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Transcriptome profiling for pericarp browning during long-term storage of intact lotus root (*Nelumbo nucifera*)

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Abstract

Lotus root (*Nelumbo nucifera*) is an edible rhizome that the consumption/production has continuously increased as more consumers demand convenient and ready-to-eat foods. However, the processing, storage, and transportation of fresh-cut fruits and vegetables promotes physiological deterioration, such as browning, which leads to a reduction in the value of the product. This study aimed to reduce the browning of lotus root pericarps during long-term storage and long-distance transport, and to clarify the functions of unigenes and browning-associated metabolic pathways using RNA-sequencing techniques. Intact lotus root cv. 'Kanasumi No.34' browning decreased after the roots were packed along with an anti-browning solution. Over 200 million short single-end reads were mapped onto the *N. nucifera* consensus coding sequence set. The significantly differentially expressed genes (DEGs) were identified. Based on the Uniprot, GO, and KEGG databases, secondary metabolism, lipid metabolism, and redox state genes were significantly upregulated in the un-packed (UP) and packed with water (PW) treatments compared to after harvest (AH) sample. Additionally, 16 expected differentially expressed genes (e.g., *PPO*, *PAL*, *POD*, *CHS*, *PDCR*, and *SOD*), which are affected by browning development, were differentially regulated in lotus root pericarp. The gene expression data presented in this study will help elucidate the molecular mechanism underlying browning development in intact lotus root during long-term storage. The results may also inform future research on improving the post-harvest life of lotus roots.

Keywords: Browning disorder, RNA sequencing, Transcriptomics, Postharvest physiology

Introduction

Lotus root (*Nelumbo nucifera*), an edible rhizome, is a popular vegetable across the globe because of its attractive white color, abundant nutrients, and crispness. In recent years, fresh cut lotus root has been attracting increasing research and industry interest as it could potentially be a novel, minimally processed vegetable. However, it is well known that the enzymatic browning of fruits and vegetables is a consequence of mechanical and physical stresses that occur during post-harvest handling, processing, storage, and transportation. These stresses promote physiological deterioration, which reduces the value of the product. Browning is the most important problem to affect lotus root during processing and storage, and the resultant deterioration has limited the development of the lotus root industry (Jiang et al. 2014)

Most anti-browning systems extend the shelf life and improve the quality of postharvest fruits and vegetables by including chemical and physical methods that inhibit polyphenol oxidase (PPO) activity. They tend to focus on the essential components of reactions, such as oxygen, copper ions, and substrates (Queiroz 2008). Thermal technology and other effective measures, including low temperature, modified atmosphere packaging, irradiation, and coating, have been applied (Ioannou and Ghoul 2013; Tinello and Lante 2018). These methods prevent moisture and aroma loss and inhibit oxygen penetration into the plant tissue. For example, in a preliminary experiment, lotus roots were harvested and stored at 5°C for 10 days to reduce development of the browning color during storage. There are numerous compounds and physical methods capable of reducing enzymatic browning, which suggests that it should be possible to use natural anti-browning agents during processing and storage. Currently, synthetic additives and natural substances that are eco-friendly, inexpensive, safe for human health, and contain several bioactive compounds have been used as anti-browning agents to reduce color change. Onions contain bioactive compounds, such as flavonoids, alkaloids, phenolic acids, coumarins, and essential oils (Amin 2005; Eissa et al. 2006; Tattelman 2005) and onion extract can inhibit enzymatic browning in mushrooms, bananas, and pears (Bernaś and Jaworska 2015; Kim et al. 2005; Lee 2007). The effect of 36 plant extracts on the inhibition of enzyme browning in fresh-cut apple was investigated by (Wessels et al. 1995).

The mechanism regulating the browning of fruits and vegetables has been investigated for several decades. Enzymatic browning of fruits and vegetables is initiated by PPO, which is a major induction (Martinez and Whitaker 1995; Lante et al. 2016). The complex processes controlling enzymatic browning also involve many other enzymes that induce or inhibit enzymatic browning, such as peroxidase (POD), phenylalanine ammonia lyase (PAL), superoxide dismutase (SOD), and catalase (CAT) (And and Watson 2016; Degl'Innocenti et al. 2007;

Droillard et al. 1987). The PAL, PPO, and POD enzymes have been shown to cause tissue browning in many species, such as apples, pineapples, sweet potato, pears, and cabbage (Murata et al. 2001; Zhou et al. 2003; Liao et al. 2006; Yan et al. 2013; Cheng et al. 2015; Banerjee et al. 2015). Sun et al. (2015) found that PPO and POD activities in fresh-cut lotus root slices were significantly inhibited after treatment with H₂S. Similar changes in POD activities have also been observed during lotus root cv. 3537 browning, and browning degree was significantly positively correlated with POD activity during storage (Jiang et al. 2014). Enzymatic oxidation of phenolics, membrane lipid peroxidation, reactive oxygen species (ROS) overproduction, and scavenger system failure have been shown to be responsible for the browning process in lotus root slices (Sun et al. 2015; Zhang et al. 2013). However, the molecular mechanisms underlying PPO, PAL, and POD involvement with intact lotus root browning during long-term storage is poorly understood.

A number of studies have used RNA sequencing (RNA-seq) to investigate the transcriptomes related to fresh cut lotus (Mellidou et al. 2014; Zhu et al. 2017) and rhizome formation by the plant (Yang et al. 2015). However, there is still little information available about the mechanisms underlying the browning of intact lotus roots during long-term storage. High-throughput transcriptome sequencing technology has recently been widely used to analyze the gene expression levels of whole organisms. The overall comprehensive characteristics of the root transcriptome from lotus were determined using the Illumina NovaSeq 6000 platform, which identified the genes and pathways involved in the browning mechanism. In this study, high-throughput sequencing technology was used to examine gene expression profiles in intact lotus root peels. We analyzed the unigene functions and the browning-associated metabolic pathways were annotated and classified.

Materials and Methods

Plant growth and sampling

Lotus roots from ‘Kanasumi no. 34’ (short shape) were harvested from a lotus farm in Ibaraki Prefecture during August 2018. They were then transported to the laboratory and the roots to be used in the experiment were selected based on uniformity of size, ground color, and freedom from defects. They were washed with tap water to remove any soil that was still attached after cutting and transferred to a pack solution. Three replicate of each treatments were; after harvest samples (AH), Unpackaged (UP), Packed without water (PNW), Packed with Water (PW), Packed with 0.1% ascorbic acid solution (PAA), Packed with 1% onion solution (PO), and Packed with 0.1% citrus peel solution (PC). These samples were then kept in a refrigerator at 5°C and 60%–80% relative

humidity for 0 hr., 6 hr., 10 days, 20 days, and 30 days. The lotus peel was collected after storage and kept at – 80°C until needed. The color was assessed after storage in all treatments. The functions of the unigenes and browning-associated metabolic pathways were clarified using RNA-sequencing techniques to analyze the intact lotus root peels from three replicate of UP and PW after 6 hr. Then the results were compared with the AH results (S1 Fig.). The key-browning-related genes in all the treatments were investigated by qRT-PCR analysis.

RNA isolation

Samples (1 g) of frozen lotus root peel were ground in liquid nitrogen to a fine powder and the total RNA was extracted using the hot-borate method (Wan and Wilkins 2011). There were three replicates of the lotus tissue extracts. The RNA integrity was evaluated using 1.0% agarose gel stained with GelStar® Nucleic Acid Gel Stain. Total RNA was quantified and examined for protein contamination (A_{260}/A_{280}) and reagent contamination (A_{260}/A_{230}) using a NanoDrop ND-1000 spectrophotometer. The high-quality total RNA samples had two distinct peaks and well defined-yield levels.

Library preparation and Illumina sequencing

Beijing Novogene Bioinformatics Technology Co., Ltd (China) was responsible for cDNA library construction and Illumina sequencing. The first step in the cDNA library construction process was to briefly enrich the lotus tissue using oligo (dT) beads. Then, the mRNA was randomly fragmented by adding a fragmentation buffer, and cDNA was synthesized using an mRNA template and a random hexamers primer. Next, a custom second-strand synthesis buffer (Illumina), dNTPs, RNase H, and DNA polymerase I were added to initiate second-strand synthesis. The double-stranded cDNA library was completed after a series of terminal repairs, A ligation, sequencing adapter ligation, size selection, and PCR enrichment. The qualified libraries were fed into Illumina sequencers after pooling according to their effective concentration and expected data volume.

Bioinformatics analysis

Pre-processing of the raw read sequences

The RNA-Seq pipeline in this study is shown in Figure 1 The raw reads were processed by Beijing Novogene Bioinformatics Technology Co., Ltd. (China). Quality control for the raw reads was performed using FastQC, and high-quality reads were obtained after adapter trimming by Cutadapt (Martin 2011). The trimmed

reads had both of 5'- and 3'-ends, but those with low quality ends, (quality scores < 30), and reads where N > 10% (N represents bases that cannot be determined) were removed. Finally, reads containing low quality (Qscore \leq 5) bases, which accounted for over 50% of the total bases, were removed. All downstream analyses were based on high-quality clean reads. The subsequent analyses were performed on high-quality clean read datasets whose quality had been confirmed by the bioinformatics analysis.

