GENE EXPRESSION ASSOCIATED WITH WOUND AND NATIVE PERIDERM MATURATION IN POTATO TUBERS

A Thesis
Submitted to The Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

Jonathan David Neubauer

In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Department:
Plant Sciences

December 2011

Fargo, North Dakota

North Dakota State University
Graduate School

Title

Gene Expression Associated with Wound and Native

Periderm Maturation in Potato Tubers

By

Jonathan Neubauer

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

ABSTRACT

Neubauer, Jonathan David, M.S., Department of Plant Sciences, College of Agriculture, Food Systems, and Natural Resources, North Dakota State University, December 2011. Gene Expression Associated with Wound and Native Periderm Maturation in Potato Tubers. Major Professors: Dr. Susie Thompson and Dr. Ed Lulai.

Potato (*Solanum tuberosum* L.) is the world's fourth largest food crop and large financial losses are incurred each year from wound and bruise related injuries. However, little is known about the coordinate induction of genes that may be associated with, or mark major wound-healing and periderm maturation events. Also, one of the key defense mechanisms for potato tubers is the robust barrier provided by the phellem (skin) of the native periderm. Many biological processes are involved in the formation of this stout tissue. However, little is known about induction of genes that may be associated with this process.

The objectives of this research were to molecularly assess the processes of wound periderm development and maturation, and native periderm maturation in potato tubers. In this study, these processes were determined in coordination with expression profiles of selected genes. The cell cycle, cell wall protein, and pectin methyl esterase genes were determined from two diverse potato genotypes and two harvests NDTX4271-5R (ND) and Russet Burbank (RB) tubers; 2008 and 2009 harvests. Cell cycle genes encoding epidermal growth factor binding protein (*StEBP*), cyclin-dependent kinase B (*StCDKB*), and cyclin-dependent kinase regulatory subunit (*StCKS1At)* expression profiles were coordinated with related phellogen formation and the induction and cessation of phellem cell formation. Genes encoding the structural cell wall proteins extensin (*StExt1*) and

extensin-like (*StExtlk)* expression profiles suggested involvement with closing layer formation and subsequent phellem cell layer formations. The coordinate induction and expression profile of *StTLRP,* a gene encoding a cell wall strengthening "tyrosine- and lysine-rich protein", suggested a role in the formation of the closing layer followed by phellem cell generation and lastly cell wall thickening in non-meristematic phellogen cells. *StPME* and *StPrePME* expression increased during periderm development, implicating involvement in modifications for closing layer and phellem cell formation.

Collectively, these results indicate that the genes monitored were involved in and their expression profiles markedly coordinated with periderm formation and the on-set of periderm maturation; results were more influenced by harvest than genotype. Importantly, *StTLRP* was the only gene examined that may be involved in phellogen cell wall strengthening or thickening after cessation of cell division.

ACKNOWLEDGEMENTS

I would like to express sincere appreciation to members of my graduate committee, Drs. Ed Lulai, Susie Thompson, Melvin Bolton and Gary Secor for the advice and direction throughout my graduate studies.

I would like to thank Dr. Ed Lulai for his advice and guidance throughout my graduate program.

Many thanks go to Dr. Ed Lulai and Dr. Jeff Suttle for giving me the opportunity of graduate work.

I thank Dr. Larry Campbell for his advice with my statistical related questions and his willingness to set aside time to help me.

Finally, I would like to give thanks to my family members for the encouragement and support throughout these studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LITERATURE REVIEW

Introduction

After the potato growing season, large financial losses can occur from the handling operations associated with harvesting, storage, and transportation of the crop. Associated wounding and bruising levels can reach up to 40% during potato harvest and handling operations (Lulai, 2007). Although some research has been conducted on wound periderm development, little research has been targeted towards determining the genes that are expressed during potato tuber periderm maturation. Histological and immunolabeling studies have shown that pectin and extensin depositions are involved in tuber periderm maturation and associated with resistance to skinning/excoriation injury (skin-set) (Sabba and Lulai, 2004, 2005, Lulai and Orr, 1993). Research to determine the genes involved in the accumulation of these cell wall polymers is a critical step in developing options to biologically assess and control the process of periderm maturation. This information in turn could be used to decrease financial losses due to wounding and bruising. The goal of this research was to identify genes related to periderm maturation, to determine the expression profiles of these genes, and to collectively obtain a better understanding of the molecular process of periderm maturation.

Potato History

Potato (*Solanum tuberosum* L.) is an edible tuber-bearing crop that is a small part of the very large genus *Solanum.* The origin of this crop is an intriguing topic that has fascinated many scientists for centuries. The diploid and tetraploid landrace populations form a monophyletic clade, which is derived from the

northern members of the *S. brevicaule* complex (Spooner et al., 2005). Primitive indigenous cultivated (landrace) potatoes are widely dispersed in the Andes from western Venezuela south to northern Argentina, on Chiloe Island, and next to Chonos Archipelago of south-central Chile. The Chilean landraces are secondarily derived from Andean races; this transitional event likely occurred after hybridization with the Bolivean and Argentinean species *Solanum tarijense.* Potato clones have different growth habits, shapes, and tuber color to name a few of the characteristics which make these landraces very diverse (Spooner et al., 2005). Geo-referencing (geographic information systems (GIS)) was used to determine that wild potatoes were from Argentina, Bolivia, Brazil, Chile, Colombia, Costa Rica, Ecuador, Guatemala, Honduras, Mexico, Panama, Paraguay, Peru, USA, Uruguay, and Venezuela (Hijmans et al., 2001). From these results, it was determined that wild potatoes are found between 38° N and 41° S. The richest area of wild species growth is predominantly high in the Central and South American tropical highlands, with clear peaks between 8 ° S and 20 ° S, also around 20 ° N. These areas are in the Andes of northern Argentina, Bolivia, Ecuador, and Peru, and central Mexico (Hijmans et al., 2001). Peru is unique since it contains a high number of wild potato species as well as rare wild species. Following this study, Spooner et al. (2005) used amplified fragment length polymorphism (AFLP) genotyping to see if potato had a single domestication event. These results pointed toward a single domestication event from a single species, or its progenitor (*S. bukasovii*), in the broad area of southern Peru.

The spread of potato outside of the area of its evolutionary origin did not occur until the 16th century A.D., when Spanish conquistadors encountered the potato while looking for gold in Peru. Soon after, the Spanish supplied potatoes on their ships and noticed that sailors that ate the potato did not suffer from scurvy. In 1597, John Gerald a botanist from England started his own cultivation practice to improve the potato (Hemphil and Thone, 1946). Also in the late 1500's, potatoes were introduced to Europe. Then in the 1840s the Irish potato famine caused by *Phytophthora infestans* (*P. infestans)* occurred, resulting in starvation, deaths, and overall abhorrence of the potato. Hence, the spread of potato propagation throughout Europe was slowed from the fear of the Irish potato famine. Russian farmers in the mid 1800's warmed up to the potato and they have been a potato producing powerhouse since.

Africa was first introduced to the potato in 1600s, and it is thought that sailors encouraged potatoes to be grown at ship ports to supply ship voyages with the vegetable. In the 1600s potatoes were introduced in the United States, but were not widely grown until the 1700s. In 1719 potatoes were planted in New Hampshire by Scotch-Irish immigrates, and from there spread across the United States. Australians were introduced to the potato in 1770s, by James Cook, an explorer, and are now grown throughout Australia. Potatoes were introduced in China and India during the 17th century, but commercialization did not occur until the 1960s when the production exploded by almost 850%. Currently, potato has became the world's fourth largest food crop, with over 223 million tonnes consumed in 2005 and over 358 million tonnes of potatoes produced (FAO, 2008).

In the 1930's, The United States Department Agriculture (USDA) teamed up with experimental stations and released 25 new varieties starting with Katahdin in 1933 (Hemphill and Thone, 1946). Also, in the 1930's the wild species *S. desmissum* was noted to have resistance towards *P. infestans.* Since *P. infestans* is known for the devastation of potatoes, the USDA developed *S. desmissum* germplasm. Another wild species (*S. chacoense*) made important contributions for processing quality. An additional wild relative *S. tarijense* had been used for chipping and cold storage qualities. In recent years, South American landraces from many sources, including a cultivated group called Phureja, contributed to red, yellow, and purple flesh potatoes. The yellow fleshed cultivar Yukon Gold is also associated with Phureja heritage (Jansky, 2009).

