Effects of Vacuum Packaging on Enzymatic Browning and Ethylene Response Factor (*ERF*) Gene Expression of Fresh-cut Lotus Root

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Abstract. Ethylene response factor (*ERF*) genes have been involved in responses to biotic and abiotic stress, including hypoxia and anaerobic stress. Vacuum packaging (a typical anaerobic stress) is an effective storage method used to delay browning of fresh-cut lotus root (*Nelumbo nucifera*). In model plants, *ERF* genes have been identified as responsive to hypoxia. Whether *ERF* is associated with browning of vacuum-packaged lotus root has not been studied. The effects of vacuum packaging on browning, phenolic content, the enzyme activity of phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), and peroxidase (POD), and *PPO*, *PAL*, *POD*, and *ERF* genes expression in fresh-cut lotus root were studied. Downregulation of *NnPAL1*, *NnPPOA*, and *NnPOD2/3* attributable to vacuum packaging coincided with increased related enzyme activities and the degree of browning of fresh-cut lotus root. The expression patterns of *NnERF4/5* were consistent with the changes in *NnPAL1*, *NnPPOA*, and *NnPOD2/3* gene expression. It has been proposed that *NnERF4/5* could have be important regulators of fresh-cut lotus root browning, and that the relationships of *NnERF4/5* and *NnPAL1*, *NnPPOA*, and *NnPOD2/3* should to be studied further.

Lotus root is an important aquatic vegetable and export vegetable in China. There are abundant nutrients and high nutritional value, mainly including starch, sugar, protein, fat, and lecithin in lotus root (Soliva-Fortuny and Martín-Belloso, 2003). Because it is easy to cut, lotus root is more suitable for processing into fresh-cut products (Du et al., 2009). Fresh-cut lotus root is a fast and convenient food, and its processing procedure includes grading, finishing, cleaning, cutting, rest, preservation, and packaging.

However, during processing and storage, fresh-cut lotus root is highly susceptible to deterioration, which directly affects its quality and shelf life. The main reason for the decline in quality of fresh-cut products has been enzymatic browning (Eissa et al., 2006; Pma, 2006; Son et al., 2015).

At present, the methods for controlling the browning of lotus root mainly include chemical treatment (Kwon and Baek, 2014; Lu et al., 2007), modified atmosphere (MA) packaging (Cheng et al., 2015), vacuum packaging (Son et al., 2015; Xing et al., 2012), heat treatment (Tsouvaltzis et al., 2011), and low-temperature storage (Min et al., 2017). Among these, vacuum packaging is a natural preservation method that can greatly enhance the shelf life and overall quality of fresh produce (McDonald and Sun, 2000). Phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), and peroxidase (POD) are involved in the enzymatic browning

of many fruits and vegetables (Banerjee et al., 2015; Cheng et al., 2015; Ji-Lian et al., 2016; Zhou et al., 2003). However, vacuum packaging can delay the browning of fresh-cut lotus roots, and samples that have been vacuum-packaged have exhibited lower total phenol contents and PPO activity as well as lower degrees of browning than samples that have been air-packaged (Son et al., 2015; Xing et al., 2012). Our previous research reported that *NnPAL1*, *NnPPOA*, and *NnPOD1–6* are the most important candidate genes involved in the browning of fresh-cut lotus root ('E Lian 6') during storage at different temperatures (Min et al., 2017). However, the effect of vacuum packaging on *PAL*, *PPO*, and *POD* genes have not been reported for fresh-cut lotus root.

Ethylene response factor (*ERF*) genes have been characterized in numerous plants in which they are involved in responses to biotic and abiotic stress, including hypoxia stress, cold stress, and heat stress (Licausi et al., 2013; Min et al., 2012; Müller and Munné-Bosch, 2015; Phukan et al., 2017). It has been reported that hypoxia-responsive *ERF* genes (*HRE*) have been characterized in Arabidopsis, with *HRE1* and *HRE2* having partially redundant roles in increasing low-oxygen tolerance (Hinz et al., 2010; Licausi et al., 2010; Yang et al., 2011). Two hypoxia-responsive *ERF* genes (*DkERF9* and *DkERF10*) were involved in regulating persimmon deastringency by separately regulating *DkPDC2* and *DkADH1* promoters (Min et al., 2012). Although the anaerobic environment produced by vacuum packaging or low temperatures is a type of unfavorable stress for plants, it has a remarkable effect on delaying browning of fresh-cut lotus root. In our previous study, we explored the role of *ERF* in delaying lotus root browning at low temperatures, and we found that *NnERF3/4/5* could be important regulators of browning of fresh-cut lotus root during storage at low temperatures. Whether *ERF* is associated with the browning of lotus roots that are vacuum-packaged requires further study.

