Pak. J. Agri. Sci., Vol. 54(1), 57-63; 2017
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
DOI:10.21162/PAKJAS/17.4862
http://www.pakjas.com.pk

DYNAMIC-CHANFING CHARACTERISTICS OF 3 KEY SECONDARY ENZYMES BETWEEN BARED- AND COMPLETED- ENDOCARP DEVELOPMENT IN WALBUTS

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Bared-nut (*Juglans regia* L) cultivars, in which the endocarp or the shell has poor development or less lignification, were characterized to determine endocarp development. The bared nut ("WEN138") and normal walnuts were sampled and tested. Fruit were sampled at several times from 50d to 92d, and three key secondary enzymes in lignin pathway were analyzed via testing the activities. The results showed that final lignin contents was lower in "WEN138" (23.2%) occurring on days of 92d than in the other cultivars (average 26.2%). The activities of phenylalanine ammonia lyase (PAL) and peroxidase (POD) reaching the peak on 64 days after full blossom, were identified with "WEN185", "XINXIN2", and "ZHIPI", and in contrast, there were no clear peaks of POD and PAL activity in "WEN138". As the signals of secondary lignin metabolism, PAL expression were investigated and demonstrated the maximal PAL expression on 71 days in "WEN138", "XINXIN2", and "ZHIPI". However, the peak lightness of PAL expression in "WEN185" was on 85d. A proposed conclusion is that bared-walnut phenotype due to lignification does not effectively take place in bared nut. The economic potential of doing the same for stone fruit is to apply this marketing possibilities for cultivating thin shell cultivars of almonds and apricot, coupled with decreased production costs for intensive processing.
Keywords: *Juglans rejia*, endocarp development, PAL activity, POD activity, CEL activity.

INTRODUCTION

Walnut (*Juglans rejia* L) is rich in protein, fat, carbohydrates, α-linolenic acid, and Ω-3 fatty acids. In addition, the nuts contain antioxidants that have been linked to befles aging and in cardiovascular health. Increases in production for the domestic and international markets in recent years has prompted rapid the development of the processing industry.

Several properties of the nut must be considered during processing. Processing is aided by a high kernel ratio and the ease by which nuts can be peeled. Processing is hampered by shells hard and poor friability. Walnut fruit develop from ovaries, while the seed is coated by an endocarp, mesocarp and exocarp. Similar to drupes such as plum and apricot, the endocarp develops from multilayered cells of the inner-wall of ovary. Over time, the endocarp cells die, acquire lignin, and eventually comprise the shell. This sequence of events involves lignification, wall thickening, and the formation of stone cells (Zuzunaga, 2001; Wu, 2005). the endocarp is composed of lignin (50%), cellulose (22%), and hemicellulose. In woody plants, the secondary cell wall lies beneath the primary wall and thickens substantially to form the secondary xylem (Mitsuda, 2007). Walnut endocarp lignin, the majority of which is guaiacyl lignin (Zheng *et al.*, 2006), is secondary thickening. Song *et al*. (2010) found that lignin synthesis occurs via the shikimate pathway for the synthesized of aromatic compounds. Phenylalanine ammonia lyase (PAL) converts L-phenylalanine to cinnamic acid, which is then methylated to form a lignin monomer. Finally, peroxidase polymerized the monomers to form lignin. Several studies have examined lignin synthesis and the endocarp hardening (Abeles and Biles, 1991; Christopher, 2010; Yang, 2009, 2010; Wang, 2001. These previous studies focused on the development of vegetative organs (plant stems and leaves) and endocarp development in the stone cells of pear fruits and drupes (Hatfield, 2001; Ralph, 2006.

Some walnut cultivars produce nuts that lack complete endocarps, and the ripe fruit have stripe shell (and produce bared nuts). We studied endocarp formation within walnut. The aim of determine the molecular mechanisms underlying

endocarp development and the production of bared nuts. Four walnut were investigated in south Xinjiang, China, and the activities and contents of key enzymes examined during endocarp hardening.

