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Functional Classification of Skinning Injury Responsive Genes in Storage Roots of Sweetpotato

Klasifikasi Fungsi Gen-gen yang Responsif terhadap Pelukaan Kulit pada Umbi Ubi Jalar

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ABSTRACT

Skinning injury in sweetpotato due to loss of skin or periderm which occurs during harvest is inevitable and account for financial loss due to dehydration, pests, and pathogens. Hence, studies on gene expression changed due to skinning injury can provide important information about this protective tissue and for improving the life of storage roots. New candidate genes involved in skinning injury were isolated with an annealing control primer (ACP). Using 20 ACP primers, a total of 103 differentially expressed genes (DEGs) were retrieved. In this study, the functional annotation of these selected 15 up-regulated DEGs (10 contigs and 5 singletons) were characterized. The results showed that these 15 "DEG-unigenes" were mainly associated with defense and stress responses, regulation and signaling, protein synthesis and fate, and metabolism may play an important role in the primary responses to skinning injury in storage roots of sweetpotato. This study showed the importance of defense and stress responses genes to the formation of wound periderm. Furthermore, this results can be used for better understanding of the molecular mechanism of skinning/mechanical injury-related genes in the storage roots of sweetpotato as well as to all stems, fruits, and roots of all plants.

Keywords: differentially expressed gene, gene function, Ipomoea batatas, wounding

ABSTRAK

Kerusakan kulit pada umbi ubi jalar akibat kehilangan kulit atau periderm yang terjadi waktu panen tidak dapat dihindari dan mengakibatkan kerugian finansial sebagai akibat dari permukaan umbi menjadi kering dan rentan terhadap hama dan patogen. Oleh karena itu, penelitian terhadap perubahan ekspresi gen akibat pelukaan dapat menghasilkan informasi penting tentang jaringan proteksi tersebut dan untuk memperbaiki daya hidup dari umbi tersebut. Kandidat-kandidat gene baru yang terlibat dalam pelukaan/kerusakan kulit diisolasi dengan menggunakan annealing control primer (ACP). Penggunaan 20 primer ACP berhasil mengidentifikasi 103 gen-gen yang terekspresi secara berbeda (DEG). Fungsi anotasi dari 15 DEG yang terseleksi dianalisis lebih lanjut. Hasil fungsi anotasi dari 15 DEG menunjukkan ke-15 DEG tersebut dikategorikan sebagai gen-gen yang berkaitan dengan: ketahanan dan respons terhadap stress, regulasi dan penyandian, sintesis protein dan lokasi akhir protein, dan metabolisme memainkan peranan penting pada respons utama terhadap pelukaan kulit pada umbi ubi jalar. Hasil penelitian ini menunjukkan bahwa gen-gen ketahanan dan respons terhadap stress mempunyai peranan penting terhadap pembentukan lapisan sekunder (feloderm). Hasil penelitian ini dapat digunakan untuk memahami lebih baik tentang mekanisme molekuler gen-gen yang berhubungan dengan pelukaan yang terjadi secara mekanis pada umbi ubi jalar dan pada cabang, buah, dan umbi dari tanaman yang mengalami pelukaan.

Kata kunci: expresi gen berbeda, fungsi gen, Ipomoea batatas, pelukaan

INTRODUCTION

Sweetpotato storage roots are underground storage organs covered by skin or periderm, a suberized layer that protects inner flesh from dehydration and pathogens. Skinning, the loss of the skin from the surface of the storage roots of sweetpotato, is responsible for significant postharvest losses due to mechanical injury, weight loss, sprouting, pests and diseases. Thus, because of its short shelf-life, in developing countries the sweetpotato storage

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roots has to be consumed or marketed within 1 to 2 weeks after harvesting (Rees *et al.*, 2008).