Ethylene can enhance rachis browning in *Vitis vinifera* and accelerate the browning of fresh-cut *Colocasia esculenta* during storage (Li and Zhang, 2015; Tan and Zeng, 2014). It is well known that *ERF* plays an important role in the realization of ethylene function (Nakano et al., 2006), suggesting that *ERF* may be involved in plant browning. *ERF* generally realizes functions by combining downstream genes. It has been reported that *DcERF1* and *DcERF2* may commit to the upregulation of *DcPAL3* promoter activity in anthocyanin-synthesizing carrot cells (Kimura et al., 2008). *GbERF1* regulated lignin biosynthesis-related enzymes, including PAL, to improve resistance to *Verticillium dahliae* (Guo et al., 2016). A study of aphid resistance indicated that downregulation of *ERF* gene expression was consistent with the lower POD and PPO activities due to high CO₂ (Guo et al., 2013). *ThCRF1* (a subfamily member of the *ERF* transcription factor from *Tamarix hispida*) has improved

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tolerance to salt-shock-induced stress by improving SOD and POD activities (Qin et al., 2017). This suggests that *ERF* may be involved in the synthesis of phenolic precursors through the transcriptional regulation of *PAL* in plants or the transcriptional regulation of *PPO* and *POD* involved in plant stress responses. Therefore, it is necessary to further study how *ERF* participates in the browning of lotus root that has been vacuum-packaged.

In this study, the effects of vacuum packaging on browning, total phenol, the enzyme activity of PPO, PAL, and POD, and the expression patterns of *PPO*, *PAL*, *POD*, and *ERF* were analyzed. Some *PPO*, *PAL*, *POD*, and *NrERF* genes were positively correlated with lotus root browning, and the possible roles of these and other *NrERF* genes are discussed.

Materials and Methods

Sample preparation

Lotus root ('E lian 5') was purchased from the commercial agricultural wholesale market (Four Seasons) in 2016; then, it was immediately shipped to the laboratory. It had a uniform size and color, and there were no defects or mechanical damage. Before samples were processed, the lotus roots were stored at 4 °C for 24 h. Then, lotus root was rinsed with clean water to remove the soil and peeled. Next, using a stainless-steel knife, it was cut into 5-mm-thick slices along the cross-section. The slices were transferred to clean water, sterilized by ozone for 5 min, and dried with clean filter paper. Some of the cymbals were vacuum-packed, and the other part was packaged with atmospheric pressure. Finally, samples were transferred to a refrigerator (4 °C).

Physicochemical parameters

Physicochemical parameters of fresh-cut lotus root slices, including the degree of browning and total phenolic content, were measured according to a previously published method (Min et al., 2017). All treatments were performed with three biological replicates.

Determination of the degree of browning. The degree of browning (Min et al., 2017) was determined using modified versions of methods referred to in the previous literature. At 4 °C, 30 mL of distilled water was mixed with 3.0 g of sliced lotus root tissue. It was homogenized and then centrifuged for 5 min at 10,000 g_n . Next, the supernatant in the centrifuge tube was collected. After incubating for 5 min in a 25 °C water bath, the absorbance was measured at 410 nm using a spectrophotometer. The browning degree was expressed as $A_{410} \times 10$.

Determination of the total phenolic content. The total phenolic content was measured according to the Folin-Ciocalteu method (Liu et al., 2018; Min et al., 2017). Three grams of sliced fresh lotus root tissue samples were homogenized with 30 mL of 60% ethanol and centrifuged at 10,000 g_n for 5 min. The supernatant (10 mL) was diluted with 40 mL of 60% ethanol for the next

measurement. The sample solution (0.125 mL) was mixed with 0.625 mL of distilled water; then, 0.125 mL of Folin phenol reagent was added. After thorough mixing (oscillation for 30 s), the mixture was permitted to stand at room temperature for 3 min; then, 1.25 mL of 7% Na_2CO_3 and 1.0 mL of distilled water were added. Finally, it was allowed to stand in a water bath at 25 °C for 90 min. Then, the absorbance was measured using a spectrophotometer at 760 nm. The standard curve of gallic acid was used to determine the total phenolic content. The result was expressed as milligrams of gallic acid equivalents per kilogram of fresh weight ($mg \cdot kg^{-1}$).