There are approximately 190 tuber-bearing wild species of potatoes grouped into the section Petota (*Tuberarium*), and subsection Potato (*Hyperbasarthrum*). Potatoes have a base number of 12 chromosomes consisting of approximately 840 million base pairs, in several ploidy levels which can be diploid, tetraploid, pentaploid, and hexaploid. The RH89-039-16 (RH), a diploid, heterozygous potato variety has been sequenced and a draft of the sequence has been released (Potato Genome Sequence Consortium, www.potatogenome.net). Wild species represent diverse gene pools that are of great importance in developing resistant and heterotic genotypes. However, a relatively low number of wild species are currently considered in breeding programs. About 75% of these wild species are diploids, differing from the autotetraploid *Solanum tuberosum*, the most cultivated species. Diploid species are found in the Andean valleys. These

species include: *S. brevicaule, S. multidissectum, S. bukasovii, S. canasense, S. soukupii, S. leptophytes, S. multiinterruptum, S. abbottianum, S. liriunianum, S. ochoae, S. spegazinii,* and *S. vidaurrei,* forming the *brevicaule* complex. Most of the members of this complex cross easily and were reported to have several natural hybrids for almost all combinations. Due to the promiscuity and morphological continuity of these combinations, some scientists have suggested that the species from South America in the *brevicaule* complex should be considered to be in the species *S. tuberosum.* The proposed evolution for potatoes begins with *S. brevicaule* as the first domesticated and selected potato germplasm to form the *S. stenotomun* complex. Following the *S. stenotomun* complex, it hybridized with an unknown species. Then sexual polyploidization; *S. tuberosum* subsp. *andigena* arose and multiple introgressions happened to produce the subsp. *andigenum* complex. Lastly, hybridization occurred with another unknown species to produce the subspecies *tuberosum* (Hancock, 2004). The *tuberosum* subspecies is the most widely cultivated species; it contains 12 chromosomes with approximately 840 million base pairs. Diploids, triploids, and pentaploids are also cultivated and preserved in the Andean region; and they belong with the group of roots and tubers inherited from the great ancient civilizations that settled in this area (Visser et al., 2009).

Raker and Spooner (2002) used SSRs to analyze S. *tuberosum* subspecies to show the difference between most of the subsp. *tuberosum* and *andigenum* landraces. Their results could explain some of the different morphological characters for day length, adaptation, and the cytoplasmic sensitivity sterility

factors frequently found in subspecies *tuberosum.* Furthermore, traditional farming systems included mixtures of wild and weedy species with cultivated potatoes, leading to a mixture of different ploidies in the Andean region. Also, farmers selected and maintained cultivars based on factors such as yield, disease resistance, storage longevity, and preferred taste. The selection process within this loose ecosystem resulted in cultivars that were genetically diverse yet agronomically versatile. Thus, the different species of potatoes were crossed and a large plastic gene pool was created. The heterogeneity is endemic and considerably large for native potatoes, making this crop difficult to divide into species (Raker and Spooner, 2002).

Synteny indicates structural similarity between different genomes, and involves the construction of linkage maps between related species. Synteny also allows functional comparisons between sexually incompatible species based on structural similarity and differences (Gebhardt, 2005). Comparing and detecting conserved genetic linkage between loci that share sequence similarity in different species is used by breeders to advance cultivars. Use of molecular markers facilitated comparison of genome structures within the three most important crop species of the Solanaceae family (potato, tomato, and pepper). Studies in comparative genetic mapping showed that the potato and tomato genomes are co-linear except for paracentric inversions of five of their chromosome arms. Pepper has a larger genome, but it is also similar to the tomato and potato genome. Another application for synteny studies involves establishing the positions of genes controlling quantitative and qualitative resistance to pathogens. These genes can

then be compared between potato, tomato, pepper, and other *Solanum* species. Furthermore, when potato's genetic map was aligned with the physical map of the sequenced Arabidopsis genome, there was 40% similarity of the potato genetic map, and about 50% of the physical map of this very distantly related plant species (Gebhardt, 2005). Additionally, Rodriguez et al. (2009) used a specific subset of markers called conserved ortholog set II (COSII). COSII are single to low-copy nuclear DNA markers that have been used for phylogenetic reconstruction in the sister clades of potato and tomato by analyzing orthologs and paralogs (Rodriguez et al., 2009). The obtained results support the hypothesis of a North and Central American B-genome origin of the tuber-bearing members of *Solanum* section *Petota*. When potatoes and tomatoes were analyzed together, the sister relationship between the potato and tomato clades is highly supported in all analyses and section *E. tuberosum* is supported as sister to both. Additionally, their results showed potatoes (*Solanum* section. Petota) form a well-supported clade (99% bootstrap support) with tomatoes (*Solanum* section. Lycopersicon) (Spooner et al., 2008). Knowledge of the genome similarities between potato and tomato can aid in the development of elite lines.

Classical breeding is time-consuming and often hampered by linkage drag. Molecular breeding appears to offer advantages for the enhancement of potato agronomics and quality. Potato breeding is aimed at improving disease, pest and virus resistance, taste and cooking qualities, tuber skin color, shape, and yield (Park et al., 2008). Applying a molecular approach, combined with classical breeding, could bring forth genetic gain at a faster rate. Utilization of wild *Solanum*

germplasm could broaden the genetic resistance base of potato and enhance advantageous traits. Furthermore, resistant (R) genes were discovered from the wild species *Solanum demissum* and used in breeding programs. For example, *P. infestans* was quick to overcome the first *R* genes from *Solanum demissum* that were incorporated into breeding programs. As a result, researchers switched to study *Rpi* genes (resistance to *P. infestans)* from other wild *Solanum* species to broaden the spectrum of resistance. Currently, molecular techniques have been used to identify *Rpi* genes and quantitative trait loci (QTL) for resistance to *P. infestans.* With these techniques, researchers discovered 12 disease *R* gene clusters dispersed over 10 chromosomes and two major *Rpi* gene clusters present on chromosome 4 and 11 in potato. A problem related to the introgression of these *R* genes between wild species and cultivated potato is sexual hybridization, which can be overcome by genetic transformation (Park et al., 2008). Another benefit of molecular techniques to potato is the use of simple sequence repeats (SSR). Fu et al. (2009) used SSRs to evaluate the genetic diversity of exotic potato accessions and focused their research to develop new potato cultivars in Canada. After using SSRs, they discovered that their germplasm harbored less variation than germplasm from seven other countries. These characterizations provided useful information for understanding genetic diversity and structure of various potato gene pools in different locations to help manage their germplasm and diversify potato breeding efforts (Fu et al., 2009).

Construction of an ultradense genetic recombination map, i.e. a global saturation of the potato genome with marker loci, provided a framework for gene

isolation to develop elite potato cultivars. Os et al. (2006) used AFLP to construct a dense genetic linkage map. The research group used 381 AFLP primer combinations and a mapping population of 130 individuals, of which 93% could be precisely assigned to a genetic position. Following the construction of the ultradense map, the research group used their results to compare to linkage groups that were found in other mapping studies. With the recent mapping of *R10* and *R11* resistance genes, it was shown that the AFLP markers could be used to identify *R* genes for *P. infestans* in other mapping efforts (Os et al., 2006). Molecular techniques combined with classical breeding methods will allow breeders to introduce desirable genes into cultivars that will complement traditional breeding efforts. Importantly, there is little information concerning the biology to support genetic approaches to improve the development and maturation of the protective barrier provided by wound and native periderm.

Periderm Maturation Research

The quality and profitability of potatoes grown for seed, the fresh market, and processing are adversely impacted by skinning type wounds. Consequently, molecular processes involved in periderm development and maturation are agriculturally and economically important. Prior to periderm maturation, the immature periderm is susceptible to skinning injury. The three distinct types of cells that make up tuber periderm are: phellem (skin), phellogen, and phelloderm (Lulai, 2002). The phellem consists of several layers of corky material, which is formed from the outwardly dividing phellogen cells (Lulai, 2007). The phelloderm is formed by inward division of the phellogen. As the periderm matures, the tuber

becomes resistant to skinning injury. The mature periderm provides a barrier that protects against both physical and environmental intrusions.

