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## Research Article

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**Posted Date:** May 7th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-479683/v1>

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# Effect of prohexadione-calcium on the shoot growth, fruits, endogenous hormones and expression of GA-related genes in ‘Fuji’ apple (*Malus domestica* Borkh.)

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**Abstract:** The shoot growth of ‘Fuji’ apple is vigorous, which causes a poor lighting condition and a high cost to prune. Prohexadione-calcium (ProCa) has been proven to regulate the shoots growth, fruits and return flowering in apple abroad but there are fewer studies on its effect on the shoot growth, fruits and return flowing in ‘Fuji’ apple in China. Moreover, the mechanism of its action is incompletely understood. So different treatments were applied as foliar sprays to dwarf self-rooted ‘Fubrax Red Fuji’/M9-T337 trees at 3, 14, 35 and 56 DAPF (Days after petal fall). The results showed that at the end of the growing season, spraying ProCa effectively inhibited the growth of shoots compared to control and one spray of 500 and 750 mg L<sup>-1</sup> ProCa, two sprays of 250 mg L<sup>-1</sup> ProCa and four sprays of 250 mg L<sup>-1</sup> ProCa showed the highest reduction. ProCa had very slight effect on yield, quality and return flowing. During the growing season, two applications of ProCa of 250 mg L<sup>-1</sup> on 3 and 14 DAPF significantly reduced the contents of GA<sub>1+3</sub>, IAA and ZR but increased the content of ABA in long terminal shoot leaves in at least one time point. The expression patterns of GA-regulated genes showed that ProCa down-regulated *MdGA3ox* transcript level at 10, 20, 30, 40 and 50 DAPF and down-regulated *MdCPS*, *MdGA20ox*, *MdRGL1a*, *MdSLY1* transcripts in at least one time point. This study provided a valuable insight to regulate the vegetative growth of ‘Fuji’ apple and explored the mechanism of ProCa action.

**Keywords:** *Malus domestica*; Prohexadione-calcium; shoot growth; endogenous hormones; GA-related genes

## 1. Introduction

Among apple cultivars in china, ‘Fuji’ is the dominant cultivars, which accounts for more than 65% of the total cultivated area. The growth of terminal shoots of ‘Fuji’ apple is vigorous, which causes a waste of labor force and a high cost to prune. Various physical and chemical means have been used to reduce vegetative growth, such as pruning and Paclobutrazol spraying. However, Paclobutrazol spraying has caused a series of problems such as fruit type change and quality deterioration<sup>1</sup>. Meanwhile, excessive vegetative growth will lead to mechanical operation difficulties, so it is necessary to regulate the growth of shoots. Fortunately ProCa, a chemical regulator, can inhibit the growth of shoots, which is efficient, economical and less toxic<sup>2-4</sup>.

ProCa is a gibberellin (GA) biosynthesis inhibitor, which blocks the conversion of GA<sub>20</sub> to GA<sub>1</sub> by acting on the enzyme involved in GA<sub>20</sub> converted into GA<sub>1</sub> pathway<sup>5</sup>. Gibberellin as an important plant hormone is involved in the regulation of vegetative growth. Seven key enzymes, including ent-copalyl diphosphate synthase (*CPS*), ent-kaurene synthase (*KS*), ent-kaurene oxidase (*KO*), ent-kaurenoic acid oxidase (*KAO*), GA 20-oxidase (*GA20ox*), GA 3-oxidase (*GA3ox*), GA 2-oxidase (*GA2ox*) involved in GA biosynthesis and deactivation<sup>6-8</sup>. First, a geranyl pyrophosphate (GGPP) is synthesized from four five-carbon isoprenoids. This step is catalyzed by ent-copalyl diphosphate synthase (*CPS*). Subsequently, GGPP is catalyzed by ent-kaurene synthase (*KS*) to ent-kaurene, an important precursor of gibberellin. Second, ent-kaurene is gradually catalyzed to ent-kaurenol, ent-kaurenal and ent-kaurenoic acid, via ent-kaurene oxidase (*KO*). Subsequently, the ent-kaurenoic acid is gradually catalyzed to ent-7 $\alpha$ -Hydroxykaurenoic acid and GA<sub>12</sub> under the catalysis of ent-kaurene oxidase (*KAO*). Third, GA<sub>12</sub> is oxidized by GA 20-oxidase (*GA20ox*) and GA 13-oxidase (*GA13ox*) to GA<sub>15</sub> and GA<sub>13</sub>, respectively, and then the synthesis process of gibberellin is divided into two main ways. The first one is that GA<sub>15</sub> is gradually converted into GA<sub>24</sub> (C<sub>20</sub>-GA) and GA<sub>9</sub> (C<sub>19</sub>-GA) under the continuous oxidation of GA 20-oxidase (*GA20ox*), and then converted into bioactive GA<sub>4</sub> under the oxidation of GA 3-oxidase (*GA3ox*). The bioactive GA<sub>4</sub> is finally degraded to inactive GA<sub>34</sub> by GA 2-oxidase (*GA2ox*). The second one

is that GA<sub>53</sub> is gradually converted into GA<sub>44</sub> and GA<sub>19</sub>, and finally converted into GA<sub>20</sub> under the catalysis of GA 20-oxidase (*GA20ox*), then which is oxidized to GA<sub>1</sub> by GA 3-oxidase (*GA3ox*). Finally, GA<sub>1</sub> is deactivated to inactive GAs due to the catalysis of GA 2-oxidase (*GA2ox*)<sup>9-11</sup>. GID1 (for *GA INSENSITIVE DWARF1*) is GA receptor protein. GAs promote stem elongation by stimulating the degradation of the growth repressing DELLA proteins via the ubiquitin-proteasome pathway<sup>12,13</sup>. The degradation of DELLA is triggered by a GA-GID1-DELLA complex, which is then identified by a specific ubiquitin E3 ligase complex (SCF<sup>SLY1/GID2</sup>) for polyubiquitination and subsequently degraded by the 26S proteasome<sup>14-17</sup>. Six genes encoding DELLA protein, *MdRGL1a/b*, *MdRGL2a/b* and *MdRGL3a/b*, have been identified in apples<sup>18</sup>. The transgenic tobacco displayed dwarf phenotypes and 75% flowered earlier than no transgenic control by the overexpression of *MdRGL1a*<sup>19</sup>. ProCa blocks the synthesis of GA<sub>1</sub>, thus inhibiting the growth of shoots, promoting the growth of reproduction, and achieving the purpose of increasing production and improving quality<sup>20</sup>. It has been suggested that the use of ProCa was a good practice for controlling vegetative, especially in apple and pear trees<sup>21-23</sup>. The applications of 125 mg L<sup>-1</sup> ProCa on the same trees for three years resulted in a 40-43% shoot length reducing. Internodes length decreased at about 30%, while the number of total nodes was unaffected<sup>24</sup>. Miller<sup>25</sup> proposed that in shoot growth control, ProCa applied as single spray or multiple low-rate sprays at petal fall (PF) or within 10 days after PF were better than at 2-3 weeks after PF, and the time of initial spray was more critical in achieving early shoot growth control than rate, but to achieve season-long shoot growth suppression, higher rate was needed. There was no effect on the quality and fruit size in the current and next year. Byers<sup>26</sup> studied that three applications of ProCa in deionized water applied to 'Fuji'/M.9 apple tree reduced shoot growth by about 25%, up to 47% if adding ammonium sulfate. The combination of ProCa and well water (high in calcium salts) made the ProCa invalid, but its effect recovered when ammonium sulfate was added.

There are a lot of experiments abroad to prove that the ProCa can inhibit the vegetative growth of apple. In China, the applications of ProCa in peanut, wheat and rice could significantly inhibit the vegetative growth of aboveground, make plants robust and promote reproductive growth<sup>2,27,28</sup>. Postharvest using with ProCa visibly reduced the occurrence of bitter pit during fruit storage<sup>29</sup>. However, there are fewer relevant studies on the effects of ProCa on apple shoot growth, fruits, endogenous hormones, the expression of GA-related genes and return flowering. In this study, the growth of terminal shoots, yield, fruit quality, the expression of GA-regulated genes and return flowering were identified. The contents of endogenous hormones in leaves were also analyzed. The goals of this research were to explore a better date and rate of ProCa application to regulating vegetative growth in 'Fuji' apple tree and the mechanism of ProCa action.