Determination of PAL, PPO, and POD enzyme activities. PAL activity was extracted and determined using a phenylalanine ammonia lyase activity assay kit (Nan Jing Jiancheng Bioengineering Institute, Nanjing, China) (Min et al., 2017). There were four reagents in the kit (reagents 1–4); 0.1 g of the sliced tissue was added to 1 mL of reagent 1 under ice bath conditions for homogenization. Then, the sample was centrifuged at 10,000 g_n for 10 min at 4 °C, and the supernatant was collected as a crude PAL extract. The reaction mixture consisted of 20 μ L of crude PAL extract, 780 μ L of reagent 2 (blank group 800 μ L), and 200 μ L of reagent 3. Afterward, the mixture was incubated at 30 °C for 30 min, and 40 μ L of reagent 4 was added. Finally, it was allowed to stand at room temperature for 10 min, and the absorbance of the sample was measured at 290 nm. One unit of PAL activity was defined as the amount of enzyme that catalyzed when the change in absorbance was 0.1 per gram of fresh sample at 290 nm.

Extraction and determination of PPO activity were based on the literature (Min et al., 2017). Under ice bath conditions, 3.0 g of sliced lotus root was homogenized in 50 mL of phosphate-buffered saline (PBS) (0.05 $mol \cdot L^{-1}$; pH = 7.0). Then, the solution was centrifuged at 3820 g_n for 15 min, and the supernatant was collected as a crude PPO extract. Next, we prepared a reaction mixture consisting of 1.0 mL of 0.1 $mol \cdot L^{-1}$ catechol and 1.5 mL of PBS. The mixture was incubated at 35 °C for 5 min; then, 1.0 mL of crude PPO was added to the mixture. Afterward, the absorbances of the mixture were measured at 420 nm over time. After the enzyme solution was added, the absorbance value was measured every 10 s during the first 1 min, and then every 30 s until 3.5 min. The absorbance value (OD) was measured over time. Finally, the enzyme activity was calculated based on the slope (OD/t) of the straight segment. An enzyme activity unit was described as the amount of enzyme leading to a change in absorbance of 0.001 per min.

Extraction and determination of POD activity refer to related articles with some modifications (Abohatem et al., 2011). Under ice bath conditions, the extraction buffer (5.0 mL; 0.2 $mol \cdot L^{-1}$) was added to 5.0 g of fresh chopped lotus root for homogenization (the extraction buffer contained 1 mM polyethyl-

ene glycol-6000, 4% polyvinyl pyrrolidone, and 1% Triton X-100). Then, the homogenized sample was centrifuged at 15,290 g_n for 30 min at 4 °C. Afterward, the supernatant was collected to determine the POD activity. Finally, 3.0 mL of 25 $mol \cdot L^{-1}$ guaiacol, 0.5 mL of enzyme solution (blank of extraction buffer), and 0.2 mL of 5 $mol \cdot L^{-1}$ dissolved hydrogen peroxide were added to the supernatant. The absorbance change in the mixture was recorded every 10 s at 470 nm. One unit of enzyme activity was defined as the amount of enzyme leading to a change in absorbance of 0.01 per min.

RNA extraction and cDNA synthesis. Total RNA content was prepared according to previous methods (Min et al., 2015). DNA traces of contaminated genomes in total RNA were cleared using TURBO Dnase (Ambion). Then, 1.0 μ g of DNA-free RNA was used for cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad) protocol. At each sampling point, RNA extraction was repeated using three samples.