MATERIALS AND METHODS

***Cultivation and sampling*:** The walnut cultivars are all originated in area of Akesu in Xinjiang, and were growing at Woody Grain and Oil Farm in Wensu County, Akesu, Xinjiang, China. The cultivars of "WEN138", "ZHIPI", "WEN185", and "XINXIN2" were collected in 2011. The bared-nut walnut germplasm were used in this study. Samples were taken every seven days, with the initial sample 50 days after full blossom and the final sample taken 42 days later. The samples (N=30) were collected from east, west, south, north, and inner. The endocarp was separated from each sample and frozen in liquid nitrogen and stored at -70°C. Endocarp formation was assessed by the determination of the contents of lignin and cellulose, the activities of phenylalanine ammonia lyase (PAL) and peroxidase (POD) and cellulase (CEL), and quantitative expression analysis of the PAL gene.

***Enzyme activity and tissue composition assays*:** PAL activity was determined as described by (Gao, 2000). POD activity and lignin and cellulose contents describe by Li (2001), and CEL activity as described by Zhao and Yang (2006).

***RNA extraction and quantitative PAL gene expression analysis*:** RNA was extracted as described by Tang (2012) and was reverse-transcribed using commercially available reagents (Fermentas Inc., MD, USA).

PCR (Polymerase Chain Reaction) was performed in 20 µl reaction volumes containing 25 ng DNA, 0.1 µM each forward and reverse primers, 2.5 mM $MgCl_2$, 2.5 mM dNTPs, 1 × Taq buffer (100 mM Tris–HCl pH 8, 500 mM KCl, 0.8% Nonidet P40), and 1U Taq DNA polymerase (Fermentas Inc., MD, USA). Amplification was performed in a PTC-200 DNA Engine Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, California, USA) in 0.2 ml tubes. Thermal cycles were as follows: initial denaturation cycle at 94°C for 3 min, 30 cycles of 94°C for 30s 57°C for 45 s, and 72°C for 2 min, and a final incubation at 72°C for 5 min. Amplified products were stored at 4°C. PCR products were separated using 2% agarose gel electrophoresis. PCR was performed on each sample three times. Primers for the *PAL* gene and the reference 18S RNA gene were as indicated in Table 1.

RESULTS

***Characteristics of walnuts from four different cultivars*:** "WEN138" was a bared cultivar (Fig. 1A). With nut weighting was 13 g and the kernel proportion was 83.2%. "ZHIPI" produced an oval nut (Fig. 1B) that was rounded at the base and had a slightly convex tip. The nut weighted 11.2 g and shell thickness was 0.8 mm. "WEN185" (Fig. 1C) produced a round nut, nut weight was 15.8 g and with shell was 1.0 mm. "XINXIN2" produced an oblong nut (Fig. 1D) with a smooth surface. The nut weighted 11.7 g and the shell was 1.2 mm.

Table 1. The primers of PAL and reference genes reference genes reference genesreference 18SRNA gene.

Gene	Genbank Reference	Primer	Primer Sequence (5′–3′)	Amplicon Length	Primer Annealing temperature (°C)
PAL	gi|399936232	PALHQF1	cgaggaagctagagccggcgtc	840bp	57
		PALHQR1	tgtggtcctagccattggggag		
18SRNA	FJ980301.1	NBHQF1	aggtttccgtaggtgaacctgc	400bp	57
		NBHQR1	agactcgatgattcgcgggattc		

Figure 1. Shell morphology in walnut in China; (A-D) WEN138, ZHIPI, WEN185 and XINXIN2; The first culture produced bared nuts.

***Endocarp development*:** "WEN138" produced bared-nut that exhibited fruit-to-fruit variation in stone formation during several growing seasons. Only small fragments of shell were produced. The majority of stone tissue was located along the margins, parallel to the suture, with additional remnants scattered throughout. Occasionally, the nut contained stone tissue within the cavity which was shaped like a stripe.