## 2. Results

### 2.1 Shoot growth

The effect of ProCa on inhibiting shoot growth was the most significant in a month after flowering as a result of the growth in this stage was more than 60% of whole year growth (Table 1). The cessation date of spring shoots was 20 May in ProCa treatments and 29 May in control. The spring mean shoot length of ProCa treatments was approximately 30.0 cm, reduced by about 30% compared with control (43.6 cm) (Table 1). The differences of spring mean shoot lengths on spraying 500 and 750 mg L<sup>-1</sup> once and 250 mg L<sup>-1</sup> four times were small (Table 1).

The shoot extension lengths of 500 and 750 mg L<sup>-1</sup> applied once, 125 and 250 mg L<sup>-1</sup> applied twice, 62.5, 125 and 250 mg L<sup>-1</sup> applied four times were 5.0 cm, 3.2 cm, 4.2 cm, 4.5 cm, 3.1 cm, 3.7 cm and 2.8 cm, respectively, from 26 May to 25 Sep. The shoot extension length was about 10.0 cm in control and other ProCa treatments. The results showed that ProCa had better effects on inhibiting autumn shoot growth with early single high concentration or multiple low concentrations.

The average shoot growth of 500 and 750 mg L<sup>-1</sup> applied once, 250 mg L<sup>-1</sup> applied four times were 29.8 cm, 28.4 cm and 28.8 cm, respectively, which were about 43.8%, 46.4% and 45.7% lower than control (53.0 cm) by Sep. The differences of the average shoot growth in other treatments were small, which indicated that the early single high

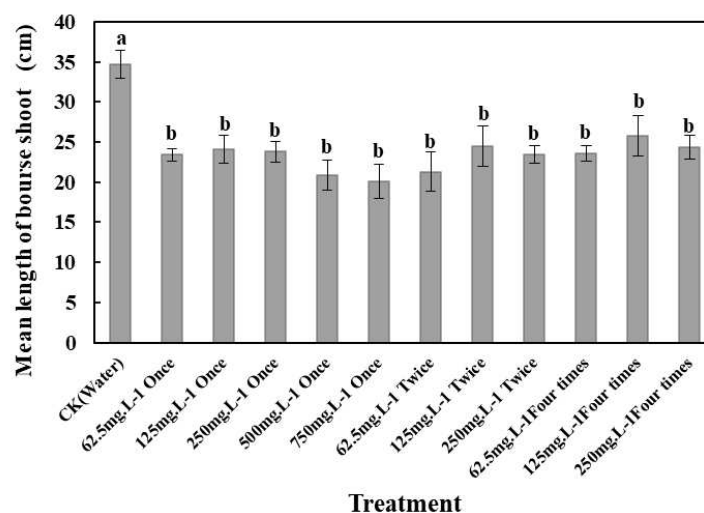
concentration and multiple low concentrations could inhibit the vegetative growth of Fuji apple tree very well (Table 1; Fig.2).

ProCa had significant effect on the growth of bourse shoot. The length of bourse shoot measured on 25 Sep in control group was about 60.0 cm and in ProCa group applied for four times was only about 20.0 cm. (Fig.3). At the end of the growing season, the mean lengths of the bourse shoot of all ProCa treatments were about 23.0 cm, which had a decrease at about 34% than control (34.7 cm) (Fig.1).

**Table 1.** Effect of ProCa application on shoot growth in Fuji apple tree.

Treatments ProCa (mg L <sup>-1</sup> )	No. applications	Mean shoot length							
		25 Apr (cm)	10 May (cm)	26 May (cm)	13 Jun (cm)	01 Jul (cm)	19 Jul (cm)	20 Aug (cm)	25 Sep (cm)
0	-	16.9 <sup>a</sup>	33.7 <sup>a</sup>	43.6 <sup>a</sup>	46.9 <sup>a</sup>	50.0 <sup>a</sup>	52.2 <sup>a</sup>	52.8 <sup>a</sup>	53.0 <sup>a</sup>
62.5	1	17.1 <sup>a</sup>	29.0 <sup>b</sup>	34.3 <sup>b</sup>	36.3 <sup>b</sup>	43.8 <sup>a</sup>	45.1 <sup>ab</sup>	45.5 <sup>ab</sup>	45.3 <sup>ab</sup>
125	1	17.0 <sup>a</sup>	26.0 <sup>bc</sup>	28.1 <sup>bc</sup>	29.7 <sup>bc</sup>	32.4 <sup>b</sup>	37.2 <sup>bc</sup>	34.8 <sup>bc</sup>	33.9 <sup>bc</sup>
250	1	16.7 <sup>a</sup>	25.5 <sup>bc</sup>	27.6 <sup>bc</sup>	30.1 <sup>bc</sup>	33.4 <sup>b</sup>	36.5 <sup>bc</sup>	36.0 <sup>c</sup>	36.5 <sup>c</sup>
500	1	16.3 <sup>a</sup>	21.9 <sup>c</sup>	24.8 <sup>c</sup>	26.0 <sup>c</sup>	28.0 <sup>b</sup>	28.3 <sup>c</sup>	29.7 <sup>c</sup>	29.8 <sup>c</sup>
750	1	15.9 <sup>a</sup>	24.2 <sup>bc</sup>	25.2 <sup>c</sup>	26.8 <sup>c</sup>	27.5 <sup>b</sup>	28.2 <sup>c</sup>	28.9 <sup>c</sup>	28.4 <sup>c</sup>
62.5	2	16.1 <sup>a</sup>	25.5 <sup>bc</sup>	27.5 <sup>bc</sup>	30.3 <sup>b</sup>	30.4 <sup>b</sup>	31.5 <sup>c</sup>	33.0 <sup>c</sup>	33.7 <sup>c</sup>
125	2	16.3 <sup>a</sup>	28.8 <sup>b</sup>	29.6 <sup>bc</sup>	30.7 <sup>b</sup>	32.2 <sup>b</sup>	33.0 <sup>c</sup>	33.5 <sup>c</sup>	33.8 <sup>c</sup>
250	2	17.1 <sup>a</sup>	26.3 <sup>bc</sup>	28.1 <sup>bc</sup>	28.2 <sup>c</sup>	30.1 <sup>b</sup>	32.1 <sup>c</sup>	32.3 <sup>c</sup>	32.6 <sup>c</sup>
62.5	4	16.8 <sup>a</sup>	26.9 <sup>b</sup>	28.9 <sup>bc</sup>	28.6 <sup>b</sup>	30.5 <sup>b</sup>	30.6 <sup>c</sup>	32.1 <sup>c</sup>	32.0 <sup>c</sup>
125	4	16.6 <sup>a</sup>	27.1 <sup>b</sup>	28.1 <sup>bc</sup>	28.4 <sup>c</sup>	29.6 <sup>b</sup>	32.4 <sup>c</sup>	32.7 <sup>c</sup>	31.8 <sup>c</sup>
250	4	15.3 <sup>a</sup>	25.6 <sup>bc</sup>	26.0 <sup>c</sup>	25.9 <sup>c</sup>	27.1 <sup>b</sup>	28.2 <sup>c</sup>	28.2 <sup>c</sup>	28.8 <sup>c</sup>

Within a column, different letters (a, b) indicate significant difference at the 0.05 level.



**Figure 1.** Mean length of bourse shoot at the end of growing season; Within each column, different letters (a, b) indicate significant differences at the 0.05 level.