Data processing and differential gene expression analysis

The high-quality clean reads were mapped against the *Nelumbo nucifera*_V1.1 reference genome sequence (<https://www.ncbi.nlm.nih.gov/Traces/wgs/APLB01/>) using the TopHat program. The tolerance parameters were the default settings, and transcripts were identified from the TopHat alignment results using Cufflinks. Only unique mappings were used in the expression analyses.

Functional annotation

The biological functions of the unigenes were predicted by aligning all the unigenes using BLAST+ (version 2.3.0; 2) and annotating them against an NCBI Nr protein database (NCBI non-redundant sequence database, a subset of Viridiplantae). BLASTX further aligned consensus sequences to a number of protein databases, such as Swiss-Prot (Bairoch and Boeckmann 1991) and TrEMBL (Bairoch and Apweiler 2000). The DEGs were mapped onto the KEGG pathways using the BLAST KOALA program (<http://www.kegg.jp/blastkoala/>) (Kanehisa et al. 2016). Then, GO annotation of the transcriptome was performed (Zdobnov and Apweiler 2001).

Reverse transcription real-time quantitative PCR (qRT-PCR)

A qRT-PCR analysis was performed to validate the mRNA abundance of 16 genes that were significantly related to browning. Total RNA was isolated from lotus root peel and cDNAs were generated from the RNA samples using a PrimeScriptTM RT reagent kit containing gDNA Eraser (Shiga, Japan). The cDNA was stored at –80°C until needed for the qRT-PCR analysis. In the qRT-PCR assay, cDNA was treated with SYBR® Premix Ex TaqTM II (Takara, Japan) according to the manufacturer's protocol and analyzed using an Illumina EcoTM 48 Real Time PCR system in a total volume of 10 μ L. The PCR cycle comprised one 15 s cycle at 95°C, followed by 40 cycles at 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. All amplified products were subjected to a melt curve

analysis. A negative control without a cDNA template was used in all the analyses to evaluate the overall specificity. The beta-actin reference gene was used to normalize the total amount of cDNA in each reaction. Amplification efficiency and relative gene expression levels were calculated using the $\Delta\Delta C_T$ and $2^{-\Delta\Delta C_T}$ methods (C_T ; cycle threshold), and the ΔC_T value of each gene was calculated by subtracting the C_T value of the endogenous control from the C_T value of the target gene. The gene-specific primers were designed using primer3plus (Untergasser et al. 2007) (Table S2).

Availability of supporting data

The data sets supporting the results produced by this study are available in the DDBJ Sequence Read Archive (DRA) (accession number PRJDB8707).

Statistical analysis

Significant changes in gene expression by qRT-PCR, height and gene expression between different treatments were evaluated by one-way analysis of variance (ANOVA) using a software package (StatView Ver.5.0, SAS Institute Inc. Cary, NC, USA). Multiple Range Tests were performed, using the Tukey's HSD with a confidence level of 95%.

Results

Effects of anti-browning solutions on lotus root browning

Development of browning was analyzed by measuring the L^* , a^* , and b^* values. The browning effect decreased when the packets contained an anti-browning solution. Only the L^* value has been reported in this paper because it represents lightness and is negatively correlated with browning intensity. The L^* value change ratio for 'Kanasumi no.34' tended to increase for all treatments after long-term storage (Fig. 2A). The L^* values change ratio for UP increased rapidly after storage and was significantly different from the other treatments ($P < 0.05$). Furthermore, PNW and the anti-browning solution treatments maintained higher L^* values that had low L^* changing ratio. This showed that they effectively inhibited browning after long-term storage. The Total soluble solids (TSS) contents had decreased by the end of the storage period in the treatments that involved packaging and were significantly different from the control (Fig. S3). The appearance of the lotus roots after storage is shown in Fig. 3 and the sensory test results showed that the PAA, PO, and PC treatments had the highest color scores

acceptant. Furthermore, the smell acceptance scores were significantly lower in the PO and PC groups, but the overall PW and PAA acceptance scores were significantly higher than AH (Fig. S3). The expression patterns for the genes involved in lotus root peel browning development during the initial storage stage were determined by analyzing the RNA extracted from peel after harvest (AH) and in the UP, and PW treatments after 6 hr. of storage.

Overview of the sequencing reads

The RNA was extracted from the peel taken after harvest (AH), and from the UP and PW treatments after 6 hr. of storage. It was then sequenced using the Illumina NovaSeq 6000 platform and an overview of the sequencing is outlined in Table S4. The Q20, Q30, and GC content were calculated using the clean data and are also shown in Table S4. After the adapter sequences and low-quality reads had been removed, there was an average of 23,268,137, 22,204,886, and 20,655,196 high-quality clean reads for AH, UP, and PW, respectively. Over 90% of the clean reads had Phred-like quality scores at the Q30 level, and the average GC content of these samples was 46.28% (Table S4). High-quality transcriptome sequence reads were preprocessed for further analysis.

Differentially expressed gene analysis

We compared and identified differentially expressed genes among the AH, UP, and PW data. Normalization was applied to the treatments to provide accurate differential expressions rather than individual quantifications. A total of 33,656, 31,674, and 32,587 unigenes were annotated from the AH vs UP, AH vs PW, and UP vs PW libraries, respectively (data not showed). The significant DEGs with FDR values < 0.01 and a one log₂ fold change were obtained by comparing the AH vs. UP, AH vs PW, and UP vs PW libraries. There were 2,091, 2,188, and 1,103 DEGs, respectively, (data not showed). The DEG distributions were further annotated using the database. DEGs were first searched against the Swiss Institute of Bioinformatics databases (Swiss-Prot) by local BLASTX (E-value cutoff was set at 1e-5) to search for the maximum number of similar genes. There were 1,177, 1,164, and 624 up and downregulated genes among the AH vs UP, AH vs PW, and UP vs PW libraries, respectively (data not showed).

Functional classification by GO distribution and GO enrichment

Over representations of GO terms in the set of DEGs for lotus peel tissue were evaluated to indicate which biological processes, molecular functions, and cellular components were most affected by the browning disorder

(Fig. 4). Several significantly induced GO terms representing molecular functions were associated with protein binding, DNA binding, and ATP binding (Fig. 4A). The biological process that was significantly enriched in the set of induced DEGs across all treatments was the oxidation-reduction process (Fig. 4B). Other over-represented biological processes included metabolic processes, regulation of transcription, and protein phosphorylation. A significant number of DEGs were also related to the transmembrane transport, signal transduction, and carbohydrate metabolic processes (Fig. 4B). In the cellular component category, the most significant DEGs were associated with membrane and integral components of the membrane (Fig. 4C). When the significant DEGs were subjected to GO enrichment analysis, most of them were significantly represented in the three main GO categories of 'biological process' (BP), 'molecular function' (MF), and 'cell component' (CC). Nucleic acid binding (in the MF category), RNA-DNA hybrid ribonuclease activity (MF), and DNA integration (BP) were significantly enriched when UP was compared with AH (Fig. 5). The RNA-DNA hybrid ribonuclease activity (MF), proteinaceous extracellular matrix (CC), and sodium ion export (BP) were the most highly enriched terms in the AH vs PW library (Fig. 5). Furthermore, aspartic-type endopeptidase activity (MF), DNA integration (BP), and sequence-specific DNA binding transcription factor activity (MF) were significantly enriched when UP was compared with PW (Fig. 5). The overall distribution of GO enrichment in this study is summarized in Table S5.

KEGG enrichment of differentially expressed genes

The KEGG pathway database records networks of molecular interactions in cells and their variants are specific to particular organisms. Pathway-based analysis helps us to understand the biological functions of gene products. The pathway information for all annotated sequences was obtained from the KEGG pathway annotations. In total, 14,977 protein (Peps) in AH vs. UP, 14,938 in AH vs PW, and 9,911 in UP vs PW were mapped to the reference pathway in KEGG using the BLAST KOALA program. The genetic information processing and environmental information processing subcategories in the functional protein family category were significantly enriched in this study. Other functional categories included carbohydrate metabolism, biosynthesis of other secondary metabolites, and lipid metabolism (Fig. S7). The KEGG Orthology (KO) list is summarized in Table S6. In this study, we focused on the phenylpropanoid biosynthesis pathway, tyrosine metabolism, lipid metabolism, and the peroxisome pathway (Fig. S7). Phenylalanine ammonia-lyase (EC:4.3.1.24), caffeoylshikimate esterase (EC:3.1.1), shikimate O-hydroxycinnamoyltransferase (EC:2.3.1.133), cinnamyl-alcohol dehydrogenase (EC:1.1.1.195), peroxidase (EC:1.11.1.7), and caffeic acid 3-O-methyltransferase (EC:2.1.1.68) were significantly

upregulated in the phenylpropanoid biosynthesis pathway (Fig. S7). The results also showed that primary-amine oxidase (EC:1.4.3.21), aromatic-L-amino-acid/L-tryptophan decarboxylase (EC:4.1.1.28 4.1.1.105), polyphenol oxidase (EC:1.10.3.1), and primary-amine oxidase (EC:1.4.3.21) were upregulated in the tyrosine metabolism pathway (Fig. S7). In particular, the polyphenol oxidase (EC:1.10.3.1) group is known to contain enzymes that play important roles in the browning mechanism. Furthermore, acyl-CoA oxidase (EC:1.3.3.6), 3-hydroxyacyl-CoA dehydrogenase (EC:5.3.3.8), and long-chain fatty acid omega-monooxygenase (EC:1.14.14.80) were upregulated in the lipid degradation metabolism pathway, and superoxide dismutase (EC:1.15.1.1) in the peroxisome metabolism pathway was the most significantly upregulated enzyme (Fig. S7).