Early in fruit development, the endocarp layer was white, and the mesocarp green. Poor endocarp formation in "WEN138" relative to the other cultivars was apparent 78 days after blossom. The most pronounced differences in endocarp formation were between "XINXIN2" and bared "WEN138". Lignin was first stained after in the endocarp 57 days after full blossom in the "WEN185" and "XINXIN2", and 64 days "WEN138" and "ZHIPI" (Fig. 2). Endocarp staining formed

Figure 2. Endocarp hardening in days after blossom. (a-g) days after blossom, 50 d; b, 57 d; c, 64 d; d, 71 d; e, 78 d; f, 85 d; and g, 92 d; (A-D) Four walnuts cultivars in China WEN138, ZHIPI, WEN185 and XINXIN2. The endocarp of WEN138 did not produce complete rings during the harden.

a complete ring in "WEN185" and "XINXIN2" but was primarily around suture margins in "WEN138" and "ZHIPI". In "WEN185", "ZHIPI", and "XINXIN2", staining progressed until the entire endocarp as stained. Similarly, hardening progressed until the stone tissue could no longer be cut with a scalpel. By contrast, "WEN138" was stained only parts of the endocarp, and this was randomly distributed. In addition, a fine network of white tissue was observed next to the "WEN138" seed that did not stain for lignin or harden.

***Enzyme activities during endocarp hardening*:** PAL is a critical enzyme controlling the synthesis of phenolic compounds and links primary and secondary metabolism. In "WEN185" and "XINXIN2", PAL activities were generally high during endocarp-hardening, with low activity in 64 days after blossom. PAL activity in ZHIP1 was generally lower than in "WEN185" and "XINXIN2", but a similar nadir was observed at 64 days. Conversely, PAL activity in "WEN138" varied little and had a slight peak 64 days after blossom (Fig.3).

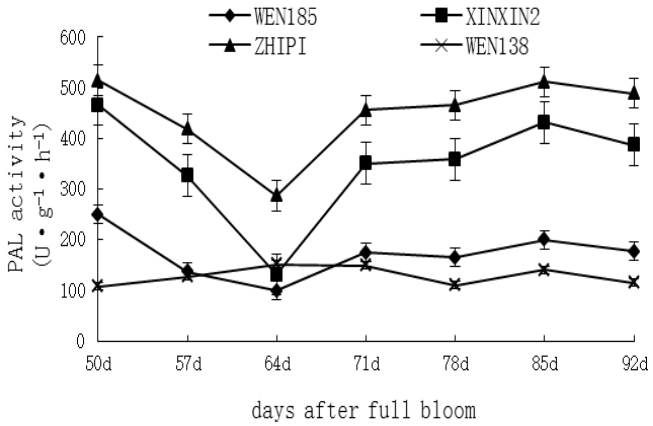

Figure 3. PAL activities during endocarp hardening in four walnut cultivars.

POD is a critical enzyme in the lignin synthesis that catalyzes the polymerization of various lignin monomers and increases deposition of monomers at the cell wall. Changes in POD activity were similar for "ZHIPI", "WEN185", and "XINXIN2". And the peak at 71 days after blossom. Activity in "WEN138" followed a different pattern. Activity levels were lower than in the other cultivars at 50 days and displayed only minimal differences in the subsequent samples. The lowest activities were seen 71 and 78 days after blossom, and the peak value at 85d, which contrasted with the 71 day nadir observed for the other cultivars (Fig. 4).

CEL activities 50 days after blossom were 16.80, 16.73, 15.43, and 21.06 $U.g^{-1}.m^{-1}$ in "WEN138", "ZHIPI", "WEN185", and "XINXIN2", respectively (Fig. 5). With the exception of "XINXIN2" on 50 days and 57 days, levels were similar across the cultivars and generally declined after 50 days. Activity in "WEN138" increased slightly between 50 days and 71 days and then declined.