**Figure 2.** The 'Fuji' apple trees for control and 250 mg L<sup>-1</sup> ProCa applied for four times.

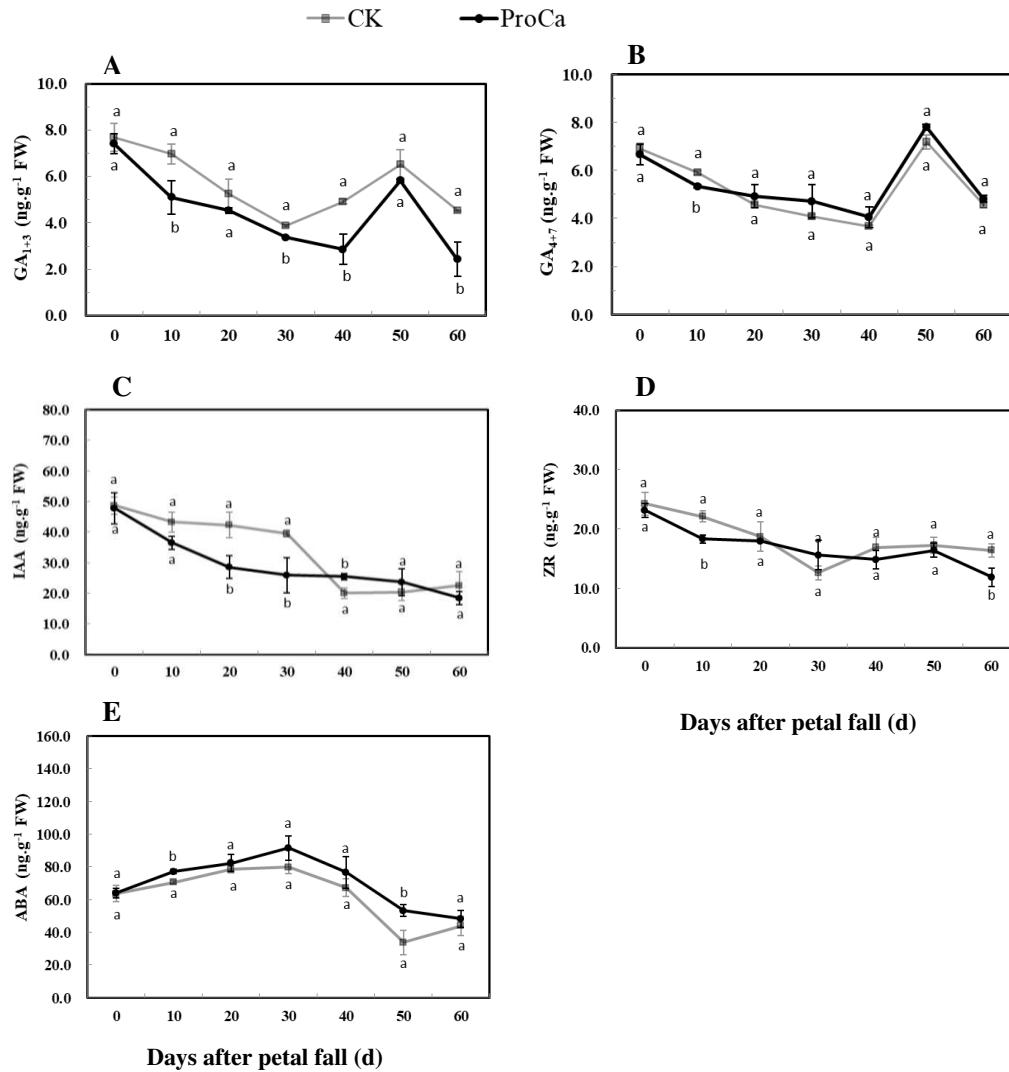


**Figure 3.** Bourse shoot length measured on 25 Sep. (A) The length of bourse shoot in control; (B) The length of bourse shoot in ProCa applications for four times.

## 2.2 Hormone contents in leaves of long terminal shoot

Several endogenous hormones, including GA<sub>1+3</sub>, GA<sub>4+7</sub>, IAA, ZR, ABA, were analyzed in leaves of long terminal shoots. In all time points during the analysis period, it can be seen from Fig. 4A that the content of GA<sub>1+3</sub> was higher in control leaves than in ProCa-treated leaves, while the content of ABA was higher in ProCa-treated leaves than in control leaves (Fig.4E). Additionally, ProCa notably reduced the content of GA<sub>1+3</sub> at 10, 30, 40 and 60 DAPF (Fig.4A) and increased the content of ABA at 10 and 50 DAPF (Fig.4E). GA<sub>4+7</sub> levels were not significantly different during the analyzed time points, except at 10 DAFB (Fig. 4B). And the results showed that ProCa significantly reduced the content of IAA at 20 and 30 DAPF (Fig.4C). Compared with control leaves, the leaves treated by ProCa had relatively stable but low ZR content, which was significantly reduced at 10 and 60 DAPF. ZR levels in Control leaves were higher than that in ProCa-treated leaves in all time points, except for 30 DAPF (Fig.4D).

From the results showed in Fig.4, we concluded that ProCa treatment dramatically affected the contents of GA<sub>1+3</sub>, ZR, IAA and ABA in at least one time point.

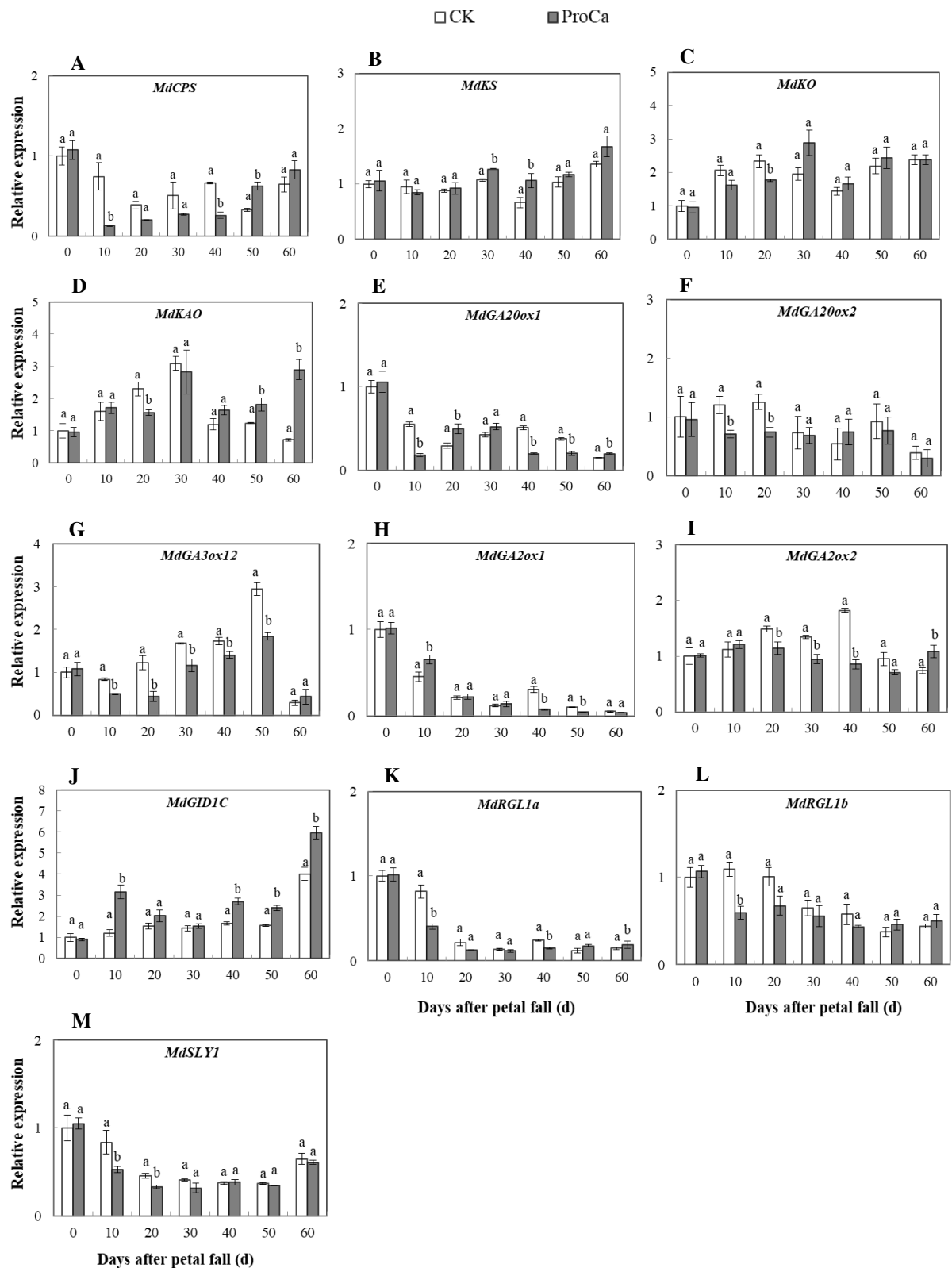


**Figure 4.** Hormone levels of zeatin-riboside (ZR),  $\text{GA}_{1+3}$ ,  $\text{GA}_{4+7}$ , IAA, abscisic acid (ABA) in leaves of long terminal shoots. Values represent means  $\pm$  SE (n=3). Different letters (a, b) indicate a significant difference at the 0.05 level.