A fully mature tuber periderm is, it is less likely to become wounded by skinning injury. Few techniques have been developed to determine resistance to skinning; none have been established as a standard. The most common experimental technique of measuring skin-set involves the tangential or torsional force required to mechanically shear the phellem (skin) from the tuber (Lulai, 2002). By using this technique to assess skin-set development, we can address physiological factors associated with resistance to skinning injury. Upon excoriation, the phellem is removed and soon thereafter a new layer of phellogen cells form as part of wound periderm development. The wound area is subjected to similar challenges and intrusions as that of the native periderm. Consequently, rapid wound-healing is essential in forming a new barrier to protect the tuber from these challenges and intrusions. During wound-healing, one of the most important biological processes is formation of the suberized closing layer. Wound induced suberization involves two stages, the first stage is the suberization of the existing cells (closing layer formation), and the second stage involves the formation of new cells including phelloderm, phellogen, and phellem, the latter of which is suberized (wound periderm formation) (Lulai, 2007) (Fig. 1). Suberin accumulates on the outside of the plants cell walls and is composed of suberin poly(phenolics) (SPP) and suberin poly(aliphatics) (SPA) that are cross-linked by glycerol, and embedded with soluble waxes. Suberin is essential for resistance to fungi and bacteria that are living amongst the tubers (Lulai, 2007). The regulation of suberin

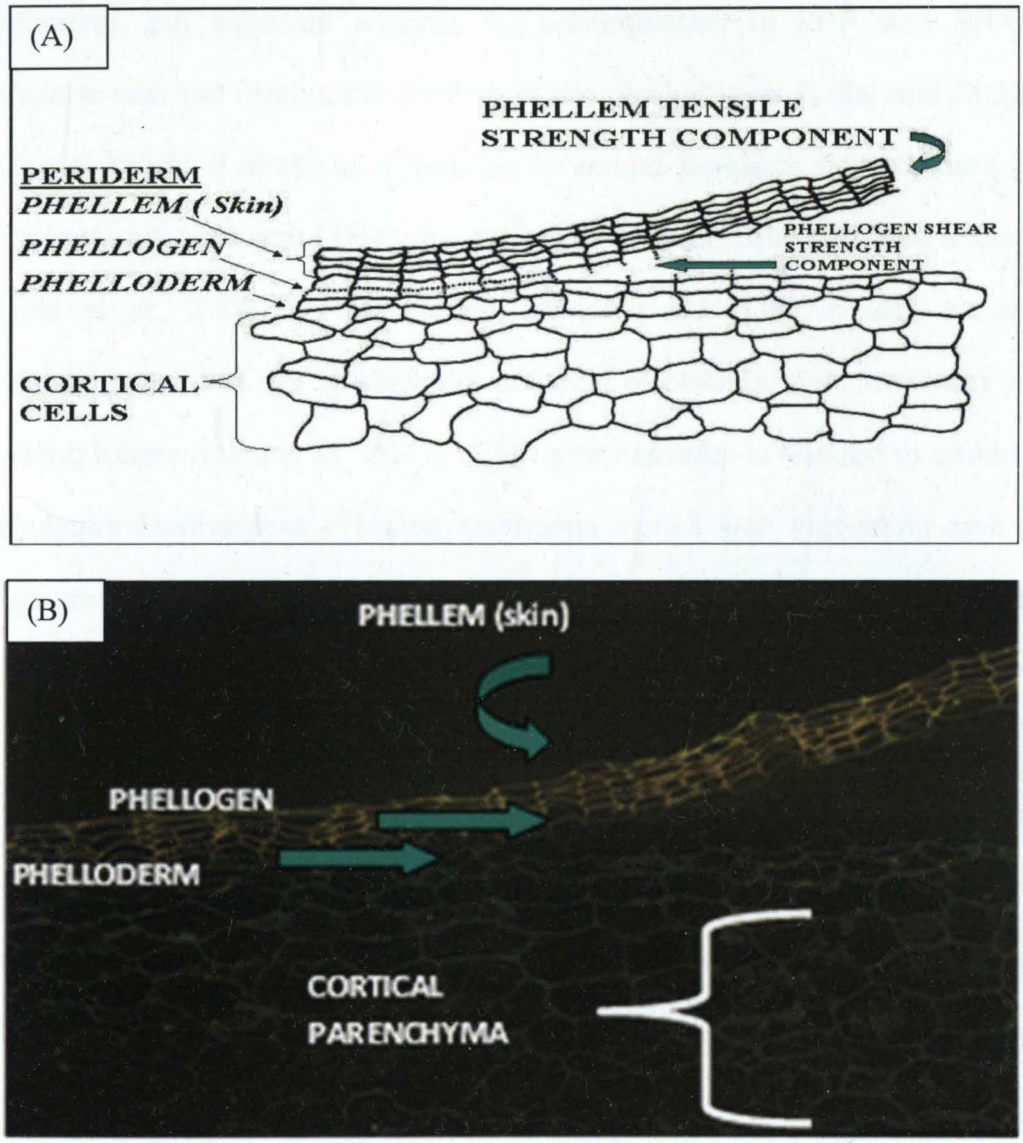

Fig. 1. Line drawing depicting tuber periderm tissues and cortical cells (A). Image of periderm tissues and cortical cells (B). *Note the separation of phellem from neighboring cells and fracturing of the phellogen cell walls upon shearing. (Images provided by Dr. Ed Lulai-USDA-ARS)

accumulation is another important step in formation of the wound periderm. The plant hormone ethylene has been known to be involved in various types of plant stresses. Research has been conducted on ethylene and its involvement in tuber wounding. Ethylene biosynthesis has been shown to be stimulated by tuber

wounding, but separate analysis for accumulation of SPP and SPA showed ethylene was not required in forming of the closing layer (Lulai and Suttle, 2004). It is not known if ethylene is required in wound periderm development. Another hormone, abscisic acid (ABA) was shown to regulate SPP and SPA accumulations (Lulai et al., 2008). Furthermore, jasmonic acid (JA) is also induced upon wounding, but it is not required for initiation of closing layer formation in wound-healing tubers (Lulai et al., 2011). Still more research is needed to understand the regulatory involvement of these hormones in cell wall thickening and periderm maturation. Results from comprehensive studies involving periderm maturation indicate that phellem/skin thickness, phellem/skin weight, or phellem histology are not related to skin-set development (Lulai and Orr, 1993). Earlier studies hypothesized that the phellogen, the first layer of fully hydrated cells within the periderm, should play an important role in periderm maturation and skin-set development. From this hypothesis, more research was focused on the phellogen cell layer. Later, it was shown that the phellogen layer of immature periderm was the source of susceptibility and resistance to skinning (Lulai and Freeman, 2001) (Fig. 2). Skin thickening and suberization were previously thought to be the main factors involving resistance to skinning. Importantly, it was determined that immature phellogen cells had thin walls and fractured easily. Furthermore, when the tuber was fully resistant to skinning, the phellogen cell walls were strengthened and thickened considerably (Lulai and Freeman, 2001). These and other results showed the importance of the phellogen layer for protection of the potato tuber (Lulai, 2002). The importance of the phellogen layer in mediating protection of the