Candidate genes involved in intact lotus root browning

This study, based on the Swiss-Prot, GO databases, and the KEGG pathway searches, focused on pathways expected to be involved or influenced by browning development during long-term storage of lotus roots, such as secondary metabolism (SM) (fourteen transcripts), lipid metabolism (four transcripts), and redox state (three transcripts) (Table 1).

Validation of differentially expressed genes based on a RNA-seq data analysis by quantitative real-time PCR

The results from the transcriptome analysis were validated by selecting 16 differentially expressed genes that were expected to be involved or influenced by browning development and summarizing them in a heatmap (Fig. 6). These DEGs were associated with secondary metabolism, tyrosine metabolism, lipid metabolism, and redox state. A qRT-PCR analysis of the selected genes was performed (Fig. 7). The eventual brown discoloration is usually the result of interactions between PPO activity and polyphenol content. The *PPO* (*XLOC_018282*) gene accumulated during browning and its expression levels were higher in all the treatments compared to AH (the control). Although *PPO* (*XLOC_018282*) expression was highest in PAA and PO after 6 h, the expression levels in these treatments were still relatively low after 30 days (Fig. 7A). In contrast, the *PPO* (*XLOC_017349*) gene increased sharply after long-term storage, which indicated that the anti-browning solutions did not significantly reduce the expression of this gene (Fig. 7B). *POD* (*XLOC_015548*) expression increased after storage, especially after 30 days in UP and PC (Fig. 7C). Furthermore, *PAL* (*XLOC_012978*) expression, the key gene in the initial step of the phenylpropanoid pathway, was significantly higher in UP after 30 days of storage (Fig. 7D). However, *4CLL9* (*XLOC_008727*) levels remained constant in all treatments except PW where it was significantly higher

after 6-h of storage. (Fig. 6E). *CHS1* (*XLOC_014927*), an important gene in the flavonoid and anthocyanin biosynthesis pathway, increased after long-term storage in all treatments except UP where it declined (Fig. 7F).

The lignin biosynthesis genes included *HST* (*XLOC_004699*), *CSE* (*XLOC_026573*), and *COMT* (*XLOC_011410*). *HST* expression was highest after the lotus root had been being packed with water (PW) and stored for 6 h, whereas its levels were lowest after 30 days in UP (Fig. 7G). The *CSE* and *COMT* genes showed similar patterns because they also decreased after storage in all treatments, apart from UP after 30 days where their expressions were higher (Fig. 7H). In addition, *SCPL50* (*XLOC_026371*) expression decreased after long-term storage in all treatments (Fig. 7J). The expressions of the lipid metabolism related genes *TOGT1* (*XLOC_000017*), *CUT1* (*XLOC_000169*), and *HOS3* (*XLOC_001264*) tended to decrease after long-term storage in all treatments. However, the UP treatment showed the lowest level after 30-days (Figs. 7K–M), which was the opposite of *PDCR* (*XLOC_021293*). The lipid degradation gene increased after long-term storage, especially in UP after 30 days, which showed the greatest upregulation (Fig. 7N). In addition, the redox state-related gene, *FMO* (*XLOC_005442*), tended to decrease after long-term storage, but its levels were high in UP after 30 days. This contrasted with *SOD* (*XLOC_005179*), which increased in PNW and PC after storage. The increase mainly occurred after 30 days (Figs. 7O, P).

Discussion

Effect of anti-browning solutions on the discoloration of intact lotus root

Intact lotus roots during long-term storage and long-distance transport require optimal post-harvest treatments to slow down physiological disorders, such as browning on the skin. In this study, the browning of intact lotus roots during long-term storage was reduced by packing them with different anti-browning solutions (PW, PAA, PO, and PC). The anti-browning solution treatments had higher L^* values, color scores, and overall acceptance values than the storage treatment control (UP). Browning is an oxidative reaction, which means that it can be reduced by removing oxygen from the surface of the root, although browning will rapidly occur when oxygen is reintroduced. Oxygen can be removed by immersing the roots in deoxygenated water, and washing away any leached phenolics and enzymes from the surface can also improve the deoxygenation process (McEvily et al. 1992). In this study, lotus roots that had been vacuum packed without water (PNW) or packed with deoxygenated water (PW) showed delayed browning development after 30 days. It has also been reported that reducing compounds, such as ascorbic acid (AA), decrease browning by reducing the 0-benzoquinones to 0-

dihydroxyphenols or by irreversible inactivation of PPO (Osuga and Whitaker 1995; Rojas-Graü et al. 2006; Tortoe et al. 2007). Similarly, Sun et al., (2010) also reported that AA had an anti-browning effect on fresh-cut apples. Onion has been found to have bioactive compounds that can reduce enzymatic browning and/or oxidoreductase activity (Eissa et al. 2006). They are particularly rich in flavonoids and alk(en)yl cysteine sulfoxides (ACSO). Sulfur compounds are also important because they strongly inhibit *PPO* activity (Griffiths et al. 2002; Lee 2007; Bernas and Jaworska 2014), and citrus powder solution effectively delayed browning in this study.

Citrus peel is an anti-browning agent and is known to be rich in polyphenolic compounds. About 0.1% citrus peel extract has been shown to effectively reduce the browning of apple slices (Park and Kim 2013). In addition, mandarin orange peel extracts are a potential source of compounds that can control browning during the storage of apple slices (Chang et al. 2011). Citrus fruit peel powder also seems to effectively protect against browning development. The natural anti-browning antioxidants can prevent the initiation of browning by reacting with oxygen. They also react with intermediate products, which breaks the browning chain reaction (Lindley 1998; Wessels et al. 2014).

Functional annotation and classification during browning development in intact lotus root

Lotus roots are an economically important vegetable in Japan and are rich in nutrients and phenolic compounds. The genes involved in lotus root browning were identified by extracting the total RNA from lotus skin. Then mRNA samples were prepared and fragmented, and used to synthesize cDNA. The cDNA was sequenced using the Illumina NovaSeq 6000 platform, and the resulting sequencing data were subjected to bioinformatics analysis. The relevant genes or proteins were identified by analyzing them using the associated Uniprot database, GO term, and KEGG pathway. Enzymatic processes were generally recognized as the main determinants of browning. In addition, lipid metabolism and the activities of antioxidant enzymes were also related to browning.

The transcriptome analysis focused on the phenylpropanoid metabolic pathway, which is involved in phenolic synthesis. Phenolic synthesis is regulated by several key enzymes, including PAL, 4CL CHS, and CHI. The pathway results in the formation of polyphenols or flavonoids, which cause browning when the plant tissue is exposed to air (Cantos et al. 2002; Saltveit 2004; Suehiro et al. 2014; Alegria et al. 2016). Zhang et al. (2019) found that the PAL, C4H, 4CL, and CHS enzymes were upregulated in fresh-cut walnut. In this study, SM played a key role in protecting plants against biotic stresses. Several genes related to SM showed differential expressions

when lotus root was subjected to long-term storage (e.g., those related to *PAL*, *POD*, *4CL*, *CHS*, *CSE*, and *COMT*; Table 1: S7), which suggested that high SM-related gene expressions promoted the activity of relevant enzymes leading to an increase in phenolic compounds derived from the phenylpropanoid pathway. This result is consistent with other studies on browning in fresh-cut fruits and vegetables (Hisaminato et al. 2014; Persic 2018; Liu et al. 2018). Furthermore, a number of lipid and membrane related DEGs identified by the GO distribution encoded key enzymes involved in fatty acid biosynthesis and the lipid degradation pathways (Table 1; Fig. 4). This confirmed that membrane lipid alterations in lotus root peels were similar to those in pears and fresh-cut apple (Saquet et al. 2003; Mellidou et al. 2014).

Pericarp browning in lotus root involves oxidation-reduction processes (Fig. 4), and antioxidant enzymes (FMO, SOD; Fig. 6) involved in the peroxisome pathway were differentially expressed. Antioxidative enzymes play a key role in scavenging reactive oxygen species (ROS) under oxidative stress conditions (Sun et al. 2010). Duan et al. (2011) reported that SOD enzymes decrease ROS-induced damage and membrane lipid peroxidation in litchi fruits (Duan 2011). Similarly, in pear and apple, *SOD* genes also showed significantly higher activities during browning development (Mellidou et al. 2014; Fan et al. 2016; Zhu et al. 2017).