### 2.3 Expression of GA-related genes in leaves of long terminal shoots

We analyzed several important GA biosynthetic and signal transduction genes, including *MdCPS*, *MdKO*, *MdKS*, *MdKAO*, *MdGA2ox*, *MdGA3ox*, *MdGA20ox*, *MdGID1*, *MdRGL1* and *MdSLY1* as showed in Fig.5A-M. Ent-kaurene is oxidized to  $\text{GA}_{12}$  by *MdCPS*, *MdKS*, *MdKO* and *MdKAO*. *MdCPS* expression was obviously down-regulated in response to ProCa at 10 and 40 DAPF (Fig.5A). Exogenous ProCa had a slightly influence on *MdKO* and *MdKS* expression levels in almost analysis time points but significantly down-regulated *MdKO* at 20 DAPF, up-regulated *MdKS* at 30 and 40 DAPF (Fig. 5C and B). A total trend of an increase in the expression of *MdKAO* prior to 30 DAPF and a decrease from 30 to 60 DAPF were observed in both group trees with a significantly decrease at 20 DAPF and increase at 50 and 60 DAPF in ProCa treated trees compared with control trees (Fig.5D). *MdGA20ox* and *MdGA3ox* involved in the conversion of non-bioactive GAs into bioactive GAs. The abundance of *MdGA20ox1* transcripts was significantly lower in response to ProCa at 10, 40 and 50 DAPF, and the *MdGA20ox2* expression level was considerably down-regulated at 10 and 20 DAPF compared with control (Fig.5E and F). Moreover, *MdGA3ox12* expression peaked at 50 DAPF and notably down-regulated from 50 to 60 DAPF in both group trees. Noticeably, ProCa had a big effect on

the expression level of *GA3ox12* which was significantly down-regulated in ProCa-treated plants at 10, 20, 30, 40 and 50 DAPF (Fig.5G). We also detected *MdGA2ox1* and *MdGA2ox2* expression patterns, the GA deactivation genes. *MdGA2ox1* expression was consistently declined from the day treated with ProCa to 60 DAPF, while *MdGA2ox2* exhibited the steadily expression level (Fig.5E and F). Additionally, the expression level of *MdGA2ox2* was significantly down-regulated in ProCa-treated plants at 20, 30 and 40 DAPF and up-regulated at 60 DAPF. A considerable increase in the expression of *MdGID1C*, encoding a GA reporter, was detected in the latter sampling dates (Fig.5J). Expression of *RGL1a* and *RGL1b* in both group leaves was initially high and then subsequently declined, notably, both of them were down-regulated at 10 DAPF (Fig.5K and L). *MdSLY1*, which involved in the degradation of DELLA proteins, exhibited a lower expression level in ProCa-treated leaves than in control leaves at 10 and 20 DAPF (Fig.5M).



**Figure 5.** Expression patterns of GA-related genes in leaves of long terminal shoots. Values represent means  $\pm$  SE ( $n=3$ ). Different letters (a, b) indicate a significant difference at the 0.05 level.



## 2.4 Fruit quality

Results showed that there were no significant differences on L/D and SSC between the ProCa treatments and the control. The SSC was 13-15 °Brix and the L/D was approximately 0.87. The flesh firmness of fruit was 6.6 -7.5 kg cm<sup>-2</sup>, which increased by 0.2 kg cm<sup>-2</sup> when spraying ProCa once at 62.5mg L<sup>-1</sup> and decreased by 0.7 kg cm<sup>-2</sup> when spraying ProCa four times at 62.5mg L<sup>-1</sup> compared with control (7.3 kg cm<sup>-2</sup>) (Table 2). L\*, a\*, b\* value are important index to evaluate the appearance of fruits, in which L\* is the brightness, the higher the value, the higher the brightness. a\* is the red and green colour, the larger the value, the higher the red colour, reversely the green colour. b\* is yellow and blue colour, the larger the value, the higher the yellow colour, reversely the blue colour<sup>30</sup>. As shown in Table 2, the maximum of L\*, a\*, b\* were 50.9 for 250 mg L<sup>-1</sup> ProCa applied once, 28.2 for 125 mg L<sup>-1</sup> ProCa applied four times, 12.9 for 250 mg L<sup>-1</sup> ProCa applied once, respectively. The minimum L\*, a\*, b\* were 45.0 for 125 mg L<sup>-1</sup> ProCa applied four times, 23.0 for 250 mg L<sup>-1</sup> ProCa applied once, 11.2 for 62.5 and 250 mg L<sup>-1</sup> ProCa applied four times, with a range of 5.9, 6.2 and 1.7, respectively. The results showed that there are no obvious differences of the value of L\* in control and ProCa treatments except for 125 mg L<sup>-1</sup> applied for four times, meanwhile, the values of a\* and b\* in control and ProCa treatments had no significant differences (Table 2).

In terms of TA, there was no significant difference between the ProCa treatments and the control. The significant difference occurred between 125 mg L<sup>-1</sup> ProCa spraying once and 125 mg L<sup>-1</sup> ProCa spraying twice. The ratio of SSC to TA in high-quality apple is 20-60<sup>31</sup>. The maximum and minimum values in ProCa treatments were 60.8 and 39.1, respectively, which meets the standard of high quality apple ratio of SSC to TA, ProCa slightly decreased the ratio of SSC to TA compared with control (63.1) (Table 2).

**Table 2.** Effect of ProCa application on fruit quality of Fuji apple tree in 2019.

Treatments ProCa (mg L <sup>-1</sup> )	No. Applications	Fruit shape (L/D)	Flesh firmness (kg/cm <sup>2</sup> )	SSC (°Brix)	TA	RSA	L	A	B
0	-	0.87 <sup>a</sup>	7.3 <sup>ab</sup>	13.6 <sup>a</sup>	0.216 <sup>bc</sup>	63.1 <sup>a</sup>	49.7 <sup>ab</sup>	24.3 <sup>ab</sup>	12.3 <sup>ab</sup>
62.5	1	0.88 <sup>a</sup>	7.5 <sup>a</sup>	13.6 <sup>a</sup>	0.223 <sup>bc</sup>	60.8 <sup>a</sup>	48.9 <sup>ab</sup>	24.5 <sup>ab</sup>	11.8 <sup>ab</sup>
125	1	0.86 <sup>a</sup>	7.3 <sup>ab</sup>	13.6 <sup>a</sup>	0.259 <sup>c</sup>	52.4 <sup>a</sup>	47.9 <sup>abc</sup>	26.3 <sup>ab</sup>	11.5 <sup>b</sup>
250	1	0.87 <sup>a</sup>	6.7 <sup>c</sup>	13.7 <sup>a</sup>	0.259 <sup>abc</sup>	52.8 <sup>a</sup>	50.9 <sup>a</sup>	23.0 <sup>b</sup>	12.9 <sup>a</sup>
500	1	0.87 <sup>a</sup>	6.6 <sup>c</sup>	13.6 <sup>a</sup>	0.270 <sup>abc</sup>	50.4 <sup>a</sup>	47.2 <sup>abc</sup>	26.9 <sup>ab</sup>	11.4 <sup>b</sup>
750	1	0.87 <sup>a</sup>	7.0 <sup>abc</sup>	14.1 <sup>a</sup>	0.276 <sup>ab</sup>	50.9 <sup>a</sup>	47.3 <sup>abc</sup>	27.1 <sup>a</sup>	11.4 <sup>b</sup>
62.5	2	0.87 <sup>a</sup>	6.7 <sup>c</sup>	13.8 <sup>a</sup>	0.319 <sup>ab</sup>	43.1 <sup>ab</sup>	46.6 <sup>bc</sup>	27.7 <sup>a</sup>	11.2 <sup>b</sup>
125	2	0.86 <sup>a</sup>	7.0 <sup>bc</sup>	13.6 <sup>a</sup>	0.348 <sup>a</sup>	39.1 <sup>bc</sup>	46.2 <sup>bc</sup>	26.6 <sup>ab</sup>	11.9 <sup>ab</sup>
250	2	0.86 <sup>a</sup>	6.9 <sup>bc</sup>	13.4 <sup>a</sup>	0.321 <sup>ab</sup>	41.6 <sup>ab</sup>	49.2 <sup>ab</sup>	25.1 <sup>a</sup>	12.1 <sup>ab</sup>
62.5	4	0.91 <sup>a</sup>	6.6 <sup>c</sup>	13.5 <sup>a</sup>	0.320 <sup>ab</sup>	42.2 <sup>ab</sup>	49.6 <sup>ab</sup>	25.7 <sup>ab</sup>	12.1 <sup>ab</sup>
125	4	0.87 <sup>a</sup>	6.7 <sup>c</sup>	13.4 <sup>a</sup>	0.229 <sup>bc</sup>	58.7 <sup>a</sup>	45.0 <sup>c</sup>	28.2 <sup>a</sup>	11.6 <sup>ab</sup>
250	4	0.89 <sup>a</sup>	6.7 <sup>c</sup>	13.5 <sup>a</sup>	0.322 <sup>ab</sup>	41.9 <sup>ab</sup>	45.8 <sup>bc</sup>	27.8 <sup>a</sup>	11.2 <sup>b</sup>

Within a column, different letters (a, b) indicate significant difference at the 0.05 level.

