

Comparison of dynamic changes in endogenous hormones and sugars between abnormal and normal *Castanea mollissima*

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Abstract

To elucidate the possible functions of endogenous hormones in the flowering of chestnut, concentrations of four endogenous hormones [indole-3-acetic acid (IAA), gibberellic acid (GA), abscisic acid (ABA), zeatin riboside (ZR)] and the soluble sugars content were measured in both normal and developmentally abnormal Chinese chestnut (*Castanea mollissima*) during flowering and fruiting stages. Our results showed that the contents of ZR, ABA, and GA exhibited a significant different pattern in normal trees from that in abnormal trees, while the contents of IAA and soluble sugars showed a similar change pattern between them. These results suggest that quantitative changes in endogenous hormones may correspond to different flowering and fruiting mechanisms.

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1. Introduction

Flowering is usually associated with hormonal and sugar changes. The transition to flowering often proves to be very challenging in research. A transition process from vegetative growth to reproductive development proceeds in response to environmental factors. These factors often exert inductive effects by consequently evoking changes in hormone and sugar levels [1]. This is a critical step in plant reproductive development because a failure of changes in hormone and sugar levels often leads to seed abortion and fruit abscission. Therefore, a better understanding of the changes during plant development is essential to the establishment of strategies for yield improvement in economically important fruit trees. Because endogenous plant hormones are understood to play a significant role in plant growth and development, a number of studies have been conducted on the relationship between the hormone

contents and flower bud formation, although there is no consistent result published for Chinese chestnut [2]. Investigations of the relationship between flower bud formation and endogenous plant hormone contents may be valuable for developing techniques for effectively controlling the amount of flowers.

As signal molecules, hormones play a pivotal role in many processes throughout the plant life cycle, including embryogenesis, lateral root development, vascular differentiation, apical dominance, climate responses, and flower development [3–5]. They are also related to sex expression in certain degree [6]. It is known that IAA exerts a strong influence on the development of the lateral buds [7–9]. ZR concentration in the bleeding sap appears to coincide with the outgrowth of auxiliary buds of *Rosa hybrida* [10]. ABA has been reported to induce flowering in some short-day plants and inhibit flowering of some long-day plants [11]. GA can increase the height of black iris (*Iris nigricans* Dinsm.) [12]. Although these hormones influence a diverse range of processes, it is still unclear how they regulate various aspects of plant growth and development.

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Many plant developmental, physiological, and metabolic processes are affected by nutrient availability. For example, alterations in the availability of soluble sugars, such as sucrose, can help regulate a diverse array of processes. Because sugars are important sources of energy and of carbon skeletons of plants, access to sugars is an important determinant of a plant's potential for growth. Additionally, it has been reported that sugar levels are the highest in tobacco (*Nicotiana tabacum*) leaves that were about to senesce, compared with younger and older leaves on the same plant, and sugar treatment can hasten the senescence of tobacco leaf discs [13,14]. Sugars are also found to help regulate the expression of a significant number of plant genes [15].

Although the relationship between floral initiation and hormones has been reported in other plants, there is no report on such relationship for Chinese chestnut. The objective of this study is to investigate the difference in endogenous hormones and flowering mechanisms between normal Chinese chestnuts that flower and bring fruits once per year and a Chinese chestnut that lives in the same orchard but flowers twice and brings fruits twice per year. The information from this study can be helpful to clarify the relationship between flower bud formation of chestnut and hormone contents.

2. Materials and methods

2.1. Plant materials

The leaf samples of developmentally abnormal tree (*Castanea mollissima*) were collected from an old plantation in Yiliang County of Yunnan Province. The leaf sample of four normal trees nearby were selected as the control. Middle-aged leaf samples were collected once per month from March to October. No sample was collected for the rest of the year because the normal trees had almost no leaf at that time. Harvested leaves were placed in an ice box immediately. After they were taken to the laboratory, these samples were then immediately dipped in liquid N₂ and stored at -80 °C until analysis.