Candidate genes for browning development during long-term storage of intact lotus root

PPO and *POD* are considered to have important roles in the enzymatic browning of fruits and vegetables (Chi et al. 2014; Dokhanieh et al. 2016). The *PPO* enzyme, encoded by members of the *PPO* multigene family, is considered to be the major factor responsible for the enzymatic browning of several fresh-cut vegetables (Degl'Innocenti et al. 2007; Chisari et al. 2008). In this study, *PPO* (*XLOC_018282*) genes in lotus roots subjected to anti-browning solutions during long-term storage increased compared to their levels immediately after harvest (Fig. 7). However, the *PPO* (*XLOC_018282*) expressions in the PAA and PO treatments were low after 30 days of storage (Fig. 7A), which suggested that AA, a reducing compound, and onion, which contains various volatile sulfur compounds, including thiols that have been reported to inhibit *PPO*, effectively reduce *PPO* expression (Negishi and Ozawa 2000; Ding et al. 2002). Similarly, Kim et al. (2005) found that pear polyphenol oxidase was also inhibited by onion extract. *POD* (*XLOC_015548*) is an enzyme that is involved in phenolic oxidation. It was upregulated in UP, but its expression was low after 30 days in the PAA and PO treatments (Fig. 7C). *PAL* has been reported to be involved in the browning of fresh-cut potatoes, carrots, lotus roots, and apples (Alegria et al. 2016; Wu et al. 2018; Gao et al. 2017) and *CHS* is involved in grape skin browning (Suehiro 2014). Our results indicated

that the relative expression levels of *PAL* (*XLOC_012978*) genes involved in the phenylpropanoid biosynthesis pathway were most highly upregulated in UP after 30 days, but their expression levels were low in the other treatments (Fig. 7D). In addition, Zhang et al. (2019) found that the *CHS* gene and two *4CL* genes were significantly differentially expressed in browned walnut husks. Chalcone synthase (CHS) plays a key role in flavonoid and anthocyanin biosynthesis. In our study, lotus roots packed without oxygen (PNW) and those packed with anti-browning solution maintained their *CHS1* (*XLOC_014927*) expression levels, but *CHS1* expression was low in UP after 30 days (Fig. 7F). In contrast to the *caffeoylshikimate esterase* (*CSE*) and *caffeic acid 3-O-methyltransferase* (*COMT*) genes, the important genes in the lignin biosynthesis pathway showed low expression levels after long-term storage in anti-browning solution, but were highly upregulated after 30 days in the UP treatment (Figs. 7H, I). Coumaric, caffeic, and ferulic acids, coniferaldehyde, and coniferyl alcohol (precursors in lignin synthesis) proved to be good substrates for POD during the browning of cut jicama (Aquino-Bolaños and Mercado-Silva 2004). Our results suggested that lotus root browning may be related to the lignification process.

During long-term storage, fruits or vegetables need mechanisms that allow them to tolerate oxidative stress and maintain membrane integrity. After the long-term storage of lotus root, the anti-browning solution treatments, especially PAA and PO, maintained *3-ketoacyl-CoA synthase* (*KCS* or *CUT1*) expression levels, whereas the UP treatment had the lowest expression levels for these enzymes (Fig. 7L). In general, very-long-chain fatty acids (VLCFAs) are essential precursors of cuticular waxes and aliphatic suberin in roots. The *VLCFAs* are synthesized by a fatty acid elongation system on the endoplasmic reticulum membrane, and *KCS* is an enzyme in the initial and rate-limiting step. This suggests that 3-ketoacyl-CoA synthase and elongation of fatty acid protein 3-like enzymes may be involved in lotus root browning. However, peroxisomal 2,4-dienoyl-CoA reductase (PDCR), a key enzyme in lipid degradation, was the most upregulated in UP after 30 days (Fig. 7P). Furthermore, *VLCFA* synthesis genes and lipid degradation genes were significantly upregulated in fresh-cut apple (Mellidou et al 2014). Fan et al. (2016) reported that significantly higher activities of antioxidant enzymes (i.e., SOD) were maintained in 'Laiyang' pear fruits undergoing browning (Fan et al. 2016). Superoxide dismutase is an important antioxidant enzyme that catalyzes the dismutation of superoxide to O_2 and H_2O_2 . In this study, SOD expression increased when lotus root was treated with an anti-browning solution and this may be because SOD reduces ROS levels. Recent proteomic studies on fresh-cut apple and luffa fruits also suggested that SOD may have a major role in the redox state system during browning (Mellidou et al 2014; Zhu et al. 2016).

Conclusion

The lotus root is an economically important vegetable in Japan and is rich in nutrients and phenolic compounds. Therefore, it is important to find post-harvest treatments that slow down the browning of intact lotus root pericarps during long-term storage and long-distance transport. In this study, the browning of intact lotus root was reduced by packing them with an anti-browning solution (PAA, PO, and PC; Fig. 8). The genes involved in lotus root browning were identified by comparing the AH, UP, and PW transcriptomes after 6 h. storage at low temperature. Significant DEGs were obtained by comparing the AH vs UP, AH vs PW, and UP vs PW libraries, which contained 2,091, 2,188, and 1,103 DEGs, respectively. Furthermore, SM, lipid metabolism, and redox state-related genes were significantly upregulated according to the results based on the UniProt database, GO databases, and KEGG pathway, especially in unpackage (UP) treatment (Fig. 8). Additionally, 16 genes that were expected to be differentially expressed (e.g., *PPO*, *PAL*, *POD*, *CHS*, *PDCR*, and *SOD*) and are influenced by browning development were differentially regulated in lotus root pericarp. The genetic resources and putative signaling pathways related to lotus root defense responses against browning may be useful in future molecular studies on *Nelumbo nucifera*.

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Supporting information

S1 Fig. Lotus root picture for RNA-seq.

S2 Table. Genes and primers for validation of RNA-Seq results by qRT-PCR.

S3 Fig. Sensory test score of lotus root cv. 'Kanasumi no 34' after long term storage at 5 °C, RH 70-80 % in effective anti-browning solution (UP unpacked, PNW Packed without water, PW Packed with water, PAA packed with ascorbic acid, PO packed with onion, PC packed with citrus powder.

S4 Fig. Overview of the sequencing reads of libraries.

S5 Table. GO enrichment in pericarp of lotus root were compared between AH_vs_UP, AH_vs_PW and UP_vs_PW

S6 Table. Statistics of KEGG enrichment (KO-list)

S7 Fig. KEGG pathway of browning related proteins

Figure captions

Figure 1 RNA-Seq Pipeline for Lotus root ‘Kanasumi no.34’

Figure 2. The color changing ratio of L^* value after storage 0-hr., 6-hr., 10, 20, and 30 days at 5 °C, RH 70-80% for ‘Kanasumi no. 34’. (UP; unpacked, PNW; Packed no-air and no-water, PW; Packed with water, PAA; packed with 0.1% ascorbic acid solution, PO; packed with 1% onion solution, PC; packed with 0.1% citrus powder solution). *, ** indicate the significant difference between weeks, based on an ANOVA and Multiple Range Test procedures with a confidence level of 95%.

Figure 3. The appearance of lotus root after storage 0-hr., 6-hr., 10, 20, and 30 days at 5 °C, RH 70-80% for ‘Kanasumi no. 34’. (UP; unpacked, PNW; Packed no-air and no-water, PW; Packed with water, PAA; packed with 0.1% ascorbic acid solution, PO; packed with 1% onion solution, PC; packed with 0.1% citrus powder solution).

Figure 4. The histograms were showing GO functional analysis. The y -axis indicates the names of the most abundant classes in each of the three main GO categories. The x -axis indicates the numbers of common DEGs.

Figure 5. The histograms were showing GO functional analysis. The y -axis indicates the names of the most abundant classes in each of the three main GO categories. The x -axis indicates z -score value.

Figure 6. Heatmap visualization of sixteen key genes associated with browning. Heatmap shows the significant browning-related genes between AH, UP, and PW with $q_value \leq 0.01$ and \log_2 -fold change ≥ 1 (red and green colors indicate up- and downregulated transcripts, respectively).

Figure 7. qRT-PCR analysis of selected sixteen genes after storage 0-hr., 6-hr., 10, 20, and 30 days at 5 °C, RH 70-80% for ‘Kanasumi no. 34’. (AH; After harvest, UP; unpacked, PNW; Packed no-air and no-water, PW; Packed with water, PAA; packed with 0.1% ascorbic acid solution, PO; packed with 1% onion solution, PC; packed with 0.1% citrus powder solution). Error bars depict the standard error of the mean for three biological replicates. Non-overlapping letters indicate significant difference between treatments, based on ANOVA analysis and Multiple Range Tests procedure with a confidence level of 95%.