## 2.5 Fruit yield in 2019 and return flowering in 2020

Contrast analysis of ProCa treatments vs control revealed that ProCa had no or little effects on the average number per tree and yield. The highest average number was 115 for ProCa applied four times at 150 mg L<sup>-1</sup>, the lowest average numbers was 88 for ProCa applied once at 750 mg L<sup>-1</sup> and four times at 250 mg L<sup>-1</sup>. The average yield was 22.2-36.7 kg with a range change of 14.5 kg. The lowest value occurred in 750 mg L<sup>-1</sup> ProCa applied once, which was 7 kg less than control (29.2 kg). Compared with the control, the average yield decreased at 250 mg L<sup>-1</sup>, 500 mg L<sup>-1</sup> and 750 mg L<sup>-1</sup>

spray once, 150 mg L<sup>-1</sup> spray twice and 250 mg L<sup>-1</sup> spray four times, which indicated that the single higher concentration and multiple low concentration applications of ProCa had negative impact on the average yield. The average fruit weight was 251.7-319.0 kg. The minimum average fruit weight appeared at 750 mg L<sup>-1</sup> spray once. In conclusion, the average number, yield and fruit weight of ProCa with higher concentration were decreased (Table 3).

In terms of the influence of ProCa on return flowing, results showed that the number of cluster per tree was from 35 to 52 and flower cluster density was 1.4-2.2. The minimum of flower clusters per tree was 35 at 500 mg L<sup>-1</sup> spray once, decrease by 18.7 % compared with control. The maximum of flower cluster density was 2.2 at 125 mg L<sup>-1</sup> spray once, increased by 15.8% (Table 3). Meanwhile, there was no significant difference on the number of clusters per tree between the ProCa treatments and the control except for an obvious increase at 125 mg L<sup>-1</sup> ProCa applied once. In addition, the difference of flower cluster density between the ProCa treatments and the control was small.

**Table 3.** Effect of ProCa application on fruit yield of Fuji apple tree in 2019 and return flower in 2020.

Treatments ProCa (mg.L <sup>-1</sup> )	No. applications	Mean number Tree <sup>-1</sup>	Yield (kg tree <sup>-1</sup> )	Fruit weight (g)	Yield efficiency (kg cm <sup>-2</sup> TCSA)	No. flower clusters per tree	Flower cluster density (flowers cm <sup>-2</sup> TCSA)
		2019				2020	
0	-	102 <sup>a</sup>	29.2 <sup>ab</sup>	285.8 <sup>abc</sup>	1.3 <sup>a</sup>	43 <sup>cd</sup>	1.9 <sup>bc</sup>
62.5	1	107 <sup>a</sup>	29.8 <sup>ab</sup>	278.7 <sup>bc</sup>	1.2 <sup>a</sup>	44 <sup>abc</sup>	1.8 <sup>bc</sup>
125	1	105 <sup>a</sup>	30.9 <sup>ab</sup>	294.0 <sup>ab</sup>	1.2 <sup>a</sup>	52 <sup>ab</sup>	2.2 <sup>ab</sup>
250	1	100 <sup>a</sup>	26.9 <sup>ab</sup>	269.0 <sup>bc</sup>	1.0 <sup>a</sup>	44 <sup>abc</sup>	1.7 <sup>bc</sup>
500	1	103 <sup>a</sup>	27.3 <sup>ab</sup>	264.8 <sup>bc</sup>	1.2 <sup>a</sup>	35 <sup>d</sup>	1.5 <sup>bc</sup>
750	1	88 <sup>a</sup>	22.2 <sup>b</sup>	251.7 <sup>c</sup>	1.1 <sup>a</sup>	39 <sup>cd</sup>	2.0 <sup>abc</sup>
62.5	2	107 <sup>a</sup>	29.3 <sup>ab</sup>	273.6 <sup>bc</sup>	1.2 <sup>a</sup>	40 <sup>cd</sup>	1.6 <sup>bc</sup>
125	2	92 <sup>a</sup>	25.5 <sup>ab</sup>	277.4 <sup>bc</sup>	1.0 <sup>a</sup>	43 <sup>bcd</sup>	1.7 <sup>bc</sup>
250	2	99 <sup>a</sup>	29.4 <sup>ab</sup>	296.7 <sup>ab</sup>	1.2 <sup>a</sup>	44 <sup>bcd</sup>	1.9 <sup>abc</sup>
62.5	4	106 <sup>a</sup>	33.8 <sup>ab</sup>	319.0 <sup>a</sup>	1.3 <sup>a</sup>	38 <sup>cd</sup>	1.4 <sup>c</sup>
125	4	115 <sup>a</sup>	36.7 <sup>a</sup>	318.7 <sup>a</sup>	1.3 <sup>a</sup>	45 <sup>abc</sup>	1.6 <sup>bc</sup>
250	4	88 <sup>a</sup>	26.2 <sup>ab</sup>	297.9 <sup>b</sup>	1.2 <sup>a</sup>	40 <sup>cd</sup>	1.8 <sup>bc</sup>

Within a column, different letters (a, b) indicate significant difference at the 0.05 level.

### 3. Discussion

#### 3.1 The effect of ProCa on shoot growth

Previous studies have shown that ProCa was an effective plant growth regulator which reduced plant height, increased thickness and enhanced lodging resistance when used in wheat and rice, increased pods and enhanced photosynthesis when used in peanuts, suppressed the length of terminal shoots when used in fruit trees<sup>28, 32</sup>. There were many researches on the application of ProCa on apple trees abroad and the results showed that ProCa had a very good effect on reducing vegetative growth of different varieties of apple trees. The early application was more important than the spraying rate to achieve a better inhibition effect and multiple sprays could effectively inhibit autumn growth. The results of this experiment showed that ProCa applied to 'Fubrax Red Fuji'/M9-T337 in different concentrations and spraying times could reduce the growth of terminal shoots and bourse shoots at different degrees as compared to control (Table 1, Fig. 1). Besides, the cessation date of terminal shoots in ProCa treatments was about 20 May and about ten

days different from control (29 May). But which had a difference of about 2-3 days in ProCa treatments. At the end of the growing season, the single high concentration in the early and multiple low concentrations had the best effects on inhibiting the growth of spring and autumn shoots, which is consistent with the results of many studies<sup>33-36</sup>. When the terminal shoots stopped growing, the top leaves of them applied ProCa became significantly smaller than that of the control (Fig.1), which was the same as the research of Medjdoub et al.<sup>37</sup>. ProCa decreased the growth of shoots by shortening the length of the nodes rather than the number of nodes<sup>38</sup>, which also been observed in our study (Fig. 2).