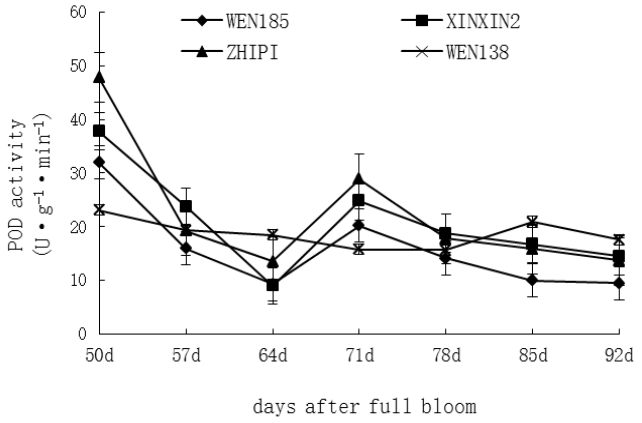

Figure 4. POD activities during endocarp hardening in four walnut cultivars.

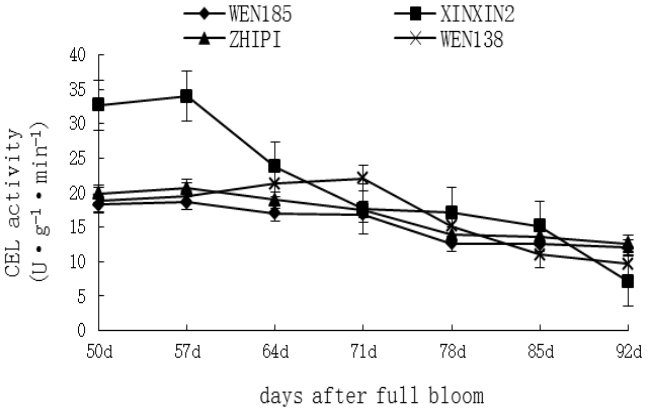

Figure 5. CEL activities during endocarp hardening in four walnuts cultivars.

***Lignin and cellulose contents during endocarp-hardening*:** Cellulose, hemicelluloses, and lignin are found within plant cell walls. Both lignin and cellulose are structural components that support plant tissues. Lignin supports the cellulose architecture and couples cells together. Lignin also enhances the mechanical strength of plant tissues, supports water transportation in conducting tissues, and increases resistance to adverse environmental conditions. Lignin composition of the endocarp increased in all cultivars after 64 days. Deposition occurred primarily between 64 day and 71 day in "ZHIPI" and XINXN2, between 64 day and 85 day in "WEN185", and between 78day and 92 day in "WEN138" (Fig. 6). Lignin deposition in "WEN138" was slower than in the other cultivars. The final lignin percentage was significantly lower in "WEN138" (23%) than in the other cultivars (26%) as assessed by ANOVA.

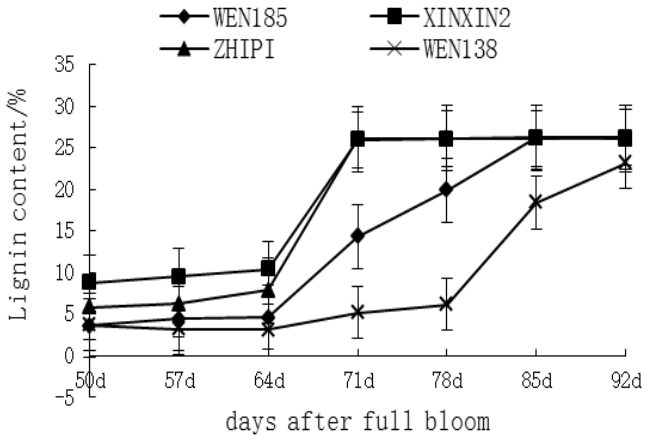

Figure 6. Lignin contents during endocarp hardening in four walnut cultivars.

Cellulose deposition was examined throughout the endocarp-hardening period. Cellulose accumulated gradually in all cultivars. The fastest accumulation was in "ZHIPI" and "XINXIN2", particularly after 78 day for "XINXIN2". Along with lignin deposition, the cellulose accumulation rate and final content was lowest in "WEN138" (Fig. 7).