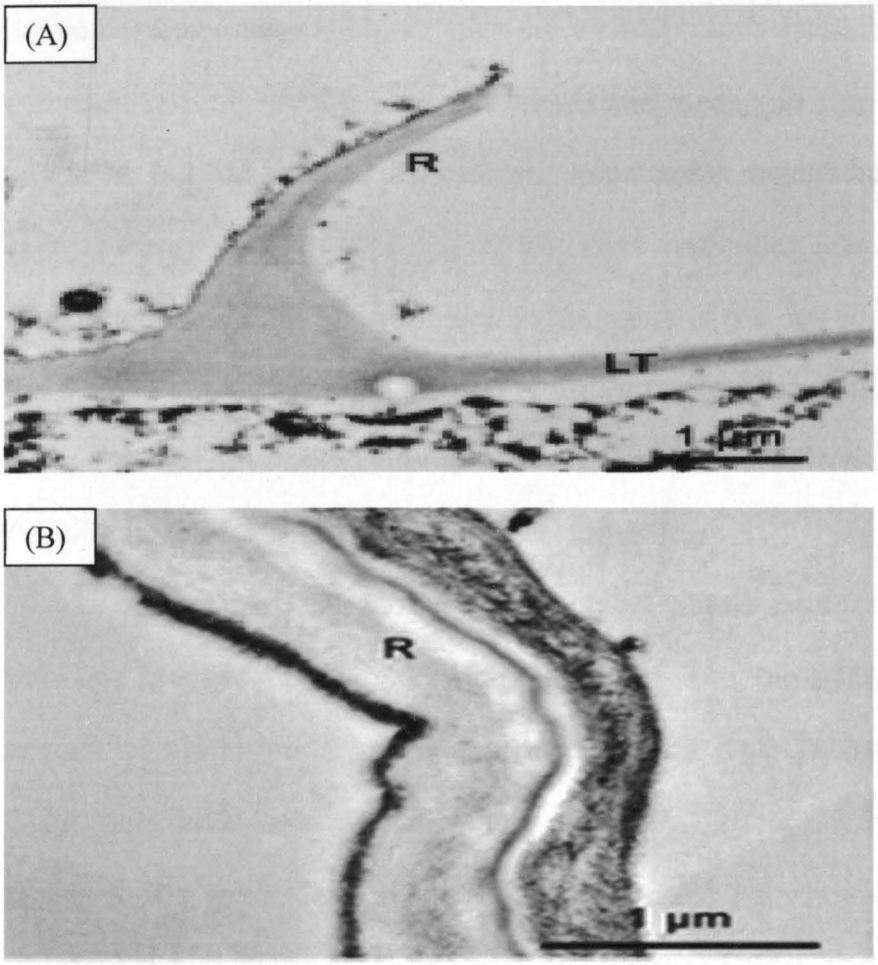

Fig. 2. Phellogen cell wall (radial (R) and lower tangential (LT)) from immature tuber periderm (A). Note that the radial wall is thin displaying a fracture and indicating the point of origin for excoriation injury. Phellogen cell wall (R) from mature tuber periderm (B). Note that the radial wall is thickened, consequently stronger and more resistant to excoriation injury. (Images provided by Dr. Ed Lulai-USDA-ARS)

potato tuber has stimulated research in this area. Histological studies and immunolabeling studies of immature and mature native periderm were conducted using immunological probes, with JIM5 and JIM7 used to bind unesterified and esterified pectin, and LM1 used to recognize extensin epitopes (Sabba and Lulai, 2005). Through these studies, it was determined that pectin and extensin accumulations are associated with the process of strengthening cell walls during

native periderm maturation and skin-set development. Resistance to skinning was accompanied by an increase in unesterified pectin in the cell walls in native periderm, but not in wound periderm. Importantly, accumulation of the extensin epitope increased dramatically upon periderm maturation (Sabba and Lulai, 2005). Both extensin and pectin are major components of the cell wall and understanding the biology of their accumulation on phellogen cell wall during periderm maturation is important in developing strategies to hasten resistance to skinning injury.

Pectins Involvement in Cell Wall Strengthening

As mentioned previously, pectin accumulations were found to be associated with the process of cell wall strengthening. The biological process of pectin accumulations includes polymerization in the *cis* Golgi, subsequently the medial Golgi then methylesterifies the pectin polymer. Next it is substituted with side chains in the *trans* Golgi (Micheli, 2001). Pectin accounts for ~35% of the dry weight in dicot cell walls. Pectins are characterized by relatively high extractability using acid or chelators and have a high content of galacturonic acid (GalUA). Pectins combined with hemicelluloses constitute the matrix in which cellulose microfibrils are embedded. This integrated combination creates the rigid yet dynamic and pliable properties of the cell wall (Harholt et al., 2010). Pectin polysaccharides are a highly heterogeneous group of polymers containing homogalacturonans (HG), xylogalacturonan (XGA), apiogalacturonan (AGA), rhamnogalacacturonan I (RGI), and rhamnogalacacturonan II (RGII) (Micheli, 2001 and Harholt et al., 2010). The ratio of these polysaccharides is variable, but HG is typically most plentiful constituting approximately 65% of the pectin and are highly

abundant in potato cell walls. Another polymer, RGI, constitutes 20% to 35% and is the second major component of pectin. On the other hand, XGA and RGII constitute less than 10% of the cell wall making them minor components (Harholt et al., 2010). Even though these are different polysaccharides they are not separate molecules, but covalently linked domains. HG is an unbranched homopolymer chain which consists of α-1,4 linked D-GalUA. HG can either be methyl-esterified or acetylated. The degree or pattern of these two chemistries varies from organism to organism. As for methylesterification, it is hypothesized to be regulated by the plant in a tissue-specific and developmental manner (Caffall and Mohnen, 2009). If more than 10 consecutive unmethyl-esterified GalA residues of HG are negatively charged and structurally coordinated, they may ionically interact with Ca^{2+}. Once this occurs, a stable gel is formed with other pectin molecules; ~70% of the pectin gel is in the cell walls of plants (Caffall and Mohnen, 2009).

The backbone of GalUA is present in high amounts in pectin, and at various positions within the biopolymer it can be substituted with xylose (XGA) and apiofuranose (AGA) (Harholt et al., 2010). XGA is formed from backbone residues of HG by substitution of xylose disaccharides at the C-3 of GalA and has been observed in many species. AGA which is found in the cell walls of aquatic plants is formed from apiose disaccharides linked to single GalA residues of HG. In water-borne plants, AGA is very abundant, suggesting a significant structural role in the cell wall structure (Caffall and Mohnen, 2009). Another polymer, RGII, is formed by clusters of complex side chains attached onto the O-2 or O-3 position of the HG

backbone. These intricate side chains are unique in plant polysaccharides. Despite the complexity of RGII, the polymer is highly conserved among vascular plants (Harholt et al., 2010). Another important aspect of RGII is that it exists as a dimer mediated by a borate ion. Since boron is an essential micronutrient and most likely contributes to RGII dimerization allowing integrity of cell wall (Harholt et al., 2010). RGI consists of disaccharide (α-1,4-D-GALA-α-1,2-L-Rha) repeats making this pectin unique. Furthermore, the structure of RGI side chains can vary considerably among plants. These disaccharide repeats (Rha residues) can be substituted with β-1,4-galactan branched arabinan, or arabinogalactan side chains. These arabinans work as spatial regulators of the HG domains and may prevent Ca^{2+} interactions, possibly preventing cell wall stiffening. A hypothesis is that the side chains act as plasticizers and undergo large remodeling during water deficient conditions. Upon hydration of these cell walls, arabinans and galactans are mobilized and may induce cell wall stiffening (Harholt et al., 2010). Further evidence pointing to the importance of the role of the RGI side chains came from research on transgenic potato plants. Ulvskov et al. (2005) showed that plants containing less galactan or arabinan had an increased susceptibility to cell wall failure.

Once there is a signal for cell wall formation or a pathogen attack, polymerization occurs. Following polymerization, these pectins are secreted into cell walls in a highly methylesterified form. These highly methylesterified pectins can be modified by pectinases, pectin methyl esterase (PME) for example, which releases acidic pectins and methanol by catalyzing demethlyesterification of

homogalacturonans (Micheli, 2001). Sabba and Lulai (2005) suggested that unesterified pectin produced by PME could be responsible for increase rigidity of cell walls via calcium bridges and showed that unesterified pectin increased in the cell walls of phellogen cells during periderm maturation. The accepted hypothesis concerning mode of action of PMEs on homogalacturonans is that PMEs either act randomly (fungi) or linearly (plants). As for fungi, the demethlyesterification releases protons that promote the action of endopolygalacturonases which contribute to the loosening of cell walls. On the other hand, in plants, PMEs act linearly giving rise to blocks of free carboxyl groups that interact with calcium creating a pectate gel. With this gel present, endopolygalacturonases action is restricted and hence cell wall stiffening occurs (Micheli, 2001). Also, ionic bridges are hypothesized to form between adjacent pectin strands which further aide in cell wall strengthening (Sabba and Lulai, 2005).