### 3.2 The effect of ProCa on fruits and return flowering

Unrath (1999) and Greene (2008) have studied that after blooming, the shoot growth was very fast and the total growth in spring reached more than 50% of the annual growth. Therefore, the early application of ProCa was very important. On the other hand, cell division was strongest during this period, which was directly related to the fruit size at harvest. So, cell division should not be reduced and a lower concentration of ProCa was recommended<sup>39-40</sup>. Our results showed that there was little difference on fruit yield between the ProCa treatments and the control except that spraying at high concentration of 750 mg L<sup>-1</sup> on 3 DAPF, and at 250 mg L<sup>-1</sup> on 3, 14, 35 and 56 DAPF reduced the yield and number per tree as compared to control. In our study ProCa slightly reduced the flesh firmness of fruits and had no effect on L/D, SSC and TA compared with control. In addition, ProCa had no facilitation on the colour of apple fruits. The specific yield was about 1.3 kg cm<sup>-2</sup> in ProCa treatments and control. Which were consistent with that ProCa application had slight effect on fruit yield in the report of Miller et al.<sup>25</sup>. The results of return flowering indicated that ProCa had no obvious effect on the number of cluster per tree and flower cluster density (Table 3).

### 3.3 The effect of ProCa on endogenous hormones

Several studies have shown that IAA, GA and ZR are growth promoting hormones, ABA are growth inhibiting hormones<sup>41-42</sup>. The interaction of GA<sub>3</sub> and IAA led to the growth of terminal shoots<sup>43</sup>. And there was a certain correlation between IAA content and the growth of terminal shoots<sup>44</sup>. In this study, GA<sub>1+3</sub> content of leaves in long terminal shoots applied ProCa at 250 mg L<sup>-1</sup> twice was significantly lower than that of the control at 10, 30, 40 and 50 DAPF, and the content of it decreased when the terminal shoots stopped growing, which was consistent with the growth rule of terminal shoots. Additionally, GA<sub>4+7</sub> has a similar trend with GA<sub>1+3</sub>, however, ProCa has slightly influence on the contents of GA<sub>4+7</sub>. Previous studies have shown that there are two ways to produce GA<sub>1</sub> and GA<sub>4</sub>, the first one is that GA<sub>15</sub> is gradually converted into bioactive GA<sub>4</sub> and the second one is that GA<sub>53</sub> is gradually converted into GA<sub>1</sub>, the difference of two ways is that the C<sub>13</sub> position of all the products involved in the second one of the routes has a hydroxyl group called C<sub>13</sub> hydroxylation path, and the C<sub>13</sub> position of all the products in the other reaction process does not carry a hydroxyl group, so it is called C<sub>13</sub> Non-hydroxylation path<sup>9</sup>. Consequently, we conclude that ProCa only has effect on the biosynthesis of GA<sub>1+3</sub>, which was similar with the study that ProCa has shown to be a useful growth regulator due to its ability to inhibit gibberellin biosynthesis by blocking 3β-Hydroxylation which involved in the pathway of GA<sub>20</sub> translated into GA<sub>1</sub><sup>6</sup>. Previous study observed that ProCa increased the content of ABA and ZR in the plant<sup>45</sup>. However, the results of our study showed that the content of ZR in control leaves of long terminal shoots was lower than in ProCa treated leaves, but only at 10 and 60 DAPF had obvious differences. ProCa significantly reduced the content of IAA at 20 and 30 DAPF, but increased the content of it at 40 DAPF. The effect of ABA on the growth was opposite to IAA, GA<sub>1+3</sub> and ZR, it inhibited the cell division and elongation, inhibited the elongation growth of new shoots. The content of ABA in this experiment in ProCa treated leaves was lower than in control leaves during the all analysis times but only at 10 and 50 DAPF ProCa significantly reduced the content of it.

### 3.4 The effect of ProCa on the expression of GA-related genes

To provide evidence for the influence of applied ProCa on the metabolism of GA in terminal shoots, we measured transcript levels of several important GA-related genes. Previous studies have found that the transcription levels of these

four key genes, *MdCPS*, *MdKS*, *MdKAO*, *MdKO* were related to the growth rate of organs and higher in the period of vigorous growth<sup>10</sup>. Itoh<sup>46</sup> found a mutation in the *KO* gene of a semi-dwarf rice, which was classified as a GA-deficient mutant. The GA level of this mutant decreased and led to semi-dwarf traits. *GA20ox* and *GA3ox* are involved in inactive GAs converted into bioactive GAs pathway. During periods of vigorous growth, the transcription level is relatively high and the amount of bioactive GA synthesized was reduced in the absence of *GA20ox* and *GA3ox*. Similarly, overexpression of *GA20ox* degraded the bioactive GA in plants, resulting in a dwarfed phenotype<sup>47-49</sup>. In our study, we discovered that ProCa inhibit the elongation of long terminal shoots by decreasing the transcript level of *GA3ox12* in almost all analysis times (Fig.5C) and interestingly, we also found that ProCa influenced the expression levels of other GA-related genes, such as down-regulating *MdGA20ox*, *MdCPS*, *MdRGL1a*, *MdKO* expression levels in at least one time point, which significantly restrained GA biosynthesis from 10 to 40 DAPF (Fig.5D, E, F and K). However, whether or not ProCa has any effect on other genes that associated to plant growth still needs to be further explored.

#### 4. Conclusion

In conclusion, the effect of single early high dose and multiple low dose of ProCa on control vegetative growth was significant, had a decrease by approximately 45% as compared to control. Meanwhile, the differences on fruit yield and quality between ProCa treatments and control were very small. According to the analysis of hormone contents, the inhibition effect of ProCa was achieved mainly by reducing the contents of GA<sub>1+3</sub>. The current study revealed that ProCa reduce the growth of shoots mainly due to down-regulated *GA3ox*. And in terms of economic benefits, it is recommended to spray ProCa at 125 or 250 mg L<sup>-1</sup> once or twice in apple orchards of the Loess Plateau and to achieve the best effect on reducing the growth of shoots the early ProCa application was very important when the length of terminal shoots was about 10.0 cm.

#### 5. Materials and methods

##### 5.1 Plant materials growth conditions and treatments

A 6-year-old research orchard of 'Fubrax Red Fuji' /M9-T337 located at Cuijiatou town (107°13 'E, 34 °37 'N) of shaanxi province, china, planted at a spacing of 1 m×3.5 m was used for this study. Trees were in uniform and treated with normal practice. And study complied with local and national regulations.

Twelve treatments applied as follows: (1) Control; (2) 62.5 mg L<sup>-1</sup> applied 3 DAPF; (3) 125 mg L<sup>-1</sup> applied 3 DAPF; (4) 250 mg L<sup>-1</sup> applied 3 DAPF; (5) 500 mg L<sup>-1</sup> applied 3 DAPF; (6) 750 mg L<sup>-1</sup> applied 3 DAPF; (7) 62.5mg L<sup>-1</sup> applied 3+14 DAPF; (8) 125 mg L<sup>-1</sup> applied 3+14 DAPF; (9) 250 mg L<sup>-1</sup> applied 3+14 DAPF; (10) 62.5 mg L<sup>-1</sup> applied 3+14+35+56 DAPF; (11) 125 mg L<sup>-1</sup> applied 3+14+35+56 DAPF; (12) 250 mg L<sup>-1</sup> applied 3+14 +35+56 DAPF. The experiment was laid out in a randomized complete block design with four trees. Single tree was treated as an experimental unit. The trees were not pruned during the course of the study. Spray treatments were applied to run-off with a low-pressure hand-wand sprayer. All treatments included ammonium sulfate as a water conditioner to adjust the PH value to about 6.5 and finally added 0.1% (V/V) Tween-20 to increase the efficiency of the chemicals. At least one guard tree was included between experimental units. The first spray was made on 24 April 2019, when average terminal shoot growth was about 12.0 cm. Petal Fall occurred on approximately 21 April 2019.

##### 5.2 Assessment of shoot growth

20 terminal shoots were randomly selected and tagged per tree, including long shoots, water sprouts and bourse shoots. Twenty shoots were measured at the time of the initial spray application and bi-weekly until the end of growing season.

##### 5.3 Determination of endogenous hormones in leaves of long shoots

Long terminal shoot leaves treated with 250 mg L<sup>-1</sup> ProCa twice and control were collected on 24 Apr. (3 DAPF) and then every 10 days, 7 times in total. All samples were immediately put into liquid nitrogen and stored in a refrigerator at - 80 °C for the determination of hormone content in leaves.