2.2. Analytical methods

2.2.1. Hormones

Fresh tissue (1.5 g) was used to measure the contents of the endogenous levels of IAA, ABA, GA, and ZR. The immunoenzymatic method of analysis, as described by Li and Meng [16], was used with some modifications. Freeze-dried powdered tissues were stirred and extracted

in 10 ml of cold 80% methanol containing butylhydroxytoluene (1 mM) as an antioxidant for 24 h at 4 °C in the dark after homogenization. The tissue samples were then centrifuged for 15 min at 10,000 rpm (Avanti 30 centrifuge, Beckman), and the supernatant was transferred to a flask. The residue was washed and extracted with 2 ml of cold methanol for another 12 h, and centrifuged under the same conditions. The supernatants were combined and decolorized using Sep-Pak C-18 cartridges twice so that the dilution curve was parallel to the curve of the standards. After filtration, elutes were vacuum-dried with a rotary evaporator at 37 °C to remove methanol. The residue was re-dissolved in a buffer containing 0.05 mM Tris, 1 mM MgCl₂, 150 mM NaCl, 0.1% gelatin, and 0.1% Tween 20. The measurements of hormone contents were carried out by ELISA according to the guide of testing set [16]. The hormone levels in each of the five plants were measured three times, and the standard errors were calculated. The antigen and antibody were purchased from Chinese Agricultural University.

2.2.2. Soluble sugars

Ethanol of 80% deposit was adopted twice, and its content was determined by the colorimetry of anthrone method [17]. Fresh leaf material (0.5 g) was homogenized with 80% ethanol using PCU (Kinematica GmbH Kriens-Luzern, Switzerland). The broken tissue was extracted in 10 ml of 80% ethanol on a boiling-water bath for 30 min. The suspension was centrifuged and the residue was re-extracted by another 10 ml of 80% ethanol and centrifuged again. The two supernatants were combined and decolorized at 70 °C. The final volume was fixed to 50 ml. Sugar content was determined by colorimetric anthrone method [17].

3. Results

3.1. Development comparison between normal and abnormal chestnut trees

Two years of careful observation showed that the abnormal chestnut tree had early bourgeons, a short dormancy stage, and twice flowered and fruited annually. The abnormal tree bourgeoned at the end of December and flowered during the early period from January to May and again during the later period from June to August. The first fruiting phase of the abnormal tree occurred from July to August, one month earlier than normal trees. The second fruiting phase was from August to September with fewer fruits than the first phase. The normal chestnut trees flow-

Table 1
The life rhythm comparison of the normal and abnormal chestnut trees

Samples	Burgeon start	Flowering	Fruit	Defoliation end
Normal Trees	March	March to June	August to September	Until the end of October
Abnormal Tree	From the end of December	June to December	July to October	Until the early of December

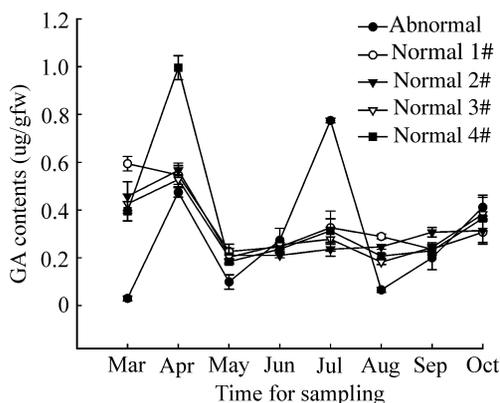


Fig. 1. GA content comparison between the normal and abnormal *Castanea mollissima*.

ered and fruited only once annually; they started to flower during the early period from January to May and produced fruit from July to August. A detailed description is given in Table 1.

3.2. Changes in four endogenous hormones levels

The monthly comparison of GA contents in leaves is presented in Fig. 1. GA contents in the leaves of the abnormal trees showed two peaks corresponding to the two flowering periods of March to May and June to August, whereas the normal trees had only one peak corresponding to the one flowering period of March to May. After that, the changes in GA contents were similar in both tree types.

There were no significant differences in the IAA amounts between the normal and abnormal trees. During the period from March to April, a sharp increase in the level of IAA was observed, which was synchronized with the flowering period. After that, the IAA level markedly decreased (Fig. 2).

The level of ZR exhibited a different pattern between the normal and the abnormal trees. In March, the ZR content in the abnormal tree was lower than that in the normal trees, whereas the situation became opposite in April. A high increase in the concentration of ZR in the control trees was observed from June to July (at 0.65 g/gfw),

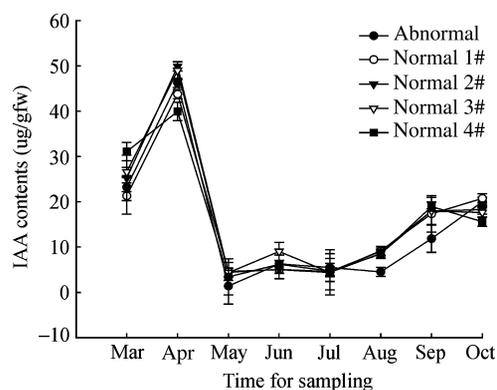


Fig. 2. IAA content comparison between the normal and abnormal *Castanea mollissima*.