Expression analysis of the *ERF* gene. Oligonucleotide primers for polymerase chain reaction (PCR) analyses were described previously (Min et al., 2015). According to previous literature, gene expression studies used the Ssofast EvaGreen Supermix kit (Bio-Rad) and CFX96 fluorescent PCR detection system for real-time PCR (Yang et al., 2018). Furthermore, 5.0 μ L 2 $\times$ Ssofast EvaGreen Supermix, 0.5 μ L upstream primer (10 μ mol), 0.5 μ L downstream primer (10 μ mol), 1.0 μ L diluted cDNA, and 3.0 μ L H_2O were used.

The PCR procedure was as follows: 95 °C for 3 min; 95 °C for 10 s for 45 cycles; and 60 °C for 30 s. Then, the melting curve analysis was completed. The relative expression of each gene was calibrated using the sample from the day 0 control group. In addition, the relative expression levels of the genes were referenced to the lotus root Actin gene, and each gene was set as a negative control with no template. Each gene expression experiment was performed in triplicate.

Data analysis

Figures were drawn using Origin 8.0 software. Statistical analyses of differences was analyzed using least significant difference.

Results and Discussion

Browning degree

The browning degree was the main indicator of the browning of fruits and vegetables (Baxter, 2010). In this study, different packaging conditions (vacuum packaging and atmospheric packaging at 4 °C) were used to store fresh-cut lotus root slices. The results showed that the browning degree during storage was significantly different between the two packaging methods (Fig. 1). Compared with atmospheric packaging, the browning degree of fresh-cut lotus roots in vacuum packaging was markedly delayed (between 0.215 and 0.285), and the browning degree of roots in atmospheric packaging

changed greatly (between 0.215 and 0.610). Therefore, vacuum packaging could effectively delay the browning process of fresh-cut lotus root; these results were similar to the results of a previous study (Shan et al., 2013).

Total phenolic content

As shown in Fig. 2, the total phenolic content of fresh-cut lotus roots was significantly different in various packaging methods. The total phenolic content of the vacuum-packaged sample at 4 °C decreased and then increased, but the content was not significantly different from beginning to end (96.891 mg/kg on day 0 to 108.981 mg/kg on day 35). The total phenolic content of atmospheric pressure-packaged samples increased greatly from 96.891 mg/kg on day 0 to 159.252 mg/kg on day 35. Moreover, the results showed that storage in vacuum packaging delayed the increase in the total phenolic content in fresh-cut slices; these results were consistent with those of a previous study (Min et al., 2017).

Fig. 1. Effects of two different types of packaging on the degree of browning of fresh-cut lotus root ('E Lian 5'). Fresh-cut lotus root was separately stored in vacuum packaging (VP) (circles) and atmospheric packaging (AP) (squares) at 4 °C. Error bars represent SEs from three biological replicates.

Fig. 2. Effects of two different types of packaging on the total phenol content of fresh-cut lotus root ('E Lian 5'). Fresh-cut lotus root was separately stored in vacuum packaging (VP) (circles) and atmospheric packaging (AP) (squares) at 4 °C. Error bars represent SEs from three biological replicates.

PAL, PPO, and POD activities

PAL, PPO, and POD enzyme activities in the two different types of packages were significantly different (Fig. 3). PAL activity in the two different packaging samples was increased, and there was no significant difference during the early stage of storage (the first 2 weeks) (Fig. 3A). However, PAL enzyme activity increased rapidly, ranging from 8.708 U/g on day 0 to 12.225 U/g on day 35 in the control group; however, vacuum-packaged samples showed no significant change in PAL activity. The PPO activity in the vacuum packaging was lower than that in the atmospheric pressure packaging during the whole storage period. The initial PPO value ranged from 0.396 to 0.604 U/g in the vacuum group and 1.178 U/g in the atmospheric pressure group on day 35 (Fig. 3B). This indicated that PPO enzyme activity was very sensitive in vacuum packaging, and that the PPO enzyme is the key enzyme for the browning of fruits and vegetables; these results were consistent with those of a previous study (Min et al., 2017). Similar to PAL, the most significant difference in activity between packaging methods became apparent after day 14: POD activity in the vacuum group samples increased from 0.528 U/g on day 14 to 0.604 U/g on day 35, whereas activity in the atmospheric group increased from 0.904 U/g to 1.178 U/g during the same time period (Fig. 3C). Finally, the PAL, PPO, and POD enzymatic activities in the vacuum packaging were lower than those in the atmospheric pressure packaging, which indicated that the vacuum conditions could effectively inhibit enzyme activity and, thus,

inhibit enzymatic browning (Xing et al., 2012).