Figure 8. Simple diagram of enzymatic browning (Adapted from Grotheer et al., 2005)

Table 1. Browning-related genes induced or repressed in the intact lotus root after long term storage

Lotus_ID_genes	Gene description	Function				FPKM		
			p_value	q_value	Log ₂ FC	AH	UP	PW
Secondary metabolism								
XLOC_004699	Shikimate O-hy droxy cinnamoyl transferase (HST)	Phenylpropanoid pathway/lignin	0.0001	0.0009	-2.0002	0	4.56	1.14
XLOC_023258	Caffeic acid 3-O-methyltransferase	Phenylpropanoid pathway	5.00E-05	0.0005	0.9652	0	29.1	56.82
XLOC_011410	Caffeic acid 3-O-methyltransferase (COMT)	Phenylpropanoid pathway/lignin	0.00105	0.003	6.8518	1.01	116.39	0
XLOC_029919	1-Cys peroxiredoxin PER1	Phenylpropanoid pathway	5.00E-05	0.0005	1.9485	0	0.79	3.07
XLOC_001024	F-box/kelch-repeat protein At1g15670	Phenylpropanoid metabolic process	5.00E-05	0.0005	-1.7937	0	6.61	1.91
XLOC_026371	Serine carboxy peptidase-like 50 (SCPL)	Phenylpropanoid pathway	5.00E-05	0.0002	-1.1862	17.82	7.83	0
XLOC_012978	Phenylalanine ammonia-lyase (PAL)	Phenylpropanoid pathway	5.00E-05	0.0002	8.0398	0.7	182.9	0
XLOC_015548	Peroxidase 12 (POD)	Phenylpropanoid pathway	5.00E-05	0.0002	2.3765	4.75	24.66	0
XLOC_018282	Polyphenol oxidase (PPO)	Tyrosine metabolism	5.00E-05	0.0002	5.7442	55.83	2992.76	0
XLOC_017349	Polyphenol oxidase (PPO)	Tyrosine metabolism	5.00E-05	0.0002	6.1217	64.38	0	4482.9
XLOC_026573	Caffeoylshikimate esterase (CSE)	Phenylpropanoid pathway/lignin	5.00E-05	0.0002	1.5909	46.89	141.25	0
XLOC_014927	Chalcone synthase 1 (CHS)	Phenylpropanoid pathway/flavonoid	5.00E-05	0.0002	-1.4134	2365.75	0	888.16
XLOC_008727	4-coumarate--CoA ligase-like 9 (4CLL)	Phenylpropanoid pathway	0.0052	0.0131	1.4714	0.95	0	2.64
XLOC_000017	Scopoletin glucosyltransferase (TOGT)	Glucosyltransferase metabolism	5.00E-05	0.0002	1.1569	15.46	34.48	0
Lipid metabolism								
XLOC_000169	3-ketoacyl-CoA synthase 6 (CUT)	Fatty acid biosynthesis	5.00E-05	0.0002	2.9322	0.98	7.46	0
XLOC_001264	Elongation of fatty acids protein 3-like (HOS)	Fatty acid biosynthesis	5.00E-05	0.0002	2.5031	5.27	29.9	0
XLOC_020194	Peroxisomal 2,4-dienoyl-CoA reductase (PDCR)	Lipid degradation/unsaturated fatty acid beta-oxidation	5.00E-05	0.0002	2.1083	20.22	0	87.18
XLOC_021293	Peroxisomal 2,4-dienoyl-CoA reductase (PDCR)	Lipid degradation/unsaturated fatty acid beta-oxidation	5.00E-05	0.0002	1.8965	11.51	42.86	0
Redox state								
XLOC_005442	Flavin-containing monooxy genase (FMO)	Oxidation process	5.00E-05	0.0002	2.2927	1.53	7.51	0
XLOC_005118	Flavin-containing monooxy genase (FMO)	Oxidation process	5.00E-05	0.0002	1.8971	1.99	0	7.43
XLOC_005179	Superoxide dismutase [Cu-Zn] (SOD)	Oxidation process	0.01715	0.0362	0.5777	108.97	162.63	0

Figures

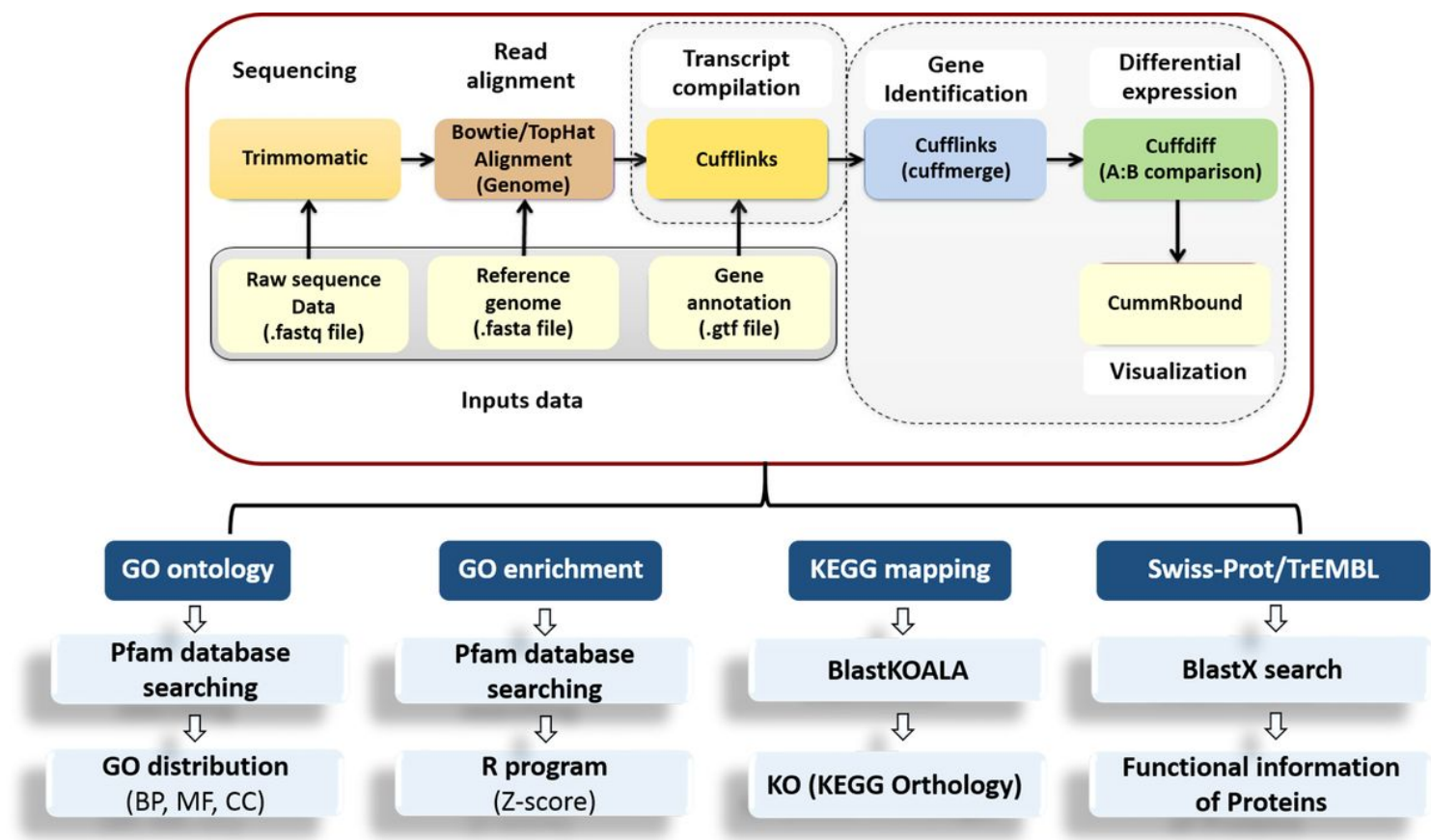


Figure 1

RNA-Seq Pipeline for Lotus root 'Kanasumi no.34'