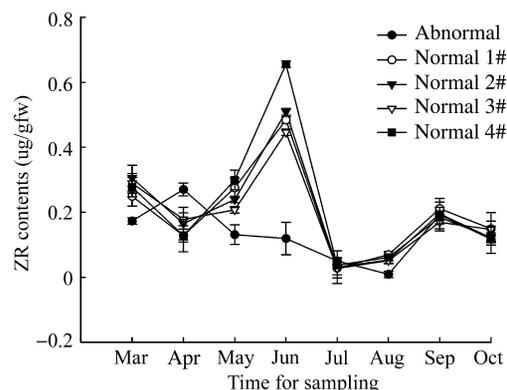


Fig. 3. ZR content comparison between the normal and abnormal *Castanea mollissima*.

whereas no increase was detected in the abnormal tree (0.12 g/gfw). After July, the changes in the contents of ZR were similar in both tree types (Fig. 3).

The level of ABA was very low in March, and changes in the ABA content in March showed a similar pattern to that in July (Fig. 4). From July to August, while the content of ABA increased slightly in the abnormal tree, it increased sharply in the normal ones, and reached its peak value at about 3 g/gfw in October, during which time the normal trees were defoliating (Fig. 4).

3.3. Changes in sugar content

There was no significant difference in the change patterns of sugar contents between normal and abnormal trees from March to October. The sugar content reached its first peak value at about 37 mg/gfw in April after bourgeoning. After July, the sugar content gradually increased; the maximum sugar content was detected in October when the trees were defoliating (Fig. 5).

4. Discussion

Based on the results of the AFLP analysis (data to be published), our experimental materials for both

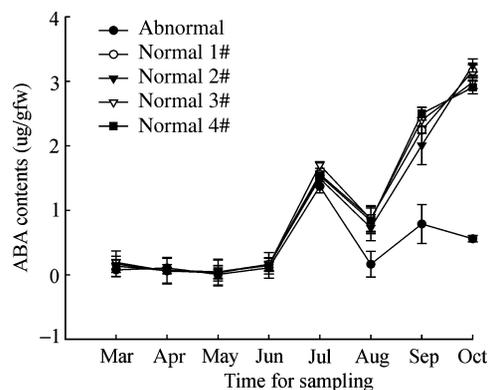


Fig. 4. ABA content comparison between the normal and abnormal *Castanea mollissima*.

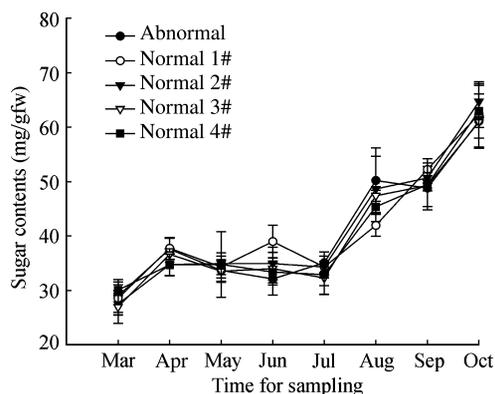


Fig. 5. Sugar content comparison between the normal and abnormal *Castanea mollissima*.

C. mollisimus types were of a common genetic background. Combined with the results of grafting experiments performed previously, we concluded that the genetic factors should not be responsible for their differences in flowering.

Hormones GA, IAA, ABA, and ZR play significant roles in cell division, cell growth, and elongation [1,3,18]. They therefore have a vital role in plant-development regulation. In this study, a significant increase in the concentration of ZR was observed from June to July in normal trees, whereas no increase was detected in abnormal ones. As described above, the abnormal tree flowered from June to July, whereas the control trees did not flower during that time. In other words, the lower level of ZR in the abnormal tree probably resulted in flowering from June to August. However, this hypothesis of ZR acting as floral repressor in Chinese chestnut needs to be confirmed in future studies. Our results are inconsistent with the results by Bernier et al. [19], in which they demonstrated that ZR not only affected processes such as cell division and vegetative bud and root formation in *Sinapis alba*, but also was considered as a determinant component of floral stimulus.