During this experiment, the changes in PAL, PPO, and POD activities under different packaging conditions were studied, and the changes were consistent with the browning of fresh-cut lotus root during storage; these results were consistent with those of previous reports (Banerjee et al., 2015; Cheng et al., 2015).

Gene expression of NnPAL, NnPPO, and NnPOD

Two different types of packaging had different effects on the two *NnPAL* genes (Fig. 4). *NnPAL1* expression in atmospheric packaging increased gradually during the whole storage period, reaching a peak on day 28. *NnPAL1* was continuously repressed by vacuum packaging, and the transcript abundance was decreased by more than 60-fold on day 28. *NnPAL2* mRNA accumulation increased in vacuum packaging, and the levels were relatively lower than those for *NnPAL1*. It was suggested that the expression of *NnPAL1* mRNA paralleled the changes in PAL enzyme activity and browning degree, and that *NnPAL1* was most likely to be involved in the synthesis of phenolic compounds in lotus root browning, which was supported by our previous work (Min et al., 2017).

NnPPOA transcripts were increased in atmospheric pressure packaging, whereas it remained constant in vacuum packaging, which suggested that vacuum packaging inhibited *NnPPOA* expression. Similar to *NnPPOA*, *NnPPOC* transcripts were increased

Fig. 3. Effects of two different types of packaging on phenylalanine ammonia lyase (PAL) (A), polyphenol oxidase (PPO) (B), and peroxidase (POD) activity (C) ('E Lian 5'). Fresh-cut lotus root was separately stored in vacuum packaging (VP) (circles) and atmospheric packaging (AP) (squares) at 4 °C. Error bars represent SEs from three biological replicates.

in atmospheric pressure packaging, whereas it decreased on day 1 and remained constant in vacuum packaging, but with much lower levels (Fig. 5). It was suggested that *NnPPOA* mRNA changes were consistent with the PPO enzyme activity and browning degree. Furthermore, *NnPPOA* was most likely involved in lotus root browning. Similar results have been reported by other studies (Min et al., 2017).

In the *NnPOD* gene family, *NnPOD2/3* mRNA were significantly decreased in vacuum packaging compared with atmospheric packaging during the whole storage time (Fig. 6). This phenomenon indicated that

vacuum packaging inhibited the expression of these two genes, which was consistent with the changes in POD enzyme activity and browning degree. *NnPOD1/5* were inhibited during early storage and upregulated during late storage in vacuum packaging. *NnPOD4/6* were induced by vacuum packaging during the whole storage period. Nevertheless, there was no significant difference in *NnPOD7* expression between the two packaging methods. It was suggested that the expression patterns of *NnPOD2/3* were consistent with the changes in POD enzyme activity and browning degree; therefore, they may be key genes involved in lotus root browning.

Our previous work found that downregulation of *NnPAL1*, *NnPPOA*, and *NnPOD2-4* was consistent with the decreased browning of fresh lotus roots at low temperatures for two different varieties ('E lian 6' and 'E lian 5') (Min et al., 2017). Changes in *NnPAL1*, *NnPPOA*, and *NnPOD2/3* expressions occurred in parallel with changes in PAL, PPO, and POD enzyme activities and the browning degree in vacuum and atmospheric packaging. Based on the present results and our previous report, *NnPAL1*, *NnPPOA*, and *NnPOD2/3* are the key genes affecting the browning of fresh-cut lotus root; however, further functional analyses should be performed to confirm these results.

ERF gene expression

Seven *ERF* genes were isolated during our previous study. These *ERF* genes showed diverse expression patterns (Fig. 7). *NnERF2*

was induced by vacuum packaging during the early stage; however, it was induced by atmospheric pressure packaging during the later stage. There was no obvious difference in the expression of *NnERF3* in the two types of packaging. *NnERF4* expression increased and then decreased during storage in the two types of packaging, but it was always lower in vacuum packaging than in atmospheric pressure packaging during the early and middle storage periods (days 7, 14, and 21), although the expression was relatively low. *NnERF5* mRNA increased gradually during the whole storage period, peaking on day 35, but *NnERF5* was significantly upregulated in vacuum packaging compared with that in atmospheric packaging. *NnERF1/6/7* were significantly induced by vacuum packaging, and the transcript abundance was increased by more than 800-, 400-, 700-fold, respectively.