Periderm formation which is characterized by secondary growth are usually found in the plants following wounding under conditions of high temperature and humidity (curing). In order to understand well about wound periderm formation, a study on the molecular mechanisms associated with its formation to extend the storage life of sweetpotato storage roots is needed. Moreover, to understand the molecular mechanisms underlying skinning injury, attempts have turned toward the isolation of genes regulated by skinning injury. This allows insight into their functions and the pathways that lead to their expression. Although several responses of plants to skinning injury, including short term metabolic and physiological changes may not require changes in gene expression, the majority are predicted to rely on alterations in gene expression. For example, wounding alters changes in gene expression in response to mechanical wounding in potato skin using a suppression subtractive hybridization library (Soler *et al.*, 2011). They showed that in response to wounding, the highest mRNA transcripts were dominated by genes involved in stress and defense followed by genes involved in signal transduction and regulations. Furthermore, they also showed the activation of genes involved in suberin and wax and cell wall in response to wounding in potato skin. Effendy *et al.* (2013) showed that intentional skinning in storage roots of sweetpotato induced the expression of genes involved in abiotic stresses, wounding responses, transcriptional regulation/signaling, and lignin and suberin biosynthesis. Other study by Lulai and Neubauer (2014) in potato skin showed the spatial and temporary wounding induced suberization genes that involved in the closing layer and wound periderm formation.

In order to understand the molecular basis of skinning injury responses, the differentially expressed genes (DEGs) in skinning-treated storage roots must be characterized. Low levels of DEGs and their transcripts are often hard to detect in abundant mRNA within tissues. A highly reliable, accurate, and reproducible specific polymerase chain reaction (PCR) amplification is a prerequisite. A novel annealing control primer (ACP)-based Reverse Transcriptase (RT) PCR technology is based on a unique tripartite structure of a specific oligonucleotide primer with its 3' and 5' ends separated by a regulator, and the interaction of each end of this primer during a two-stage PCR. Because of the high-annealing specificity during PCR, the application of the ACP to DEG identification generates reproducible, accurate, and long (100 bp to 2 kb) PCR products that are detectable on agarose gels. This method specifically targets the template sequence for hybridization through a polydeoxyinosine linker (Hwang *et al.*, 2003). Here, ACP-based RTPCR is performed to identify genes up-regulated in storage roots of sweetpotato using cDNA from skinned storage roots to determine the common genes. Freshly harvested sweetpotatoes were used for this approach. Briefly, this work presents 15 DEGs that could be used to further investigate their role in skinning injury and wound healing responses at the molecular level. The objective of this work was to describe transcriptional changes in LA 07-146 subjected to skinning injury in storage roots of sweetpotato at 2, 4, 8, and 12 h.

MATERIAS AND METHODS

Place and Time of the Research

This research was carried out in the Molecular Biology Laboratory, School of Plant, Environmental, and Soil Sciences, Louisiana State University Agricultural Center, Baton Rouge, Louisiana, United States of America from August to October, 2011.

Plant Materials and Skinning Treatment

Freshly harvested storage roots of sweetpotato cultivar LA 07-146 were washed, blot-dried, and carefully scraped with a razor scraper (Titan 11030; Star Asia-USA, Renton, WA) to remove the thin outer pigmented skin as detailed in Effendy *et al.* (2013).

RNA Isolation, cDNA Preparation and ACP-based Gene-Fishing PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) following manufacturer's instructions (Effendy *et al.,* 2013). First-strand cDNA synthesis was performed using a GeneFishing™ DEG premix kit (Seegene, Rockville, MD) as previously described (Seegene, Rockville, MD). Second strand cDNA synthesis and subsequent PCR amplification were performed in a single tube using the protocol as detailed in the GeneFishing™ DEG premix kit manual (Seegene, Rockville, MD).

Cloning and Sequencing of DEGs

Based on intensity or presence/absence) between control and skinning were performed following the manufacturer's instruction (QIAquick® Gel Extraction Kit Qiagen, Valencia, CA).

Nucleotide and Deduced Amino Acid Sequencing Analyses

The DEGs were cut from the gel and cloned into pGEM®-T Easy vector (Promega, Madison, WI). Muzuni *et al*. (2010) has successful using this vector to clone fragment cDNA H^+-ATPase from *Melastoma malabathricum*. The positive colonies from DEGs were confirmed by colony PCR using M13F and M13R primers. Plasmids isolated from these clones were single-pass sequenced with T7 primer in an ABI 3730x1 genetic analyzer.

DNA sequences were processed manually to clean the vector backbone and the poly (A) tail and searched against the non redundant nucleotide and protein database of National Center for Biotechnology Information using BLASTN and BLASTX interference (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al*., 1997).