0.3g Frozen leaves were ground to power in liquid nitrogen. The contents of Indole acetic acid (IAA), zeatin riboside (ZR), abscisic acid (ABA), GA<sub>1+3</sub> and GA<sub>4+7</sub> calculated with an indirect enzyme-linked immunosorbent assay by the Phytohormones Research Institute (China Agricultural University) as described by Yang et al.<sup>50</sup>

#### 5.4 Expression of GA-related genes in leaves of long shoots by quantitative RT-PCR

The relative expression levels of GA-related genes were detected by qRT-PCR using a previously described method<sup>51</sup>. The qRT-PCR specific primers are listed in Table 4. MdACTIN served as a reference gene to normalize mRNA expression levels. Relative gene expression levels were calculated by the 2<sup>-ΔΔCt</sup> method<sup>52</sup>.

**Table 4.** Primers designed for the GA-associated genes in ‘Fuji’ apple.

Gene accession no.	Primers(forward/reverse)	Sequence(5'-3')
MDP0000155229	MdGA2ox1-F	GCTTCTTTCAGCCTTGTGCTAGA
	MdGA2ox1-R	GCAATGTGGTGGGTAGCGAT
MDP0000309451	MdGA2ox2-F	CTATTTTCAGGCATGGAGCAACA
	MdGA2ox2-R	CTCTTGGACTTGCCGCCTAT
MDP0000239572	MdGA3ox12-F	TGCCAACTTCTGCGATGCAAT
	MdGA3ox12-R	AGCAATGAGGAGTCCGTGTG
MDP0000248981	MdGA20ox1-F	GACTTTAGGCACTGGCCCTC
	MdGA20ox1-R	CCCGTTTGAAAGAGCCATGAA
MDP0000136940	MdGA20ox2-F	GGCAGGTTCTCCTCCAAACT
	MdGA20ox2-R	TGGTAAACCTTCCCGAAATCTCT
MDP0000299804/MDP0000246689	MdKO-F	ATTTGGAGCCGGAAGAGGGTGTG
	MdKO-R	GCATTGGATAGAGTTTGCGAGTGG
MDP0000828007	MdKS-F	AGGCTGTAGGAAGTTCCGAAT
	MdKS-R	TAACACCGTTGCCATGAATC
MDP0000147908	MdCPS-F	AGAAAGGGCAGGGACTCATT
	MdCPS-R	CAGAAGCCACTTTTCCCAAG
MDP0000326359	MdKAO-F	CGGATTAGTGC GTTCCATCT
	MdKAO-R	AACCCTTCCAGATGGGATAA
MDP0000319522	MdGID1c-F	CAACGTCAATGAATCCAAGAAGG
	MdGID1c-R	AAAACCCCGTCAACCGGAA
MDP0000237978	MdRGL1a-F	GCTCAGGAAGATGGACTC
	MdRGL1a-R	AGGTGATGTGCTAATGGAT
MDP0000640034	MdRGL1b-F	GATAACTCCGACCACCTT
	MdRGL1b-R	CCACTGATAGCACCTTCT
MDP0000314394	MdSLY1-F	TTTACCTGTTTGCCCTCTGCCG
	MdSLY1-R	GCTGGTGAGTGTGAATCTGTG
MDP0000912745	MdActin-F	TGACCGAATGAGCAAGGAAATTACT
	MdActin-R	TACTCAGCTTTGGCAATCCACATC

#### 5.5 Fruit quality and yield

10 fruits were collected randomly for each tree at the harvest time. Average fruit diameter and length/diameter (L/D) were determined, fruit firmness, soluble solids concentration (SSC) and titratable acidity (TA) were also measured, using a pressure tester (EPT-1; Lake City Technical Products), a tiltrotor (PAL-BXIACID 5; Atago), and a refractometer (PAL-1; Atago), respectively. L\*, a\*, and b\* color space coordinates were measured on each fruit. The yield and total number of per tree were also recorded at harvest.

#### 5.6 Assessment of flowering

All flower clusters were counted per tree in full bloom stage and flower density was expressed as the number of flower clusters per trunk cross-sectional area (TCSA). The TCSA was measured at 1.0 m from the ground level.

### 5.7 Statistical analysis

Data were subjected to an analysis of variance by Duncan's multiple range test at the 5% level using the SPSS 11.5 software package (SPSS, Chicago, IL, USA). Statistical processing of plant hormone contents and the expression levels of GA-related genes were performed in Microsoft Excel (2010). Diagrams were generated in Microsoft Excel (2010).

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## Acknowledgements

This research was funded by the earmarked fund for China Apple Research System (CARS-27).

## Author Contributions statement

L.W and L.Z. designed and interpreted of all experiments. Y.S, F.C and D.K participated in the data analysis. L.W wrote the manuscript. All authors have read and approved the manuscript.

## Additional Information

**Competing interests:** The authors declare no competing interests.



Figures

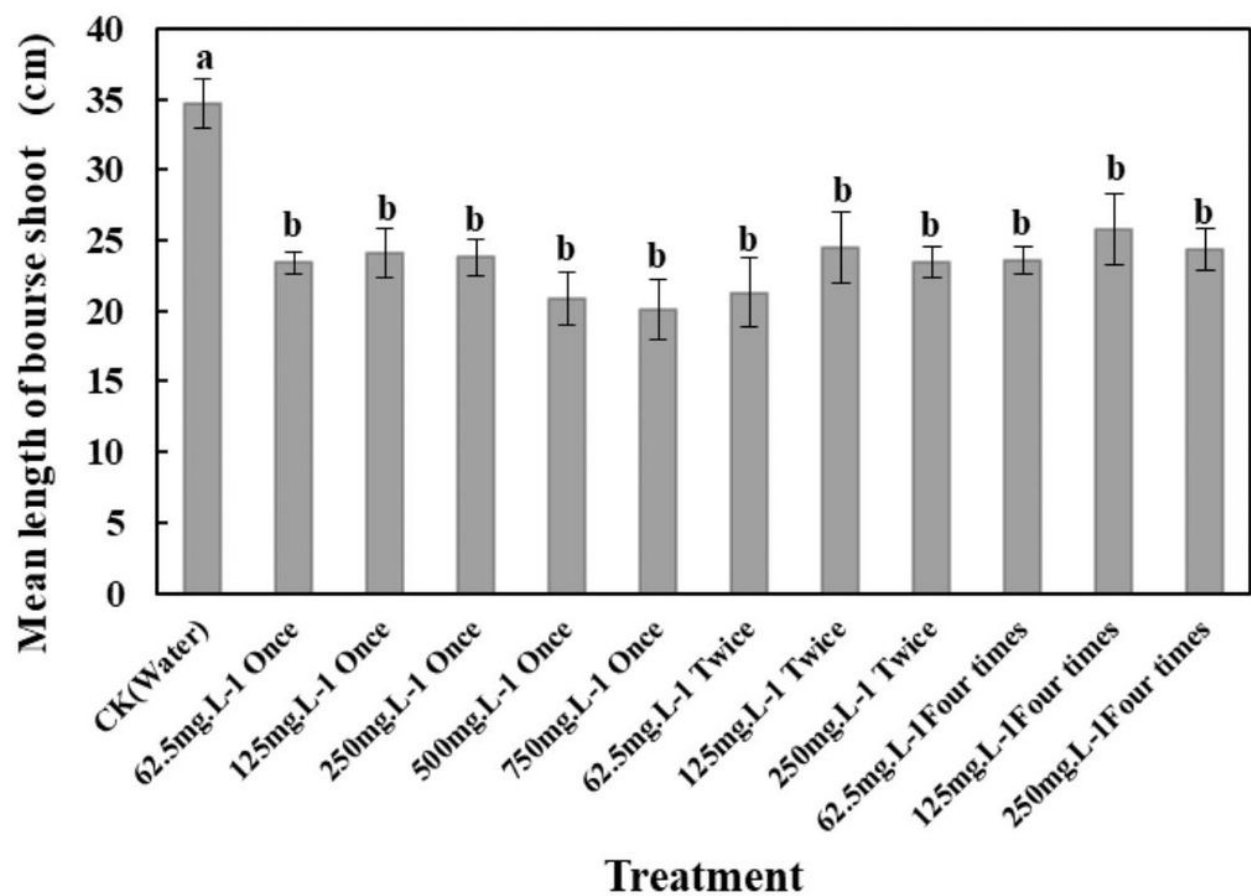


Figure 1

Mean length of bourse shoot at the end of growing season; Within each column, different letters (a, b) indicate significant differences at the 0.05 level



**Figure 2**

The 'Fuji' apple trees for control and 250 mg L-1 ProCa applied for four times.