Recently, it has been shown that the activity of PME also depends on pH and degree of methylesterification of the pectins (Micheli, 2001). At a given pH, some PME isoforms are more catalytically active than others on highly methylesterified pectins. Furthermore, cations enhance PME activity; trivalent cations are more effective than bivalent cations, which are more effective than monovalent cations (Micheli, 2001). Regulation of PME is another important aspect in cell wall strengthening. ABA enhanced PME activity in tomato seeds, but inhibited activity of PME during seed germination of yellow cedar (*Chamaecyparis nootkatensis*). On the other hand, (GA3) stimulated PME activity on yellow cedar seeds (Micheli, 2001). Another observation was that overexpression resulted in

increased stem elongation in the transgenic plants in the early stages, but later in the growth cycle the rates were reversed (Micheli, 2001). Furthermore, wild type plants produced tubers with higher tuber weight than mutant plants. This growth trend possibly suggests that adaptations occurred in mutant plants to account for the overexpression of PME. Also noted was that increases in PME activity enhanced calcium-binding capacities in tuber cell walls, directly affecting the number of binding sites for the respective cation (Pilling et al., 2000). These results suggest that PME has a direct impact on plant development.

Sabba and Lulai (2004) used the antibodies JIM5 and JIM7 to determine corresponding epitopes specific to different degrees of esterification in pectin. JIM5, which binds to relatively unesterified pectin, and JIM7, relatively esterified pectin, labeled phelloderm cells of both immature and mature native periderm. JIM7 produced a stronger label on the phellem cell walls than JIM5. On the other hand, the walls of the wound periderm were labeled poorly. An interesting observation was that the phellogen layer of immature native periderm had weak labeling, but was strongly labeled in mature native periderm. As for wound periderm, a similar pattern was observed, but the signal was weaker (Sabba and Lulai, 2004).

Structural Proteins

The plant cell wall also includes five structural protein classes. These protein classes include: glycine-rich proteins (GRPs), proline-rich proteins (PRPs), solanaceous lectins, arabinogalactan proteins (AGPs), and hydroxyproline-rich glycoprotein (HRGPs). All of these proteins may be evolutionarily related, since

they have similar sequences (Showalter, 1993). Furthermore, Deepak et al. (2010) suggests that transcription of these proteins occurs in the nucleus following translation and post translation occurs in the cytoplasm. Although there are more proteins, these five classes generally represent the most abundant plant cell wall proteins (Showalter, 1993).

GRPs are characterized by their primary structure which contains up to 70% glycine repeated units (Showalter, 1993). Various developmental and stress conditions cause the expression of GRPs. These cell wall proteins are broken down into two classes. One class is developmentally regulated and found in the cell wall. As for the second class, they are located in the cytoplasm and induced by a range of stress conditions including wounding. GRPs may also have an association with cell wall lignifications (Showalter, 1993). TLRPs which have similar function of GRPs, are present in roots, stems, and leaves of tomato plants (Domingo et al., 1994). Since tomatoes are related to potatoes, tomato genes involved in wounding could be present in potatoes too. Domingo et al. (1994) concluded that plants have evolved regulatory mechanisms allowing genes involved with biosynthesis of structural cell wall proteins to be expressed in plant cell wall tissues. Different environmental cues can cause expression of these genes. Additionally, this research showed that TLRPs contain nine tyrosine residues, which account for 14% of the total amino acid residues of the protein. This high tyrosine content is a major characteristic of structural cell wall proteins. Domingo et al. (1999) also demonstrated that TLRPs are expressed during the process of lignification and hypothesized that TLRPs participate in mediating the

final building and shaping of lignified secondary cell walls. Furthermore, tyrosine, as a structural component of TLRP, is proposed to participate in the cross-linking of proteins to cell walls and can be linked to aromatic side chains of lignin. This cross-linking facilitates integration of TLRPs with other components of the cell walls (Domingo et al., 1999).

Another class of cell wall proteins, PRPs, are categorized into two classes; (1) components of normal plant cell walls and (2) nodule cell walls produced from infection by nitrogen-fixing bacteria. Both classes of PRPs are characterized by the occurrence of Pro-Pro repeats amongst an array of other larger repeat units. PRPs are expressed during development and upon wounding, yielding tissue and cell specific patterns of expression (Showalter, 1993). As for GRPs and PRPs, these proteins are insolubilized in the cell wall overtime. It has also been shown that there is an interaction between GRPs with PRPs and with other cell wall components to overall strengthen the wall (Showalter, 1993).

The solanaceous lectins are another class of cell wall proteins that are unique since they are glycoproteins of diverse origin. These proteins are limited to *Solanaceous* plant species and have unique amino acid and carbohydrate composition which is made up of hydroxyproline arabinose (Showalter, 1993). Furthermore, these proteins have the ability to agglutinate oligomers of *N*-acetyl glucosamine and have a predominantly extracellular location. In potato, lectin is a glycoprotein, which is made up of 50% protein and 50% carbohydrate (Showalter, 1993). These proteins are also expressed upon wounding and involvement of cell to cell communication has been suggested (Showalter, 1993).

AGPs cell wall protein class are highly glycosylated and usually soluble, making them unique. These proteins are widely distributed and comprise two to 10%, by weight, of the total protein found in plants (Showalter, 1993). With a small amount of their weight as a protein, D-galactose and L-arabinose carbohydrates account for most the components of these AGPs. A predominantly extracellular location and abundance in the middle lamellae has been suggested for location of AGPs. These proteins are also involved in cell-to-cell recognition (Showalter, 1993). Showalter et al. (1993) has proposed that AGPs could act as glues, lubricants, and humectants based on their biochemical and physical properties.

Extensins are a family of HRGPs which are highly abundant and are major protein components in cell walls of dicots. In dicots, these proteins are characterized by having a high content of hydroxyproline and serine, with some combination of valine, tyrosine, lysine, and histidine. Also, extensins usually have a pentapeptide motif of Ser-Hyp_4, which is repeated throughout. Furthermore, these Ser-Hyp_4 motifs are amongst hydroxyproline residues that are glycosylated with one to four arabinosyl residues. Serine residues with a single galactose unit are also among the structure (Showalter, 1993). These carbohydrates are considered to have a significant role in maintaining the linear, rod-like form of these molecules. Extensin accumulation is tissue specific and expression is elicited by both cellular and environmental factors (Ahn et al., 1998). After extensin is secreted into the cell wall it is rapidly insolubilized by formation of intra- and intermolecular cross-linkages such isodityrosine (IDT) bridges. This cross-linking phenomenon can occur quickly by an oxidizing reaction involving

peroxidase and hydrogen peroxide (Deepak et al., 2007). Additionally, cross-linking provides added resistance to cell wall degrading enzymes that are secreted by pathogens (Deepak et al., 2010). According to Dey et al. (1997), extensins are known for important physiological functions, such as secondary cell wall thickening. Showalter (1993) also suggested it is possible that extensin could act as cell wall "fly paper", meaning extensin is capable of immobilizing certain plant pathogens. Deepak et al. (2007) showed that there was a higher level of HRGPs of both transcript and protein levels in a cultivar of pearl millet resistant to *Sclerospora graminicola*. Another study showed that the extensin accumulation increased dramatically upon meristematic inactivation of the phellogen, and is associated with strengthening of cell walls (Sabba and Lulai, 2005). Therefore, extensin gene expression could be a key component in phellogen cell wall strengthening during periderm maturation.

Bown et al. (1993) screened a cDNA library for Extensin like (Ext-like) cDNA's. Through this study they determined that a separate Ext-like gene existed which is very similar to extensin. Another interesting fact Bown et al. (1993) noticed was that Ext-like mRNA increases when a potato tuber was wounded. Further evidence of an Ext-like protein was found in Arabidopsis, where the *AtEPR1* gene has extensin like structure; this gene was expressed during seed germination, hence cell expansion. Dubreucq et al. (2000) hypothesized that this gene is involved in morphogenic control of cell-wall architecture during the process of cellular differentiation. Later, nematode resistance was found to be in single clusters that encode proteins with high homology to extensin-like protein of potato

(Finker-Tomsczak et al., 2011). Furthermore, Ext-like proteins are induced upon interaction with nematodes, which is a 'wounding type reaction'. Microarray analysis showed that extensin genes are up-regulated during a cyst nematode infection. Finker-Tomsczak et al. (2011) suggested that these proteins may be involved in strengthening cell walls in defense of a nematode infestation. These data suggest that the Ext-like gene may have a pertinent role in wound-response, similar to extensin. These studies provided insight on some of the components that are important in the formation of the cell wall.