NnERF4/5 expressions were significantly suppressed by vacuum packaging, which was consistent with *NnPAL1*, *NnPPOA*, and *NnPOD2/3* changes and browning. This indicated that in lotus root, the decreased expressions of *NnERF4/5* were concurrent with decreases in browning; however, the specific relationship between *ERF* and browning is unclear and should be studied further. In our previous study, we found that *NnERF3/4/5* were continuously downregulated by low temperatures and were associated with the browning of fresh-cut lotus roots under low-temperature conditions. Therefore, it was proposed that *NnERF3/4/5* could be important regulators of fresh-cut

Fig. 4. Messenger RNA (mRNA) from phenylalanine ammonia lyase (*PAL*) genes in response to different temperature treatments. Fresh-cut lotus root was separately stored in vacuum packaging (VP) (black) and atmospheric packaging (AP) (red) at 4 °C. The day 0 sample values were set at 1. Error bars represent SEs from three biological replicates.

Fig. 5. Messenger RNA (mRNA) from polyphenol oxidase (*PPO*) genes in response to different temperature treatments. Fresh-cut lotus root was separately stored in vacuum packaging (VP) (black) and atmospheric packaging (AP) (red) at 4 °C. Error bars represent SEs from three biological replicates.

Fig. 6. Messenger RNA (mRNA) from peroxidase (*POD*) genes in response to different temperature treatments. Fresh-cut lotus root was separately stored in vacuum packaging (VP) (black) and atmospheric packaging (AP) (red) at 4 °C. Error bars represent SEs from three biological replicates.

Fig. 7. Messenger RNA (mRNA) from ethylene response factor (*ERF*) genes in response to different temperature treatments. Fresh-cut lotus root was separately stored in vacuum packaging (VP) (black) and atmospheric packing (AP) (red) at 4 °C. Error bars represent *SES* from three biological replicates.

lotus root browning. We further studied the effects of vacuum packaging on browning and *ERF* gene expression in lotus root. *NnERF4/5* were highly correlated with the browning process of lotus root, which further supported the previous findings. Therefore, *NnERF4/5* were proposed to be important regulators of fresh-cut lotus root browning. Moreover, because of the significantly different expression levels of *ERF4* and *ERF5* during storage, and because of the differences in the response to vacuum packaging compared with *ERF4*, it seemed that *NnERF5* was more positively correlated with browning, and that *ERF4/5* might have different roles in lotus root browning. *ERF4* mainly participated in lotus root browning during early storage, whereas *ERF5* functioned during the whole storage period. The relationships of *NnERF4/5* and *NnPAL1*, *NnPPOA*, and *NnPOD2/3* during lotus root browning should be the subject of further research. *NnERF1/6/7* were significantly increased during vacuum storage, which indicated that they are more sensitive to hypoxia stress (vacuum storage). The processing of fresh-cut fruits and vegetables promotes physiological and biochemical changes in the product (Toivonen and Brummell, 2008). These changes may cause significant decreases in color, texture, and flavor. Fresh-cut peppers in vacuum packaging had more noticeable ethanol and acetaldehyde contents, which are essential to the flavor of fresh-cut products (González-Aguilar et al., 2004). Therefore, *NnERF1/6/7* may be related to the loss of quality of fruits and vegetables, especially the loss of flavor. Similar studies involving

two hypoxia-responsive *ERF* (*DkERF9* and *DkERF10*) genes showed acetaldehyde and ethanol synthesis in persimmon were separately regulated by *DkPDC* and *DkADH* promoters (Min et al., 2014).

Conclusions

Browning and PAL, PPO, and POD enzyme activity and gene expression results of this study showed that vacuum packaging effectively delayed lotus root browning. The downregulation of *NnPAL1*, *NnPPOA*, and *NnPOD2/3* in vacuum packaging coincided with the increased related enzyme activities and the browning degree of fresh-cut lotus root. Furthermore, the expression patterns of *NnERF4/5* were consistent with the changes in *NnPAL1*, *NnPPOA*, and *NnPOD2/3* gene expressions. It has been proposed that *NnERF4/5* could be important regulators of fresh-cut lotus root browning.

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