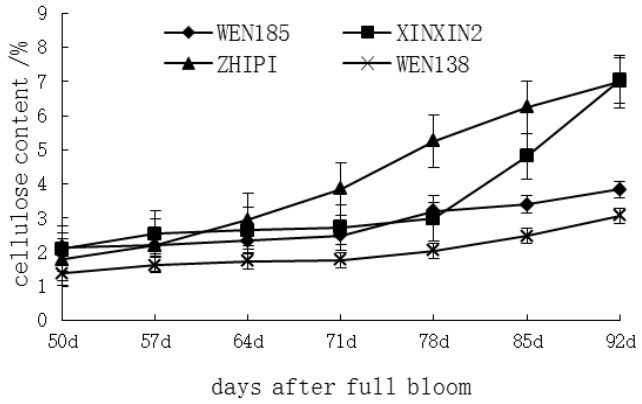

Figure 7. Cellulose contents during endocarp hardening in four walnut cultivars.

***Correlations between enzyme activities and the contents of lignin and cellulose*:** The activities of PAL and POD were at their lowest 64 day after blossom (Figs 3 and 4). POD and PAL activities rose after 64 days, corresponding to the increase in lignin. However, lignin accumulation did not correlate with the activities of POD or PAL. Cellulose accumulation was negatively correlated with cellulase activity in all cultivars. Cellulose deposition correlated significantly with lignin accumulation in all cultivars except "XINXIN2" (Table 2).

Table 2. The correlation coefficient between contents and enzyme activity during walnut endocarp-hardening periods.

Cultivars	lignin-PAL activity	cellulose-cellulose activity	lignin-POD activity	cellulose-POD activity	PAL-POD activity	cellulose-lignin
WEN185	0.197	-0.97**	-0.532	-0.601	0.212	0.960**
XINXIN2	0.319	-0.81*	-0.330	-0.473	0.649	0.605
ZHIPI	0.212	-0.98**	-0.509	-0.590	0.413	0.870*
WEN138	-0.136	-0.87**	0.059	-0.221	-0.310	0.950**

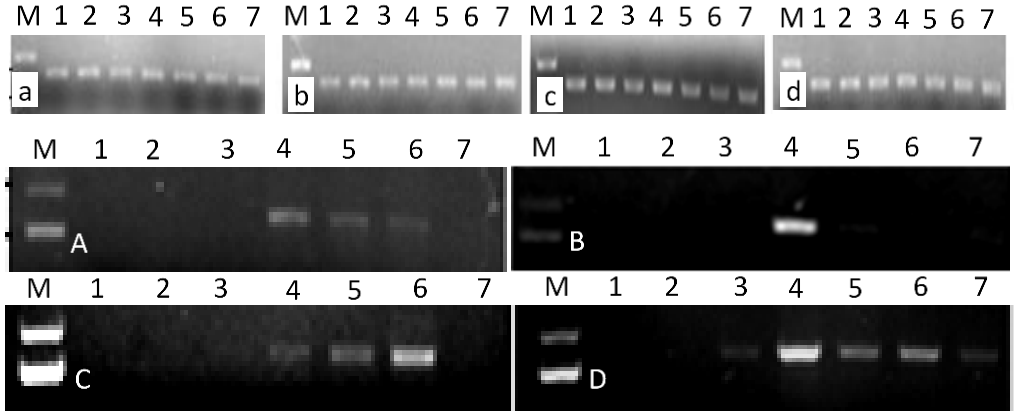

Figure 8. Quantitative analysis of PAL expression during endocarp development in four walnut cultivars.(a–d), expression of 18s rRNA reference gene. A–D, expression of PAL: A, WEN138; B, ZHIPI; C, WEN185; and D, XINXIN2. Lanes 1-7 are 50, 57, 64, 71, 78, 85 and 92 days after blossom.