We found a similar pattern of IAA contents in the two lines. During the period from March to April, a sharp increase in IAA level was observed in synchronization with the developing buds. The results showed that there may be a correlation between endogenous IAA and flower buds formation of chestnut. A significant increase in endogenous IAA content during bud outgrowth was also reported in other plant species [20,21]. Beatriz [22] also suggested that newly synthesized IAA increased when the bud differentiation of *Ananas comosus* began.

The GA content in the leaves of the abnormal tree showed two peaks that corresponded to the two flowering periods between March and May and between June and August, whereas the normal trees had only one peak between March and May. The high GA levels coincided with the presence of floral primordia. During the period from July to September, a marked increment in the level of GA was detected in the abnormal tree, which coincided with the beginning of the second period of floral primordia formation in the abnormal tree. Interestingly, the period of

nonflowering in the normal trees coincided with the lower levels of GA from July to September, during which period the abnormal tree had high GA levels and was again flowering. Based on our experimental data, we hypothesized that the changes of GA content had a direct effect on flower bud formation. An obviously high GA content was observed when Chinese chestnut was flowering. Wijayanti et al. [23] postulated that exogenous GA induced flowering in *Pharbitis nil*. Nadia et al. [24] also reported that exogenous GA promoted flowering in the black iris (*I. nigricans* Dinsm.), which may be attributed to the growth-promotion effect of GA in stimulating and accelerating cell division, increasing cell elongation and enlargement, or both [25]. Other studies have also revealed the effectiveness of GA in inducing flowering [26–28], although Garner and Armitage [29] observed no effect of GA on stem quality of caspia (*Limonium* ‘Misty Blue’). However, many previous studies indicated that exogenous GA inhibited flower bud formation, e.g. in citrus [30], in apples [31], in pears [32], and in cherries and peaches [33].

It is interesting to note that, in our present study, change patterns of GA and IAA contents were similar from March to May. Presumably, there may be a functional relationship between these two hormones, and the maintenance of low GA/IAA has correlation with the chestnut flowering, although further evidences are necessary to validate this opinion. The relationships between IAA and GA seem to be more associated with the induction of the phase change than their respective absolute values [34]. At the same time, an obvious phenomenon is the maintenance of high GA/ZR from March to May and from June to August in accordance with the flowering time of abnormal tree. Therefore, we presumed that the high level of GA/ZR was positively related to the chestnut flowering although which need to be further documented. Many other previous studies also indicated that the ratio of different hormones was important to plant flower development, e.g. in fig [35], in loquat [36], in vanilla [37].

Our results showed a similar pattern of ABA contents in the two lines from March to August. Interestingly, after August, the rate of increase of ABA levels in the abnormal tree was lower than that in the control. Changes of ABA levels in normal trees coincided with post-flowering of adult trees, and the growth rate of ABA increased because of the maturation effect. ABA was reported to be involved in the regulation of transition from vegetative to reproductive phase [38], and higher level of ABA was the characteristic of maturity [39]. Our results are in agreement with those of other authors [40] who postulated that ABA affects plant senescence and is involved in maturation. These authors reported that the mature phase was characterized by high ABA levels, whereas the juvenile state showed lower ABA quantities. Bernier et al. [41] had claimed that the effect of ABA on flowering was diverse and species-dependent. Until now, we could not conclusively determine the role of ABA in chestnut. Further experiments are needed to elucidate the exact roles of

ABA because the influence of ABA on flowering varies even within a single species. In *P. nil*, a short-day plant, ABA has been reported to induce flowering [42,43], promote flowering [44], and inhibit flowering [45].

Sugars showed an identical pattern throughout maturation. Despite differences between individuals, similar values were observed when comparing sugars in the abnormal tree with those in control under conditions of the same age, or equivalent pre- or post-flowering state. It had been reported that sugar levels were the highest in tobacco (*N. tabacum*) leaves that were about to senesce, as compared with younger and older leaves of the same plant [13,14]. Then, the increasing sugar could be seen as a sign of senescence. Therefore, in this study, sugars appeared to be irrelevant to the flowering of the abnormal chestnut.

5. Conclusions

When investigating changes in hormone levels in abnormal and normal chestnuts during flowering, our results exhibited different patterns in the changes in the endogenous contents of ZR, ABA, and GA in the normal and abnormal trees. The contents of IAA and soluble sugars showed similar change patterns. The data presented in this paper indicate that quantitative changes in endogenous hormones may correspond to different flowering and fruiting mechanisms.

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