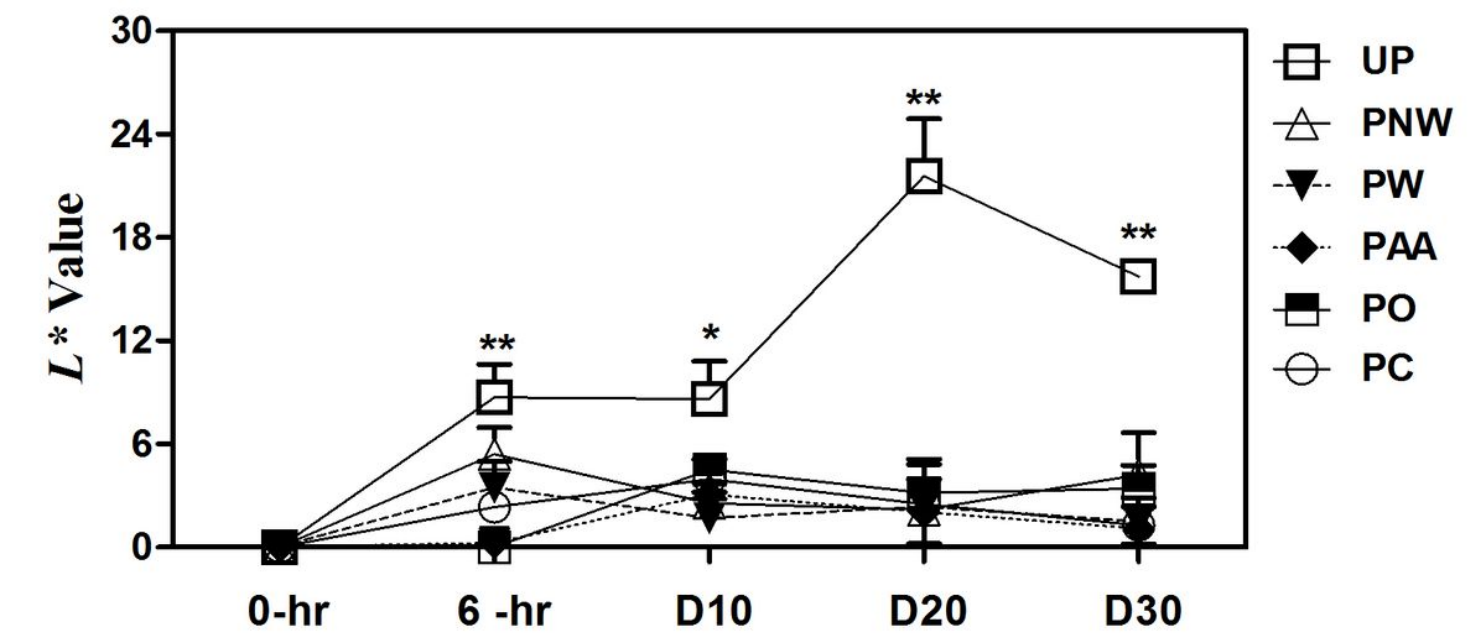


Figure 2

The color changing ratio of L* value after storage 0-hr., 6-hr., 10, 20, and 30 days at 5 °C, RH 70-80% for 'Kanasumi no. 34'. (UP; unpacked, PNW; Packed no-air and no-water, PW; Packed with water, PAA; packed with 0.1% ascorbic acid solution, PO; packed with 1% onion solution, PC; packed with 0.1% citrus powder solution). *, ** indicate the significant difference between weeks, based on an ANOVA and Multiple Range Test procedures with a confidence level of 95%.

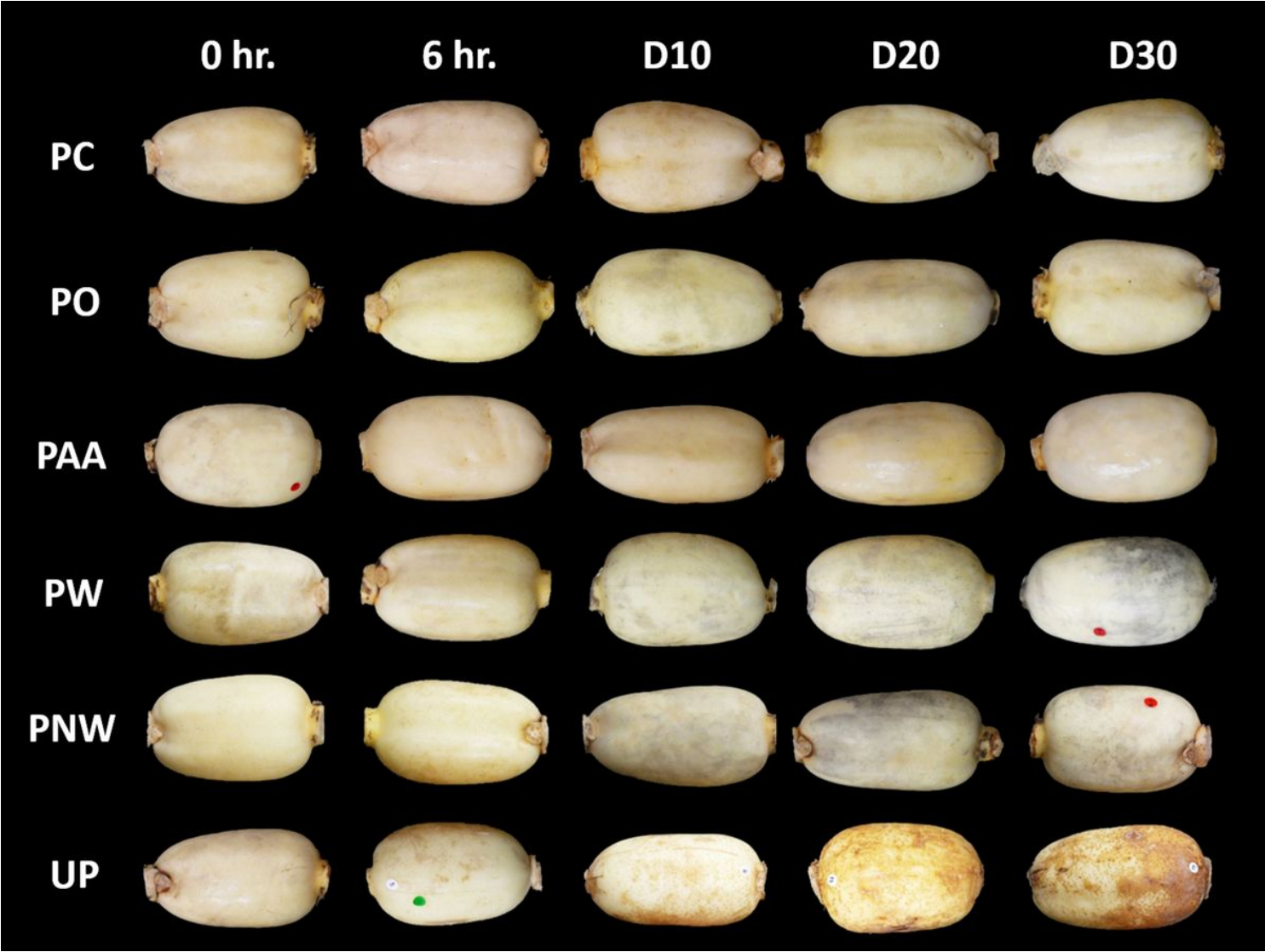


Figure 3

The appearance of lotus root after storage 0-hr., 6-hr., 10, 20, and 30 days at 5 °C, RH 70-80% for 'Kanasumi no. 34'. (UP; unpacked, PNW; Packed no-air and no-water, PW; Packed with water, PAA; packed with 0.1% ascorbic acid solution, PO; packed with 1% onion solution, PC; packed with 0.1% citrus powder solution).