Cell Wall Biosynthesis

The process of cell wall biosynthesis and the molecular mechanisms involved in cell wall formation are important in understanding and elucidating the complete biology of the cell wall. The cell wall harbors many vital functions such as structural stability, counterbalance of internal turgor pressure, and protection from both mechanical and pathological injury (Deepak et al., 2010). Plants have many types of cell walls which can be grouped into two functional classes, primary or secondary walls. The formation of these types of cell walls depends upon if the cell wall is growing, or if cellular expansion has ceased. Primary cell walls are formed during growth, whereas secondary cell walls are constructed after cellular expansion has terminated (Liepman et. al, 2010). In flowering plants, primary cell walls can be separated into two broad groups. Type I cell walls have a structure which includes cellulose fibres encased in a network of xyloglucan, pectin, and structural proteins and are found in dicots, non-commelinoid monocots, and gymnosperms. Type II cell walls have a structure consisting of cellulose fibres

encased in glucuronoarabinoxylans, high concentrations of hydroxcinnamates, low concentrations of pectin and structural proteins (Deepak et al., 2010). This research focuses on potato tuber cell walls, i.e. a dicot which is characterized with type I cell walls.

Cellulose is the main component of the cell wall, constituting 30-40% of the wall mass. This major component of the cell wall contains 1,4-β-glucan chains, and is regarded as the major load-bearing constituent of the cell wall. Cellulose is synthesized from biological machinery located in the plasma membrane. This machinery enacts cellulose biosynthesis via cellulose synthase complex, a large multi-protein complex, synthesizing 18-36 glucan chains bonded together in a single microfibril (Liepman et al., 2010). According to Liepman et al. (2010), the location of this complex allows access to intracellular UDP-glucose and the ability to extrude microfibrils directly into the cell wall. The orientation of the microfibrils determines shape, while the matrix controls growth rate (Boyer, 2009). The microfibrils are the foundation for construction of the cell wall. Another component of the cell wall is hemicellulose, which is composed of cross-linking glycans that form a network and bind cellulose microfibrils. Xyloglucan, the most common hemicellulose, is made up of a 1,4-β-linked glucan backbone that can have 1,6-α-linked xylosyl residues or side chains of xylosyl, galactosyl, and fucosyl residue substitutions (Liepman et al., 2010). The cellulose-xyloglucan network has been suggested to regulate cell expansion and be the main contributor to the load-bearing capacity of the primary cell wall. Combined, the hemicelluloses, cellulose network, pectins, and structural proteins are integrated constituents of the cell wall.

All of these components essentially interlock with each other forming this exceptional structure.

Cell Cycle

Through the cell cycle and accompanying cell division, new periderm cells are formed. The cell cycle, active within the meristematic cells of the periderm, has four phases: chromosomal segregation mitosis (M), gap 1 (G1) occurring after (M) leading to DNA synthesis (S) phase, and gap 2 (G2) which separates (S) phase from (M) (Dewitte and Murray, 2003). It has been suggested (G1) monitors the environment and cell size, while (G2) ensures that genome duplication has been completed. The (G1) to (S) phase transition is regulated through phosphorylation of retinoblastoma-related proteins. The cell cycle process appears to be pushed along by the retinoblastoma (RB)-E2F pathway. After this pathway is induced, stimulus of the (G1)-(S) phase transcription factors occur leading to the start of DNA synthesis (Veylder et al., 2003). As the cell cycle advances forward to the (G2) to (M) transition, cyclin-dependent kinases (CDK), CDKA and CDKB, are probably involved, since these CDKs transcriptional levels peak during mitosis and (G2). Veylder et al. (2003) also suggest that plants have a MSA-mediated mechanism that controls specific genes allowing the (G2) to (M) transition.

After mitosis is initiated, a preprophase band is formed from cortical microtubules rearranged into a narrow cortical ring, the future divisional plane. Following the dissolving of the preprophase band, a mitotic spindle is constructed and migrates to opposite ends of the cell (prophase). Then spindle fibers attach to

centromeres and align at the equatorial plate (metaphase). After the spindle fibers shorten and the centromere splits, segregation occurs (anaphase). Following segregation, the telophase emerges and daughter nuclei are formed. During this process, a phragmoplast is created from the microtubule rearrangement. The phragmoplast functions to organize the synthesis of a new cell wall between the daughter cells (cytokinesis). In most cases, two identical daughter cells are formed from this progression. If endoduplication occurs, the mitosis step is skipped and repeated S phases occur; this results in multiple ploidy levels in the same cell. Dewitte and Murray (2003) stated that this cell cycle process for ploidy increase only transpires after cells have ceased normal mitotic cycles. In addressing the selective induction of these cell cycle phases, Inze and Veylder (2006) propose that there is a Mitosis-Inducing Factor (MIF) in the cell which acts as a mechanism to continue replication or undergo endoreduplication.

These phases are partially controlled by the key regulators, CDKs complexes. In plants, CDKs have been divided into: CDKA, CDKB, CDKC, CDKD, and CDKE. Each CDK has its independent role in the cell cycle. According to Mironov et al. (1999), only A and B classes of CDKs are well defined in the plant kingdom. Their research showed that *CDKB* accumulated transcripts in either S or G2, or in G2 and M phases. *CDKB* kinase activity is predominantly linked to mitosis (Mironov et al, 1999). Inze and Veylder (2006) stated that the CDKB transcription pattern and kinase activity reaches a maximum during mitosis and is down regulated at the onset of endoduplication. Collectively, these results suggest that changes in *CDKB* transcripts should be detectable during periderm

maturation. Importantly, through *in situ* hybridization, CDKB expression was shown in actively dividing tissues (Dewitte and Murray, 2003). Another factor, the CDK regulatory subunit (*CKS1At*), was shown to be at peak transcript level when cell division was at maximum. Richard et al. (2001) showed that *CKS1At* was highly expressed in their cell suspensions from beginning to end; suggesting *CKS1At* could be another good candidate gene for studying cell cycle aspects in periderm maturation.

Upon further investigation of the cell cycle, Horvath et al. (2006), found a plant growth regulatory gene, *EBP1* that is expressed in developing organs and promotes cell proliferation. In addition to cell proliferation, *EBP1* is required for the expression of *CDKB1:1* a form of *CDKB*. Expression profiles were used to determine that *EBP1* was functioning during tuber development, and that it is also expressed in all actively growing tissues. An expression test was conducted in which Horvath et al. (2006) determined *EBP1* to be a dose-dependent regulator, since reduction in transcript affected the growth of leaves, roots, and tubers. Collectively, this research provides insight to identify various candidate genes for expression profiling during periderm maturation and skin-set development.

The cell cycle is a key component in understanding wound healing, since cell cycle re-initiation occurs after injury (Veylder et al., 2007). By knowing the cell cycle timing or transition, one could possibly provide tools to determine potential cell number and corresponding size of the organ (Horvath et al., 2006). Also, plants are sessile and therefore cannot escape unfavorable conditions. Therefore, they have evolved to deal with conditions that come their way. To deal with

environmental stresses, plants have evolved a high number of cell cycle genes, allowing them to colonize different environments. Thus, it could be possible to fine-tune the cell cycle to benefit crops. Upon wounding, new periderm is needed to protect the tuber; hence oligopotency is essential for the survival of the tuber. This characteristic is the potential ability to build new individuals from existing cells (Veylder et al., 2007). In wound periderm, dedifferentiation occurs since parenchyma cells must reprogram to form the phellogen layer which divides outward and inward to form a new periderm.

Conclusion/Future Aspects

The global advancement of agriculture depends on new emerging technologies as well as the incorporation of historical technologies. Researchers with an understanding of old and new technologies and how to integrate and apply them will be better prepared for challenging research goals. New technologies that give insight into the genetic and molecular mechanisms of important traits provide researchers with the scientific tools to overcome agricultural problems.