***Quantitative analysis of PAL expression during endocarp hardening*:** RNA was extracted from the endocarp samples, reverse-transcribed, and used for amplification of the 18S rRNA gene (Fig. 8, A–D). The results of 18S rRNA amplification were used to equalize input cDNA for PAL amplification (Fig. 8, A–D). PAL expression in "WEN138", ZHIPI, and "XINXIN2" was highest in 71 day after blossom and was highest in "WEN185" at 85 day. Peak *PAL* expression was lower in "WEN138" than in the other cultivars.

DISCUSSION

Our data support the hypothesis that bared nuts do not form a complete endocarp. The deficiency was apparent as early as 64 day after blossom. Less lignin and cellulose were deposited in the bared nuts than in the full nuts. Lignin is deposited on the cellulose matrix within a cell. The lower cellulose levels were found in "WEN138" and this might explain the lower levels of lignin deposition and the poor shell development. Lignification is a normal plant development is fundamental to the formation of secondary xylem.Lignification is important in walnut fruit development and protected the seed. Previous research by Wu *et al*. (2008) and Yoshinaga *et al*. (2012) showed that PAL was a key rate enzyme in lignin biosynthesis. POD and PAL activities were positively correlated with lignin deposition in bamboo tissue (Luo *et al*., 2008). In addition, Wang (2002) showed that PAL and POD were critical for lignification and that their activities were correlated with the lignifications in bamboo. Christopher *et al*. (2010) and Cao *et al*. (2009) studied peach endocarp development and showed that the yield of 3-aromatic alcohol monomers, key building blocks for lignin, was influenced by PAL activity. In addition, PAL expression increased during the early stages of endocarp hardening in peach and then decreased during the later stages. Research by Biener (2009) showed that lignin biosynthesis was adversely affected by inhibition of PAL and the resulting decrease in synthesis of lignin monomers. However, other compounds synthesized in the benzoic acid pathway were more sensitive than lignin to loss of PAL. In addition, research by Sewalt *et al*. (1997) showed that plants grew abnormally, and adaptability and resistance declined when PAL activity was inhibited.

In contrast with previous research,we found that the activities of PAL and POD decreased over 64 day after blossom and then increased. This was probably as a result of the long lignification period in walnut and feedback regulation governing the accumulation of phenolic polymeric material. PAL activity did not correlate with lignin content in walnut endocarp. This indicated that PAL was not rate-limiting enzyme in the lignin deposition metabolic pathway in the walnut endocarp harden. Formation of the walnut endocarp involves the synthesis and deposition of lignin and cellulose. Zheng *et al*. (2006) found that the major components of the walnut endocarp were lignin, cellulose, and hemicelluloses. PAL is critical for the synthesis of the initial lignin monomers that are important for the initial stages of lignification. PAL forms a link between primary and secondary metabolism, and expression levels were affected lignin biosynthesis (Sewalt *et al*., 1997). The scaffolds for lignin deposition are primarily cellulose microfilaments. Hemicelluloses and lignin then build upon this skeleton. In walnut, cellulose and lignin deposition increased as the endocarp developed. Lignin content reached approximately 26% in the compared cultivars, but the contents to "WEN138" is 23%. Expression of *PAL* was maximal 71 days after blossom in "WEN138", "ZHIPI", and "XINXIN2", corresponding with the increase in lignin deposition. However, *PAL* expression was highest 85 days after blossom in "WEN185".

The bared-nut walnut germplasm "WEN138" provide an opportunity to examine the development and molecular factors underlying inception and progression of this species endocarp formation. Elucidation of these mechanisms will provide detail of main results of bared nuts and the endocarp lignification.

***Acknowledgments*:** This research was jointly supported by the Doctoral Fund of the Xinjiang Production and Construction Corps (2011BB006), the Natural Science Foundation (31260469), Huazhong Agricultural University-Tarim Scientific Research Union Foundation Project (HNTDLHI401), and the Corps Industry Project (2014BA014).

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