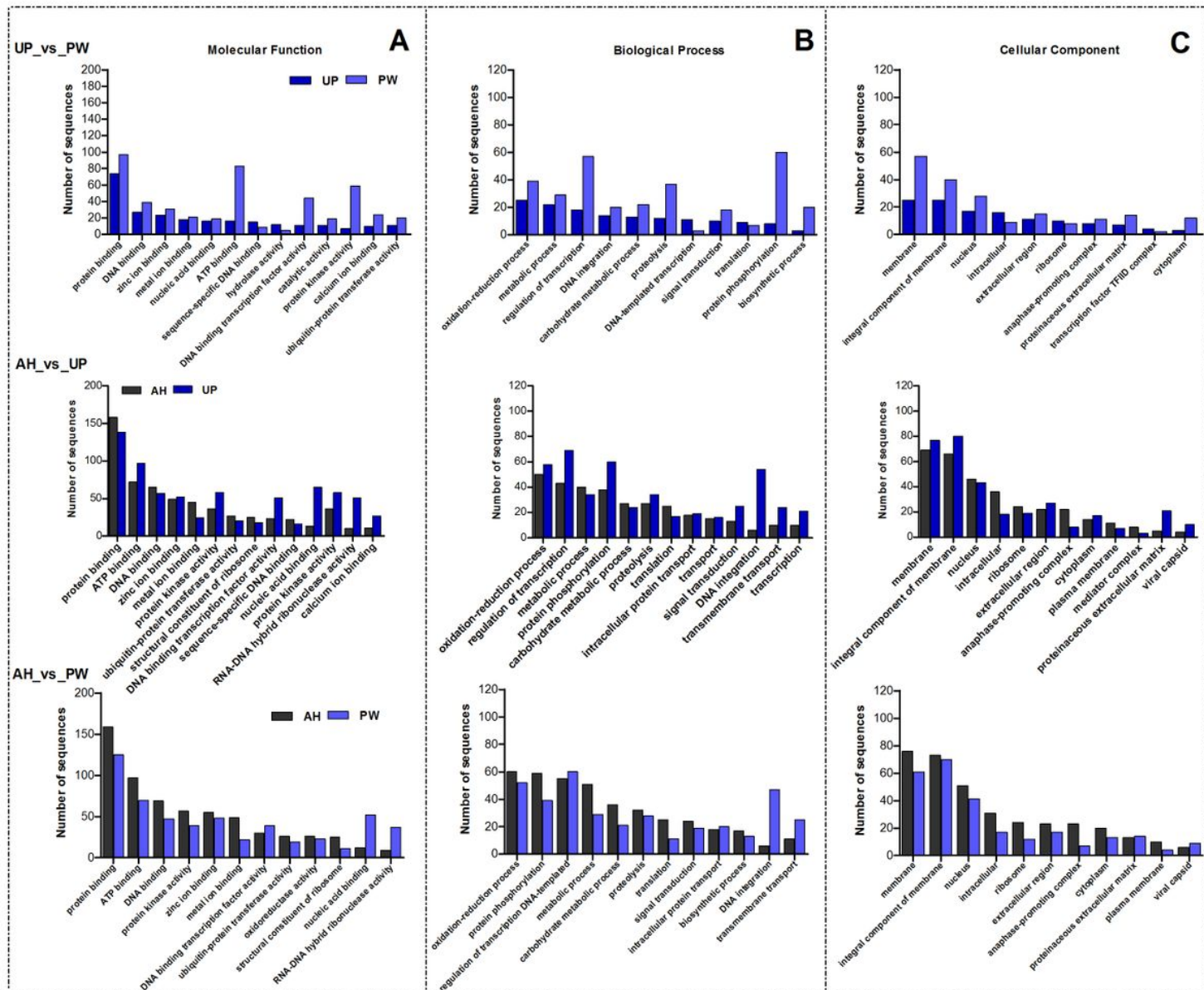


Figure 4

The histograms were showing GO functional analysis. The y-axis indicates the names of the most abundant classes in each of the three main GO categories. The x-axis indicates the numbers of common DEGs.

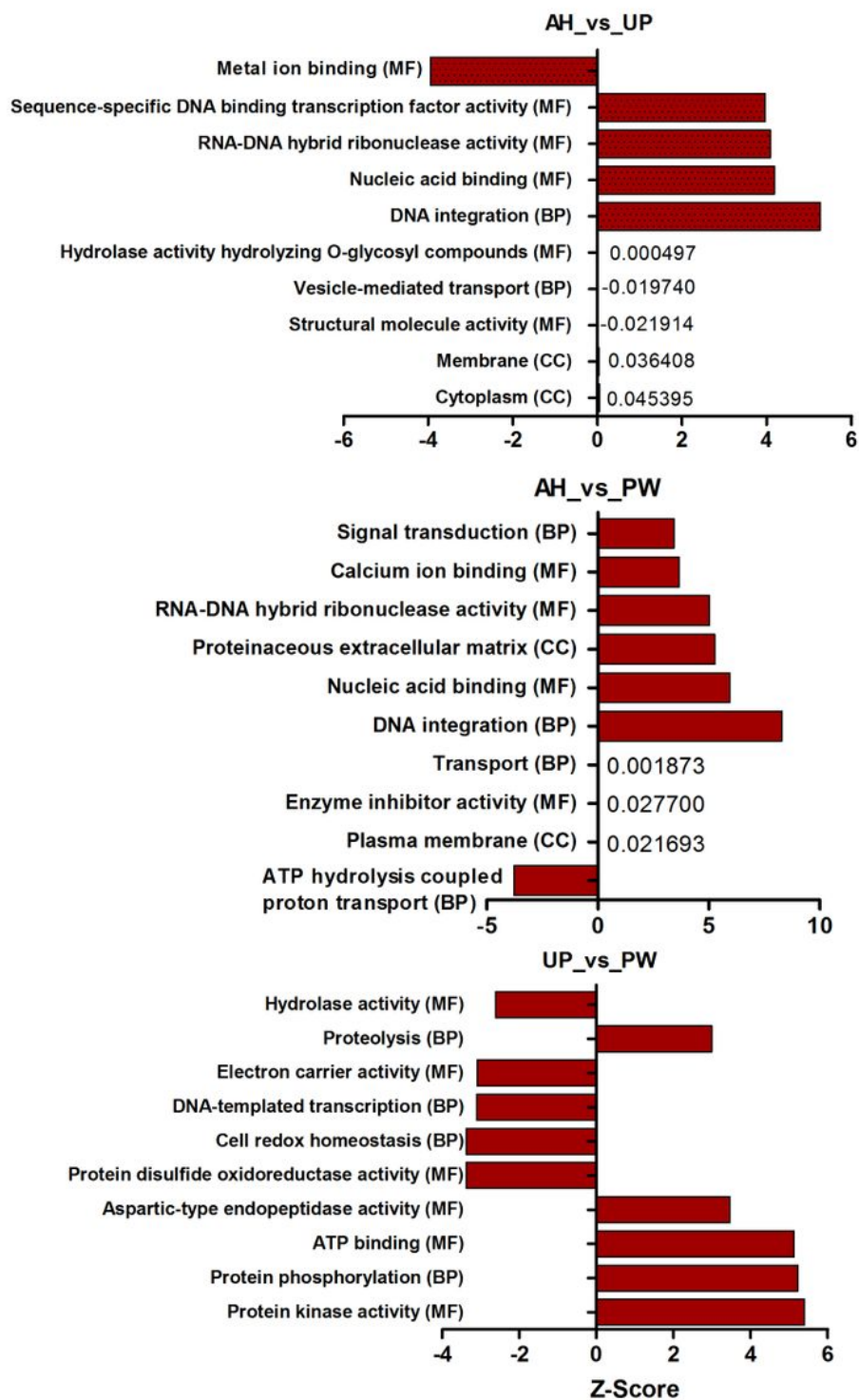


Figure 5

The histograms were showing GO functional analysis. The y-axis indicates the names of the most abundant classes in each of the three main GO categories. The x-axis indicates z-score value.

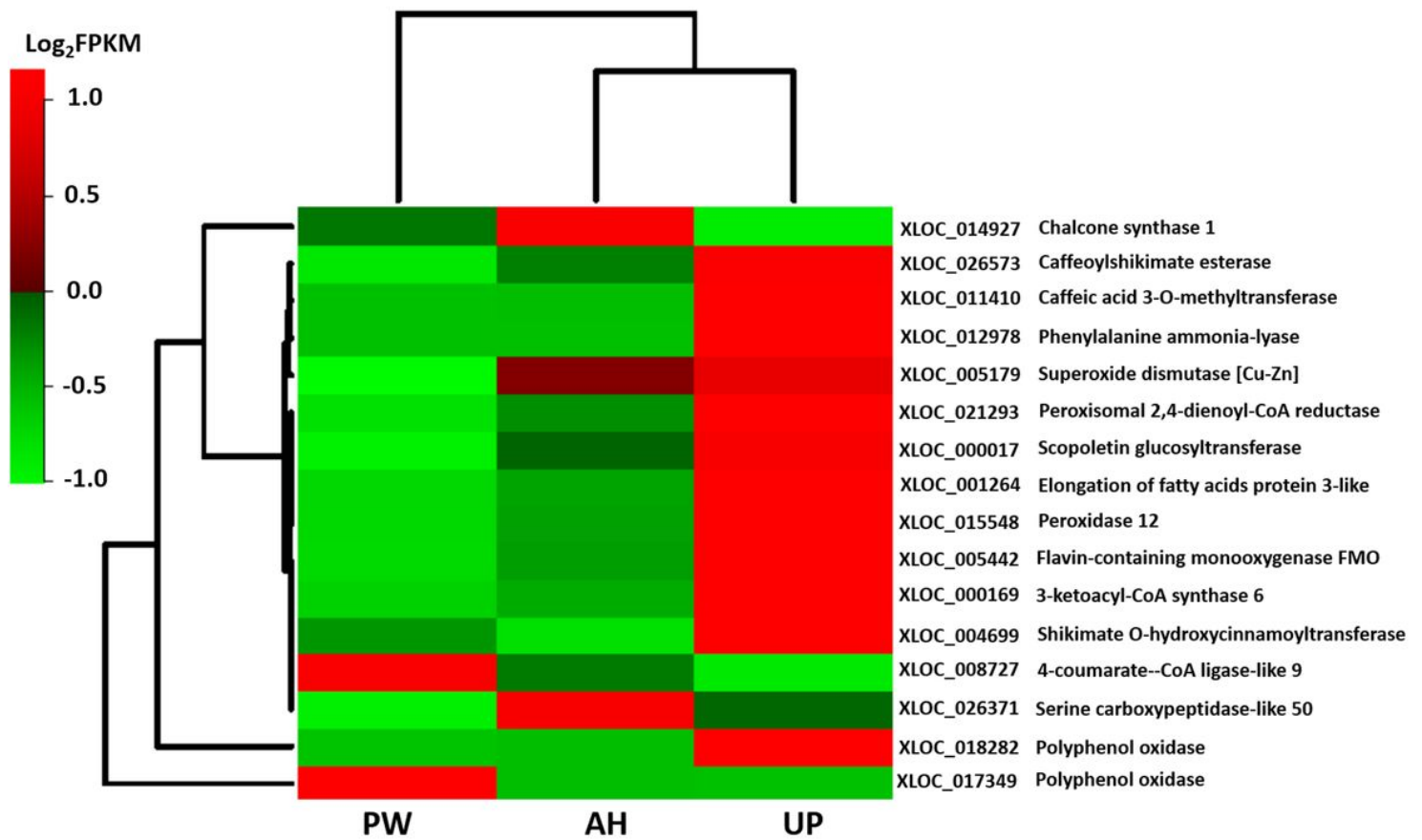


Figure 6

Heatmap visualization of sixteen key genes associated with browning. Heatmap shows the significant browning-related genes between AH, UP, and PW with $q_value \leq 0.01$ and $\log_2\text{-fold change} \geq 1$ (red and green colors indicate up- and downregulated transcripts, respectively).

