *fasciated* in leaf and apical shoot was same in 'FL1' than in 'MLK1' (Figure 2A and B). This is consistent with the result of Tanksley (2008). At the beginning of the flower bud differentiation, the difference of mRNA level was larger in leaf than in apical shoot between the two varieties. Therefore, there is need for further study to explain the difference.

The relationship of GA_3 and fasciated in regulation of the locule formation

Fasciated and the locule number present negative correlation

The lower the *fasciated* mRNA level, the higher the number of locules (Tanksley, 2008). The application of exogenous GA_3 and PAC causes the locule number to grow high and decline. According to the relationships of the *fasciated* transcripts and the locules, GA_3-treated and PAC-treated plants exposed to *fasciated* mRNA levels experience growth and decrease.

Our study showed that with the apical shoot of 'MLK1' or 'FL1', GA_3-treated enhanced the *fasciated* levels on the 1st day, but observably restrained it at a lower level than in the control on the 3rd and 5th day. PAC-treated increased the *fasciated* transcripts in the apical shoot of 'MLK1' at a higher level on the 1st and 3rd day. In 'FL1' apical shoot, the *fasciated* transcripts increased, especially, on the 5thday. In the leaf of 'MLK1' variety, GA_3-treated inhibited the transcripts at an extremely lower level while PAC enhanced the level, especially, on the 3rd day, the results were the same in trend 'FL1', but the extent of the inhibiting effect was lower than in 'MLK1'. The increased and decreased levels of *fasciated* transcripts were different between the two varieties after treatment. Our results show that this is caused by the differences in the plant material, which makes it easier for the locule number to be regulated in the 'MLK1' than in 'FL1'.

Feedback and feedfoward are required by plants to maintain the bioactive gibberellins concentrations within a limited range (Hedden and Kamiya, 1997; Ross et al., 1999; Zhong et al., 2001; Olszewski et al., 2002). However, this homeostatic mechanism may be circumvented when the increased production of bioactive GAs is desired during plant development. Increasing the endogenous GAs activity, the relative biosynthesis enzymes activity or genes transcript levels would be changed. For example, the mRNA levels of GA20-oxidase and GA3*β*-hydroxylase was increased by the application of bioactive GAs (Hedden and Kamiya, 1997; Rebers et al., 1999). The recently cloned gene GA2-oxidase (the main gene of degradate biological activity GAs) was also regulated by bioactive GAs (Thomas et al., 1999) and as such, these genes encoded GAs relative enzymes by way of changing their own transcripts to maintain the endogenous GAs at a relatively steady level. Our data showed that GA_3-treated increased the *fasciated* transcript level on the 1st day in apical shoots of the two varieties; this is similar to the result in rice (Dai et al., 2007). This indicates that *fasciated* is associated with GAs relative enzymes in our plant which needs further study for it to be proven testify. But in the long term, GA_3 repressed *fasciated* transcript ion and that is the reason GA_3 can increase the locule number. In leaf of the two varieties, the feedback effects were not observed and it is possible that the feedback is a short period effect. In our previous study, we found that the GA_3 treatment time should be at the beginning of the flower bud differentiation which can make an important effect. The increasing of *fasciated* transcription level on the 1st day could not decrease the locule number. The important factors about the effects of exogenous GA_3 on biosynthesis enzymes and their signal transduction components, requires further study.

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