RESULTS AND DISCUSSION

Effect of Skinning Treatment on Storage Root RNA Populations

To examine the effect of skinning treatment on storage root RNA populations, freshly harvested storage roots were exposed to skinning treatment. In order to determine how rapidly changes in the mRNA population occurred following the onset of a skinned treatment, time course experiments were carried out. RNA was isolated from sweetpotato storage roots for 0 (control), 2, 4, 8+12 h following the onset of a skinning treatment and used for ACP-based RTPCR. The RNAs results obtained were then used for ACP-based differential display (DD)-RTPCR with 20 ACP primers. Differentially expressed genes were used to examine and compare the patterns of expressed mRNAs present in skinning injury treated storage roots. Subsequently, a number of skinned-induced partial cDNAs were isolated and examined in roots subjected to skinning. The results showed that skinning induced changes in the accumulation of a number of different DEGs (Table 1); indicative of a skinned-induced activation and repression of gene expression. A number of changes in mRNA accumulation that occurred in skinned-treated roots occurred rapidly within 2 h after subjected to skinning injury. In previous studies, it was observed that a wounding treatment resulted in the altered synthesis and accumulation of a number of genes in roots of potato (Soler *et al*., 2011; Boher *et al*., 2013; Effendy *et al*., 2013; Lulai and Neubauer, 2014). In the present study, skinned-induced changes in the accumulation of a number of different RNAs was demonstrated (Table 1). A substantial proportion of the changes in RNA accumulation that occurred in skinning-treated storage roots were transient in nature and many occurred rapidly within 2 h of skinning (Table 1). The rapid induction of gene expression is consistent with previous studies with the wound-enhanced accumulation of genes expression in roots in which the earliest changes were observed within 2 h after wounding in storage roots of sweetpotato (Effendy *et al*., 2013).

Cloning and Sequencing of Skinning Injury Responsive DEGs

ACP-based DD-RTPCR clearly demonstrated that changes in mRNA population of storage roots occurred during a period of skinning injury. A total of 70 DEGs of interest were excised and 119 independent clones (from 42 DEGs) were successfully re-amplified and cloned. The majority of these represented DEGs products with and induced or enhanced accumulation in skinning-treated storage roots. The inserts of these clones were sequenced and this revealed that among the 119 cDNA clones, 101 sequences (consisted of 44 singletons and 19 contigs) contained distinct sequences which matched to plant-specific cDNA sequences. Fifteen DEGs (10 contigs and 5 singletons) were further selected to study their functions in plants (Table 1).

Table 1. Accumulation of ACP-gene fishing products corresponding to RNA from storage root of sweetpotato subjected to skinning injury

Group	Type	0h	2h	4h	8h+12h
Abiotic stress					
IbSIn04 (TH2)	Induced	–	++	+++	+++
IbSIn 21 (SRP)	Up-regulated	+	+	++	+
IbSIn61a (ELIP3)	Induced	–	+++	++	+
IbSIn61b (GDC)	Induced	–	+++	++	+
Wound stress					
IbSIn 29 (PPI CYP40)	Induced	–	+	+++	++
IbSIn46 (TAL)	Induced	–	–	+	+++
IbSIn 48 (Cyc)	Up-regulated	+	++	+++	++++
IbSIn 59 (TCTP)	Up-regulated	++	+	++++	+++
IbSIn60 (Spor B)	Up-regulated	++	++++	+++	+
IbSIn40 (nifU)	Up-regulated	+	++	++++	+++
Lignin and suberin					
IbSIn30b (Cyt P450)	Up-regulated	+	+	++	+++
Regulation and signaling					
IbSIn15 (RPK)	Induced	–	–	+	++
IbSIn30a (bHLH)	Up-regulated	+	+	++	+++
IbSIn 56 (RNP)	Up-regulated	+	+++	++	+++
IbSIn69 (HRS)	Down-regulated	++++	+	++++	++

Functional Annotation of Selected DEGs

The genes obtained in the storage root ACP Gene-Fishing library were manually sorted into functional categories taking into account the main metabolic and cellular processes in the periderm. Four functional categories: stress and defense, regulation and signaling, protein synthesis and fate, and metabolism were identified.