Historical technologies have played an important role in the advancement of agriculture. The crops we grow and consume have been domesticated throughout time. Knowing where crops originated and the evolutionary and transitional processes involved is crucial for advancement of a species. In the beginning of agrarian cultures, farmers probably did not realize that they were inadvertently selecting a mutant. Over time, farmers began to realize that these mutants had important traits for human consumption, so they consciously selected for these mutations. Historical evidence points towards the tropical or subtropical areas as

the center of origin for most species. Knowing the place of origin is crucial in being able incorporate original plant material. Original cultivars may contain resistance genes or other important traits, since domestication reduces genetic diversity of a species. One prime example is the Irish potato famine where *P. infestans* wiped out entire potato crops resulting in starvation and death. Knowledge of the origin of potatoes has led to discovery of resistant genes towards *P. infestans,* aiding in defense to this disease. Moving forward in time, the cloning and the construction of linkage maps aided in the advancement of crops. An invertase gene in tomato was cloned leading to the exploitation of this gene showing the gene is directly involved in sucrose accumulation in the fruit. The construction of molecular markers allows for high and low density molecular linkage maps. RFLPs were used in potato and tomato, as well as many other crops, to develop molecular linkage maps. These maps lead to synteny studies showing that certain loci are shared within a species. Therefore, researchers have a better understanding of where certain traits are located within a species, allowing for the transfer of genomic information among species. Furthermore maps can be used for map-based cloning. Map-based cloning has been used in tomato to provide evidence that a *Pto* gene confers resistance *Pseudomonas syringae*. This technique is a valuable resource for cloning and exploiting resistance genes in plant species.

New problems continue to emerge with the globalization of agriculture. The development of new technologies is essential in addressing these tribulations. Recently, faster sequencing has led to whole genomes being published, allowing researchers access to this valuable information. Furthermore the combination of

older molecular markers (AFLP, RFLP, RAPD, and isozyme) with new markers (SNPs and microsatellites) have allowed for saturation of a genome. A major benefit of a saturated genome is that the connection between gene and phenotype can be determined. These techniques allow researchers to use "model" organisms (rice, arabidopsis, etc.) to benefit related organisms. Molecular markers also facilitate comparative mapping among similar genomes that are not published and possibly allow QTL discovery. Researchers conducting phylogenetic studies have used molecular markers along with statistical approaches. Approaches such as STRUCTURE, principle component analysis, neighbor joining, and linkage disequilibrium (LD), to name a few, aid in interpreting the generated data. These statistical programs have been used in many species to determine the variability of certain alleles within geographical origin and if certain traits are related to each other. Another benefit of this technology is that the origin of a certain trait/QTL can be determined; this provides a means to go back to the original species. Even though QTL analysis is not a new technology, new approaches to incorporate QTL data have been crucial for genetic gain. Single marker analysis, interval mapping, or composite interval QTL mapping have been used for analyses of QTLs. Also QTL analysis has been used to resolve genotype by environment interactions. Association mapping is one of the most recent approaches of determining marker linkages to a trait. Association mapping uses STRUCTURE and a mixed linear approach for controlling population structure, and the association of a phenotype.

All of these technologies have aided researchers to better understand the genetic and molecular mechanisms in plants that lead to certain traits/phenotypes.

Furthermore, this project involves gene expression studies which could be used with classical breeding techniques toward the improvement of potato cultivars. Gene expression studies also could lead to molecular markers, which have allowed for discovery of genes and QTLs. Through an understanding of these new and old technologies, researchers will have the necessary tools vital to develop elite cultivars.

References

Ahn JH, Choi Y, Kim SG, Kwon YM, Choi YD, Lee JS. Expression of a soybean hydroxyproline-rich glycoprotein gene is correlated with maturation of roots. Plant Physiol 1998;116:671–9.

Bown DP, Bolwell GP, Gatehouse JA. Characterization of potato *(Solanum tuberosum* L.) extensins: a novel extensin-like cDNA from dormant tubers. Gene 1993;134:229-33.

Boyer JS. Cell wall biosynthesis and the molecular mechanism of plant enlargement. Functional Plant Biol 2009;23:383-94.

Caffall KH, Mohnen D. The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. Carbohydrate Research 2009;344:1879-1900.

Deepak S, Shailasree S, Kini RK, Hause B, Shetty SH, Mithofer A. Role of hydroxyproline-rich glycoproteins in resistance of pearl millet against downy mildew pathogen *Sclerospora graminicola*. Planta 2007;226:323-33.

Deepak S, Shailasree S, Kini RK, Muck A, Mithofer A, Shetty SH. Hydoxyproline-rich glycoprotein and plant defence. J Phytopath 2010;158:585-93.

Dewitte W, Murray JAH. The plant cell cycle. Ann Rev Plant Biol 2003;54:235–64.

Dey PM, Brownleader MD, Pantelides AT, Trevan M, Smith JJ, Saddler G. Extensin from suspension-cultured potato cells: a hydroxyproline-rich glycoprotein, devoid of agglutinin activity. Planta 1997;202:179-87.

Domingo C, Gomez MD, Caíias L, Hernandez-Yago J, Conejero V, Vera P. A novel extracellular matrix protein from tomato associated with lignified secondary cell walls. Plant Cell 1994;6:1035-47.

Domingo C, Sauri A, Mansilla E, Conejero V, Vera P. Identification of a novel peptide motif that mediates cross-linking of proteins to cell walls. Plant J 1999;20:563-70.

Dubreucq B, Berger N, Vincent E, Boisson M, Pelletier G, Caboche M, et al. The *Arabidopsis AtEPR1* extensin-like gene is specifically expressed in endosperm during seed germination. Plant J 2000;23:643-52.

Finkers-Tomczak A, Bakker E, Boer J, Van der Vossen E, Achenbachen U, Golas T, et al. Comparative sequence analysis of the potato cyst nematode resistance locus *H1* reveals a major lack of co-linearity between three haplotypes in potato (*Solanum tuberosum* ssp.). Theor Appl Genet 2011;122:595-608.

Food and Argiculture Organization of the United Nations (FAO). International year of the potato. 2008. Available at http://www.potato2008.org/en/index.html (verified 10 Feb. 2010). FAO.

Fu Y, Peterson GW, Richards KW, Tarn TR, Percy JE. Genetic diversity of Canadian and exotic potato germplasm revealed by simple sequence repeat markers. Am J Potato Res 2008;86:38-48.

Gilber, HJ. The biochemistry and structural biology of plant cell wall deconstruction. Plant Physiol 2010;153:444-55.

Gebhardt C. Potato Genetics: Molecular Maps and More. In: Lörz H, Wenzel G, editor. Molecular marker systems in plant breeding and crop improvement. Nagata T, Lörz H, Widholm JM, editors. Biotechnology in Agriculture and Forestry. Springer-Verlag Berlin Heidelberg, 2005. p 215-27.

Hancock JF. Plant evolution and the origin of crop species, Second Edition. CABI International, Wallingford, United Kingdom. 2004. p 215-17.

Harholt J, Suttangkakul A, Vibe Scheller H. Biosynthesis of pectin. Plant Physiol 2010;153:384-95.

Hemphill J, Thone F. Well-bred potatoes. Society for Science and Public 1946;49:314-15.

Hijmans RJ, Spooner DM. Geographic distribution of wild potato species. Am J Bot 2001;88:2101-12.

Horvath BM, Magyar Z, Zhang Y, Hamburger AW, Bako L, Visser RG, Bachem CW, Bogre L. EBP1 regulates organ size through cell growth and proliferation in plants. EMBO J 2006;25:4909-20.

Inze D, Veylder LD. Cell cycle regulation in plant development. Ann Rev Genet 2006;40:77-105.

Jansky S. Potato improvement using exotic germplasms: prospects and challenges. Pamphlet. 16 Nov. 2009. USDA-ARS and UW-Madison.

Liepman AH, Wightman R, Geshi N, Turner SR, Scheller HV. Arabidopsis-a powerful model system for plant cell wall research. Plant J. 2010;61:1107-21.

Lulai EC. The roles of phellem (skin) tensile-related fractures and phellogen shear-related fractures in susceptibility to tuber-skinning injury and skin-set development. Am Potato J 2002;79:241-48.

Lulai EC. Skin-set, wound healing, and related defects. In: Vreugdenhil D, editor. Potato Biology and Biotechnology: Advances and Perspectives. Elsevier Limited, 2007. p 471-96.

Lulai EC, Freeman TP. The importance of phellogen cells and their structural characteristics in susceptibility and resistance to excoriation in immature and mature potato tuber (*Solanum tuberosum* L.) periderm. Ann Bot 2001;88:555-61.