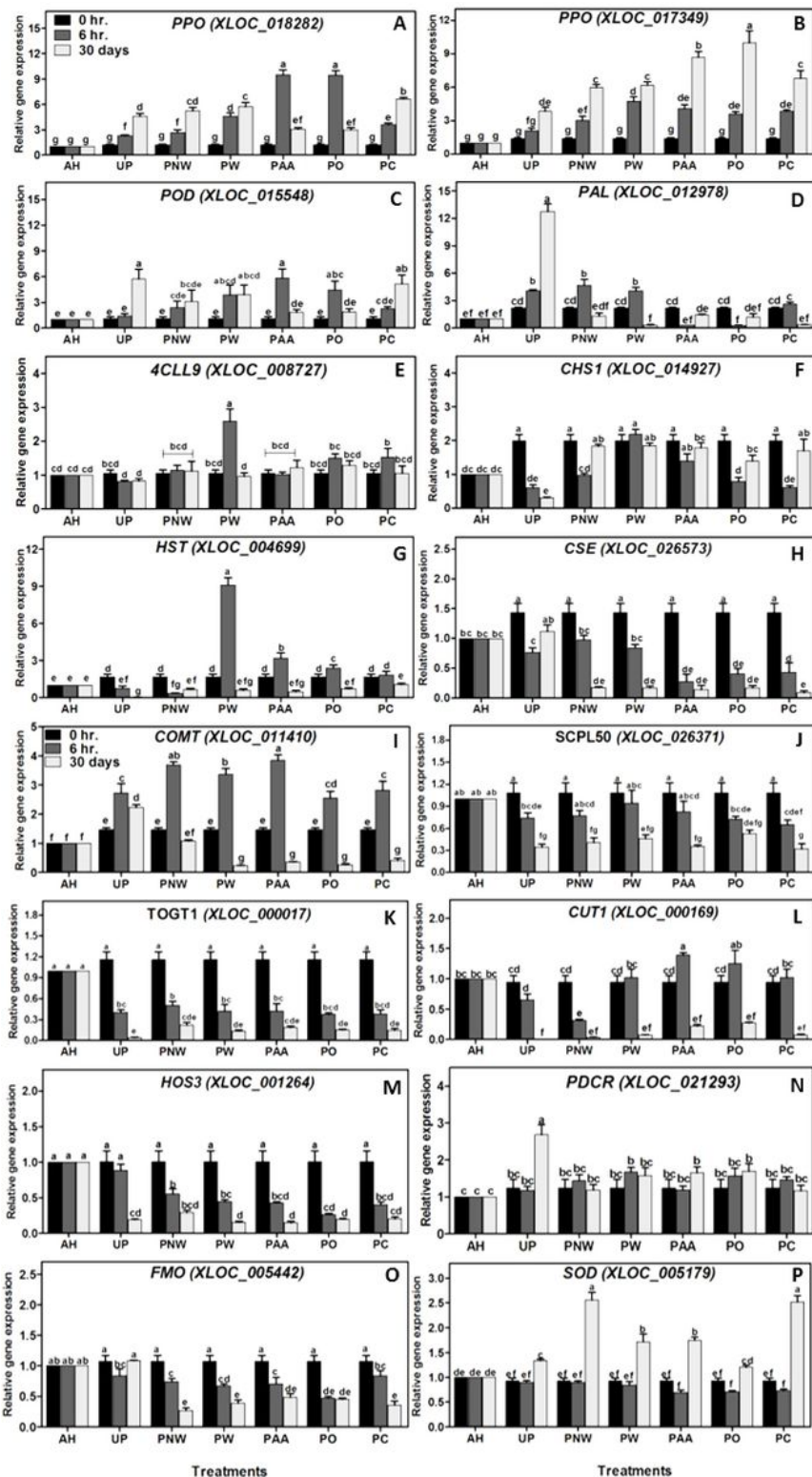


Figure 7

qRT-PCR analysis of selected sixteen genes after storage 0-hr., 6-hr., 10, 20, and 30 days at 5 °C, RH 70-80% for 'Kanasumi no. 34'. (AH; After harvest, UP; unpacked, PNW; Packed no-air and no-water, PW; Packed with water, PAA; packed with 0.1% ascorbic acid solution, PO; packed with 1% onion solution, PC; packed with 0.1% citrus powder solution). Error bars depict the standard error of the mean for three

biological replicates. Non-overlapping letters indicate significant difference between treatments, based on ANOVA analysis and Multiple Range Tests procedure with a confidence level of 95%.

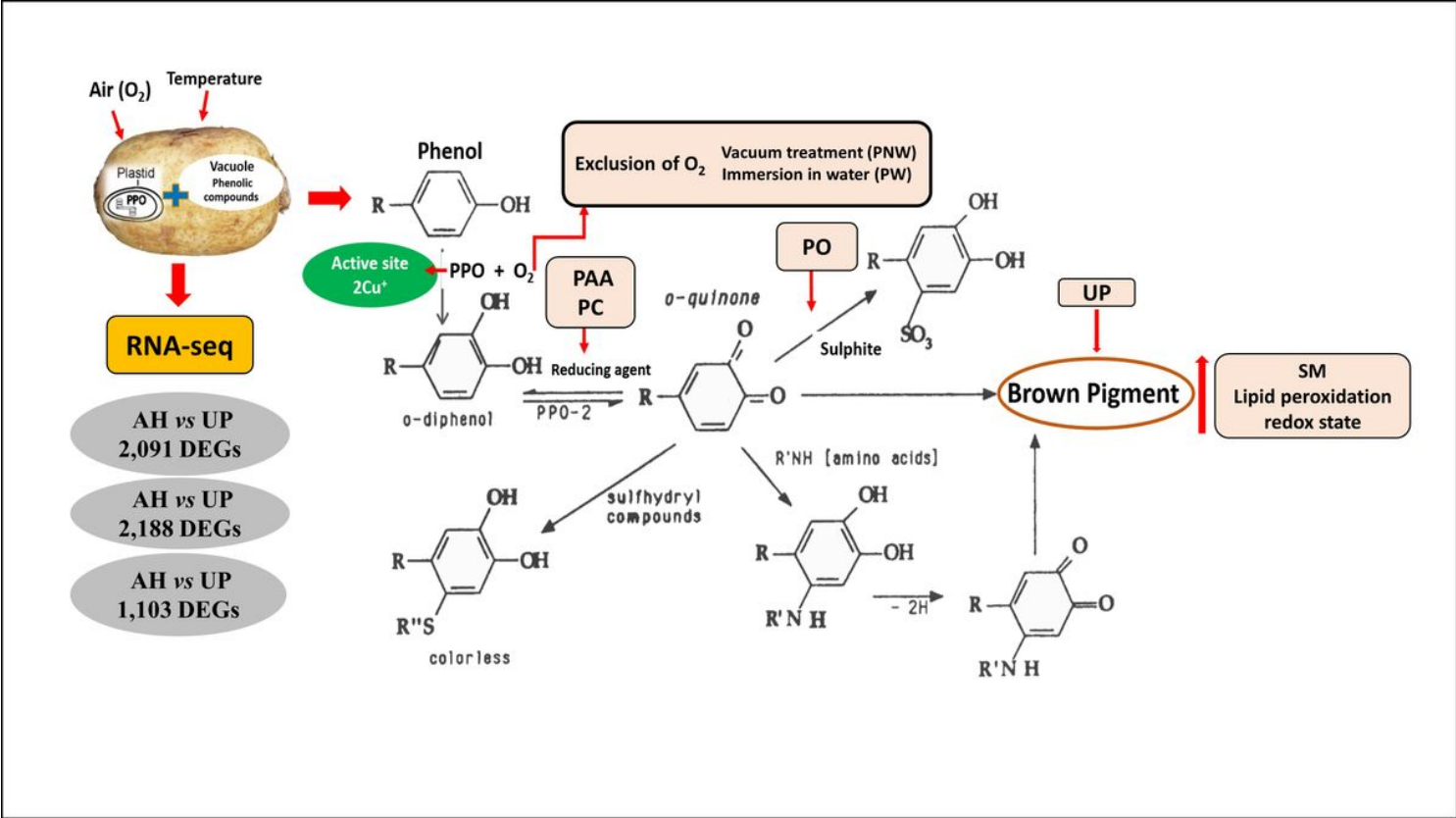


Figure 8

Simple diagram of enzymatic browning (Adapted from Grotheer et al., 2005)

Supplementary Files

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- [Supplementaryfiles.pdf](#)