DEGs accumulation related to wounding responses as a result of skinning injury have the highest DEGs, with 6 DEGs (6 contigs), followed by the accumulation of genes involved in regulation and signaling with 4 DEGs (2 contigs and 2 singletons) (Table 2). Skinning injury also induced genes involved in general abiotic stress response due to skinning injury with 4 DEGs (1 contigs and 3 singletons) and lignin and suberin 1 DEG (1 contig) (Table 2). A list of selected genes with their annotation based on similarity to databases is shown in Table 2.

ACP-based DD-RTPCR produced varied transcript length. DEGs length search from 15 DEGs (5 singletons and 10 contigs) was conducted using BLASTX. The lowest transcript length (<=20 amino acid; 11.11% DEGs) and the longest sequence length (160-179 aa; 5.56% DEGs). The highest number of transcripts (36.11% DEGs; 40-59 aa) and null number of transcript (0% DEG; 120-159 aa) (Figure 1).

Most of BLASTX hit sequences (12 DEGs; 33.33%) showed sequence identity of 80-89%, followed by 10 DEGs (27.78%) with sequence identity of 90-99% and even 5 DEGs (13.89%) showed sequence identity of 100%. The rest of BLASTX hit sequences were: 3 DEGs (each 50-59 and 60-69% identity); 2 DEGs (40-49% identity); 1 DEG (70-79% identity); and 0 DEG (<= 40% identity) (Figure 2).

Of 36 BLASTX-hit transcripts, sweetpotato and *Ricinus communis* have the highest hit (each six hits; 16.67%), followed by *Nicotiana sylvestris*, *Tarenaya hassleriana* (each three hits, 8.33%), and *Amborella trichopoda, Capsicum annuum, Elaeis guineensis, Hordeum vulgare,* and *Medicago truncatula* (each two hits, 5.56%) and eight species (each has one hit, with the total hit 22.22%) (Figure 3). The sweetpotato genes identified in this study relative higher when compared to the study by Tao *et al.* (2012).

Of 36 DEGs studied, TCTPs were the most abundant DEGs (5 hits, 13.80%), followed by Cyt P450 and TAL (each 4 hits, 11.11%), the third most abundant DEGs were RPK, PPI CYP450, Spor B and ELIP3 (each 3 hits, 8.33%), and a group of DEGs consisted of NifU, Cyc, and RNP (each 2 hits, 5.56%), and the rest consisted of 5 single DEGs (each 1 hit with the total hit 13.89%) (Figure 4).

Table 2. Functional annotation of fifteen DEGs in response to skinning injury

Number of DEGs	Putative gene expression[a]	Organism	Function[b]
5	Translationally controlled tumor protein	*Ipomoea nil*[c]	Protein synthesis and fate, signal tranduction
4	Cytochrome P450 76C4-like	*Vitis vinifera*[c]	Metabolism, cell rescue, defense and virulence
4	Transaldolase (TAL) gene	*Solanum lycopersicum*[c]	Wound response
3	Receptor protein kinase, putative	*Ricinus communis*[c]	Signal transduction
3	Peptidyl-prolyl cis-trans isomerase CYP40-like	*Vitis vinifera*[c]	Protein folding chaperon
3	Sporamin B	*Ipomoea batatas*[c]	Storage protein, defense response
3	Early light-inducible protein (ELIP3)	*Populus trichocarpa*[c]	Stress responses, desiccation
2	NifU-like protein	*Medicago truncatula*[c]	Redox balance
2	Cyclophilin	*Ipomoea batatas*[c]	Protein folding
2	Ribonucleoprotein complex subunit 3-like protein	*Zea mays*[c]	rRNA processing; RNA methylation
1	Thioredoxin H2	*Ipomoea batatas*[c]	Redox signaling
1	Serine-rich protein-related	*Theobroma cacao*[c]	Signal tranduction
1	Basic Helix loop helix (bHLH)	*Nicotiana sylvestris*[c]	Transcription factor
1	Glutamate decarboxylase-like	*Solanum lycopersicum*[c]	Metabolism
1	TOM1-like protein 2 isoform X1	*Nicotiana tomentosiformis*[c]	Protein fate signaling

Note: [a]Putative description for sweetpotato genes were obtained using Basic Local Alignment Search Tool (Altschul *et al*., 1997). [b]Functional classification was done according to the Munich Information Center for Protein Sequences (Schoof *et al*., 2002).[c]Functional annotation was confirmed by qRT-PCR (Effendy *et al.,* 2013)