**A**



**B**



Figure 3

Bourse shoot length measured on 25 Sep. (A) The length of bourse shoot in control; (B) The length of bourse shoot in ProCa applications for four times

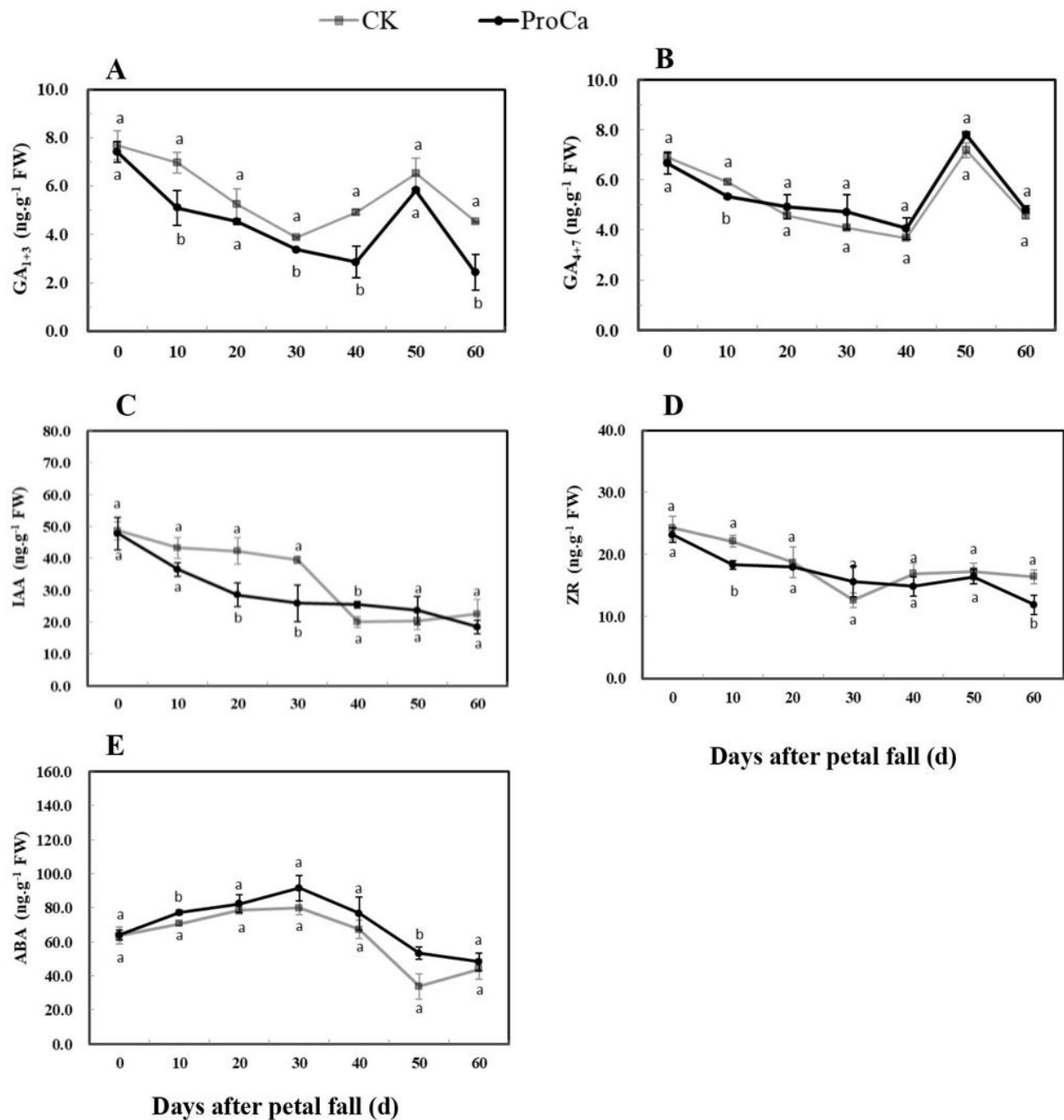
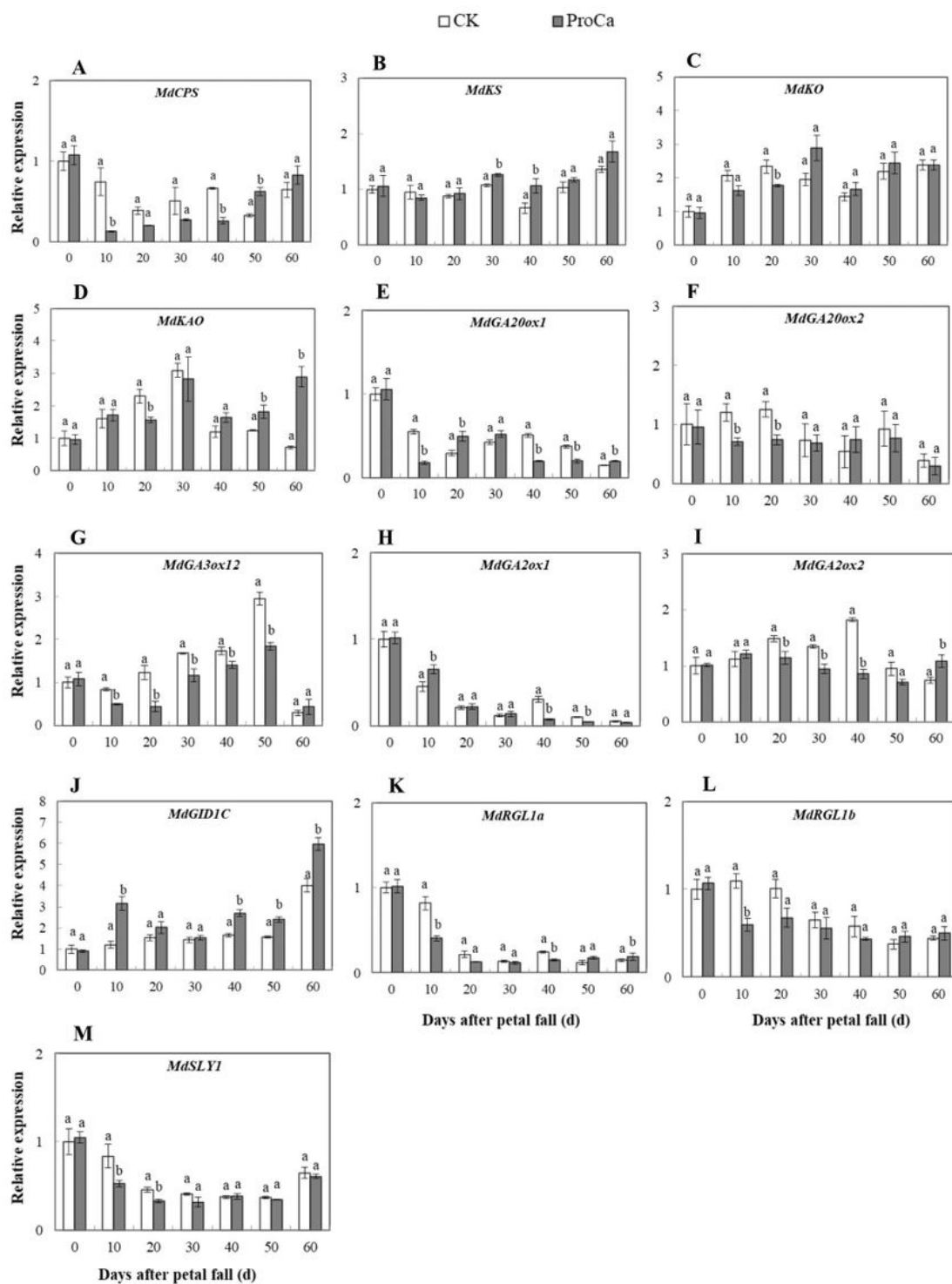


Figure 4

Hormone levels of zeatin-riboside (ZR), GA<sub>1+3</sub>, GA<sub>4+7</sub>, IAA, abscisic acid (ABA) in leaves of long terminal shoots. Values represent means  $\pm$  SE (n=3). Different letters (a, b) indicate a significant difference at the 0.05 level



**Figure 5**

Expression patterns of GA-related genes in leaves of long terminal shoots. Values represent means  $\pm$  SE ( $n=3$ ). Different letters (a, b) indicate a significant difference at the 0.05 level.