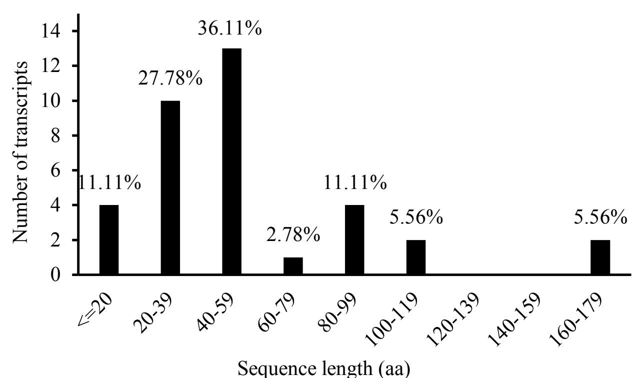

Figure 1. Distribution of DEGs length and percentage of transcripts with BLASTX hits. Thirty six DEGs with transcripts ≥ 100 bp of final assembly were used for BLASTX search. The X-axis represents the sequence length in amino acid (aa) and the Y-axis represents the number of transcripts

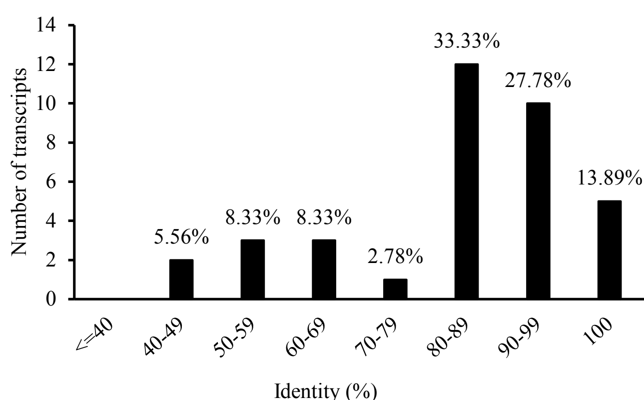

Figure 2. Sequence identity distribution. The X-axis represents the DEGs identity (%) and the Y-axis represents the number of transcripts

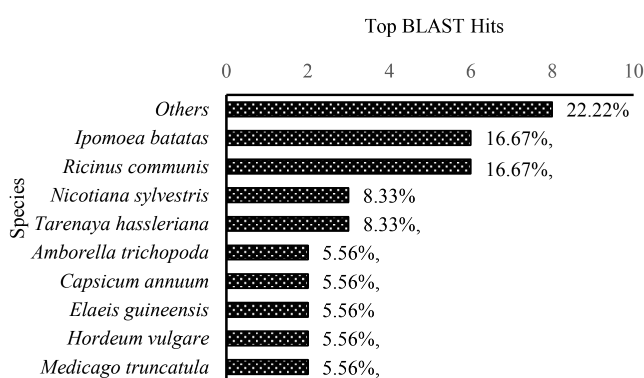

Figure 3. Top-Hit species distribution. The X-axis represents top blast hits and the Y-axis represents the species

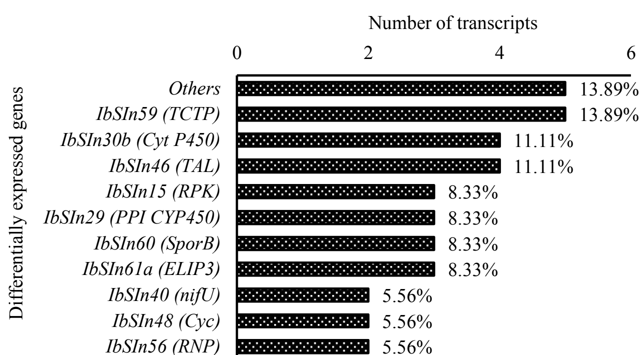

Figure 4. Top-Hit DEGs distribution. The X-axis represents the number of transcripts and the Y-axis represents DEGs hit

IbH34 showed 90% similarity with a translationally controlled tumor protein (TCTP). We also found that TCTP is the only full length gene found using ACP-based DD-RTPCR. Our study also showed that TCTP is the most abundant DEGs constitutes of 13.89% transcripts. Studies by Rho *et al.* (2011) showed that TCTP has an anti-apoptotic activity and can prevent apoptosis. Furthermore, TCTP is an important component of TOR (target of rapamycin) signaling pathway, a major regulator of cell growth. Berkowitz *et al.* (2008) showed that silencing TCTP caused slow vegetative growth and reduced leaf expansion. Furthermore they showed that knockout TCTP caused impaired pollen tube growth.

In present study, one DEG IbF61 showed similarity with transaldolase (TAL). Only few studies report on expression of TAL in plants. Plant TAL genes are induced by abiotic stress conditions including wounding (Caillau and Quick, 2005). Caillau and Quick (2005) reported that TAL gene (*ToTal1*) accumulation has been observed in root, stem and red fruit of tomato. Another study by Yang *et al.* (2014) showed that rice TAL was expressed in leaf, panicle, stem, knot, and root. A knockdown TAL plant using a gene-specific RNA interference (RNAi) construct in rice, showed reduction of TAL transcript in leaves of TAL-RNAi line. They found that knockdown TAL gene leads to changes in number and pattern of vascular bundles (Yang *et al.*, 2014). TAL up-regulation may enhance the flux to shikimate pathway (Misra *et al.*, 2010) from primary to secondary metabolites enabling synthesis of aromatic amino acid, flavonoids, and lignin.

Sporamin, a major storage protein in sweetpotato storage roots belong to soybean trypsin inhibitor superfamily can be grouped into two subfamilies Spor A and Spor B. Tao *et al.* (2012) showed that Spor B expressed the highest levels in roots and barely detectable in young leaves, mature leaves and stem. On the contrary, Spor A expressed in all tissues measured (young leaves, mature leaves, stem, fibrous roots and storage roots at three developmental stages at 1.5, 3, 5 months after planting). Studies by Rajendran *et al.* (2014) showed that sporamin expression is induced by biotic and abiotic stresses. They showed that endogenous level of jasmonic acid and salicylic acid can regulate the sporamin expression in response to mechanical wounding or herbivory attack. These studies suggest that sporamin can function as storage proteins as well as signaling pathway when subjected to both stresses.

In the present study, IbF82 showed 98% similarity with cyclophilin (Cyc) from *I. batatas* and was induced in response to skinning as early as 2 h after skinning treatment. Cyps, ubiquitous proteins of immunophilin superfamily,

contain peptidyl-prolyl cis-trans isomerase (PPIase) activity. Genomic studies by Mainali *et al.* (2014) identified 62 CYP (Cyclophilin) genes in the soybean genome. Studies have demonstrated the importance of many PPIases in plant biology, but no genome-wide analysis of the CYP gene family has been conducted for a legume species. CYP transcripts are commonly up-regulated in response to salt stress (Ruan *et al.*, 2011), osmotic stress and high light condition (Kim *et al.*, 2012), desiccation and salt stress (Ahn *et al.*, 2010, Trivedi *et al.*, 2012), low temperature, abscisic acid (ABA). Various CYPs have been found in cytosol, nucleus, chloroplast, secretory, and mitochondria (Mainali *et al.*, 2014) suggesting a substantial role in a wide array of plant defense and cellular processes such as protein folding, mRNA processing, protein degradation and signal transduction which are crucial during development and stress response (Laxa *et al.*, 2007).

CONCLUSIONS

In conclusion, ACP-based DD-RTPCR provides an effective way to explore transcript profiles of skinning-injury responsive genes in storage roots of sweetpotato. The DEG data presented here to the best of our knowledge is the first overview of genes that are expressed in response to skinning injury in sweetpotato. Our study is focusing on native storage root intentional skinning and it indicates that, in addition to its function as a protective layer, the skin responds to plant stress and defense responses by altered gene expressions. The presence of these DEGs in this study implied that tolerance of sweetpotato to skinning injury can be achieved by an up-regulated expression of skinning related genes. Periderm formation which is characterized by secondary growth are commonly found in plants following wounding. Periderm formation, development, and anatomy are highly similar in all plant species, therefore the functional characterization of genes that are expressed during storage root skinning injury may have implications for other periderm systems in all plants as well. These up-regulated genes identified in this research can be exploited further to unravel regulatory networks involved in skinning injury responses in plants and to develop strategies towards impaired skin healing.

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