Lulai EC, Huckle L, Neubauer J, Suttle J. Coordinate expression of AOS genes and JA accumulation: JA is not required for initiation of closing layer in wound healing tubers. J Plant Physiol 2011;168:976-82.

Lulai, EC, Morgan WC. Histochemical probing of potato periderm with neutral red: a sensitive cytofluorochrome for the hydrophobic domain of suberin. Biotech Histochem 1992;67:185-95.

Lulai EC, Orr PH. Determining the feasibility of measuring genotypic differences in skin-set. Am Potato J 1993;70:599-09.

Lulai, EC, Suttle J. The involvement of ethylene in wound induced suberization of potato tuber (Solanum Tuberosum L.) Postharvest Biol Tech 2004;34:105-12.

Lulai EC, Suttle JC, Pederson SM. Regulatory involvement of abscisic acid in potato tuber wound-healing. J Exp Bot 2008:59;1175-86.

Micheli F. Pectin methylesterases: cell wall enzymes with important roles in plant physiology. 2001. Trends in Plant Science Vol. 6 No. 9.

Mironov, V., L.D. Veylder, M.V. Montagu, and D. Inzé. Cyclin-dependent kinases and cell division in plants-the nexus. Plant Cell 1999;11:509-21.

Narvaez-Vasquez, J. and C.A. Ryan, Jr. 2002. The systemin precursor gene regulates both defensive and developmental genes in *Solanum tuberosum*. Proc Natl Acad Sci USA 2002;99:15818-21

Os H, Andrzejewski S, Bakker E, Barrena I, Bryan GJ, Caromel B, et al. Construction of a 10,000-marker ultradense genetic recombination map of potato: providing a framework for accelerated gene isolation and genome wide physical map. Genetics 2006;173:1075-87.

Park TH, Vleeshouwers VGAA, Jacobsen E, Vossen E, Visser RGF. Molecular breeding for resistance to *Phytophthora infestans* (Mont.) de Bary in potato (*Solanum tuberosum* L.): a perspective of cisgenesis. Plant Breed 2009;128:109-117.

Pilling J, Willmitzer L, Fisahn J. Expression of a *Petunia inflate* pectin methyl esterase in *Solanum tuberosum* L. enhances stem elongation and modifies cation distribution. Planta 2000;210:391-99.

Potato Genome Sequence Consortium. 2010. Available at www.potatogenome.net. (verified 5-23-11).

Raker CM, Spooner DM. Chilean tetraploid cultivated potato, *Solanum tuberosum*, is distinct from the Andean Populations: Crop Sci 2002;42:1451-58.

Richard C, Granier C, Inze D, Veylder LD. Analysis of cell division parameters and cell cycle gene expression during the cultivation of *Arabidopsis thaliana* cell suspensions. J Exp Bot 2001;52:1625-33.

Ridley BL, O'Neill MA, Mohnen D. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. Phytochemistry 2001;57:929-67.

Rodriguez F, Wu F, Ané C, Tanksley S, Spooner DM. Do potatoes and tomatoes have a single evolutionary history, and what proportion of the genome supports this history? BMC Evolutionary Biology 2009;9:1-16.

Sabba RP, Lulai EC. Immunocytological comparison of native and wound periderm maturation in potato tuber. Am Potato J 2004;81:119-124.

Sabba RP, Lulai EC. Immunocytological analysis of potato tuber periderm and changes in pectin and extensin epitopes associated with periderm maturation. J Hort Sci 2005;130:936-42.

Showalter AM. Structure and function of plant cell wall proteins. Plant Cell 1993;5:9-23.

Spooner DM, McLean K, Ramsay G, Waugh R, Bryan GJ. A single domestication for potato based on multilocus amplified fragment length polymorphism genotyping. PNAS 2005;102:14964-99.

Ulvskov P, Wium H, Bruce D, Jorgensen B, Qvist KB, Skjot M, et al. Biophysical consequences of remodeling the neutral side chains of rhamnogalacturonan I in tubers of transgenic potatoes. Planta 2005;220:609-20.

Veylder LD, Joubes J, Inze D. Plant cell cycle transitions. Current Opinion in Plant Biol 2003;6:536-43.

Veylder LD, Beeckman T, Inze D. The ins and outs of the plant cell cycle. Nature 2007;8:655-65.

Visser RGF, Bachem CWB, Boer JM, Bryan GJ, Chakrabati SK, Feingold S, et al. Sequencing the potato genome: Outline and first results to come from elucidudation of the sequence of the world's third most important food crop. Am J Potato Res 2009;86:417-29.

PAPER 1. WOUNDING COORDINATELY INDUCES CELL WALL PROTEIN, CELL CYCLE, AND PECTIN METHYL ESTERASE GENES INVOLVED IN TUBER CLOSING LAYER AND WOUND PERIDERM DEVELOPMENT

Abstract

Little is known about the coordinate induction of genes that may be involved in agriculturally important wound-healing events. In this study, wound-healing events were determined together with wound-induced expression profiles of selected cell cycle, cell wall protein, and pectin methyl esterase genes using two diverse potato genotypes and two harvests (NDTX4271-5R and Russet Burbank tubers; 2008 and 2009 harvests). By 5 d after wounding, the closing layer and a nascent phellogen had formed. Phellogen cell divisions generated phellem cell layers until cessation of cell division at 28 d after wounding for both genotypes and harvests. Cell cycle genes encoding epidermal growth factor binding protein (*StEBP*), cyclin-dependent kinase B (*StCDKB*) and cyclin-dependent kinase regulatory subunit (*StCKS1At)* were induced by 1 d after wounding; expression profiles of these genes coordinated with related phellogen formation and the induction and cessation of phellem cell formation. Genes encoding the structural cell wall proteins extensin (*StExt1*) and extensin-like (*StExtlk)* were dramatically up-regulated by 1 d to 5 d after wounding, suggesting involvement with closing layer formation and later phellem cell layer formation. The coordinate induction and expression profile of *StTLRP,* a gene encoding a cell wall strengthening "tyrosine-and lysine-rich protein", suggested a role in the formation of the closing layer followed by phellem cell generation, and lastly cell wall thickening in non-

meristematic phellogen cells. Wounding up-regulated pectin methyl esterase genes (*StPME and StPrePME*); *StPME* expression increased during closing layer and phellem cell formation, whereas maximum expression of *StPrePME* expression occurred at 5 d to 14 d after wounding, implicating involvement in later modifications for closing layer and phellem cell formation. Collectively, these results indicate that the genes monitored were wound-inducible and their expression profiles markedly coordinated with closing layer formation and the indices for meristematic activity of the phellogen layer during wound periderm development; results were influenced by harvest, but not genotype. Importantly, *StTLRP* was the only gene examined that may be involved in phellogen cell wall strengthening or thickening after cessation of cell division.

Introduction

After the potato growing season, large financial losses can occur from the handling operations associated with harvesting, storage, and transportation of the crop. Associated wounding and bruising levels can range from 9 to 40% (Lulai, 2007a), and as such, losses can easily surpass $100 million annually (Brook, 1996). The quality and profitability of potatoes grown for seed, the fresh market, and processing are adversely impacted by skinning and other wound-related injuries. The biological processes regulating wound healing are poorly understood, including a lack of knowledge of the expression profiles of genes induced during formation and maturation of the wound periderm (Lulai, 2007b; Ginzberg et al., 2009; Soler et al., 2011). Research to determine the genes involved in the accumulation of cell wall polymers associated with wound periderm formation and

maturation is a critical step in developing options to biologically assess and control these processes. Information on these molecular processes is agriculturally and economically important in decreasing financial losses due to wounding and bruising.

Wounding induces an array of biological responses to protect and heal the damaged area of the tuber. Among the initial responses is development of a suberized closing layer; i.e. suberization of the existing cells at the wound surface, which is closely followed by formation of a meristematic layer of cells referred to as the phellogen or cork cambium. In the initial response, suberin poly(phenolics) (SPP) are the first suberin biopolymers to accumulate on the walls of exposed and neighboring cells followed by suberin poly(aliphatics) and glycerol (Lulai and Corsini, 1998; Bernards, 2002; Lulai, 2007a). The presence of SPP on wound responding cell walls is easily detected by autofluorescence microscopy and is often used to monitor the initiation and progress of these wound-healing processes. Following closing layer development, the wound periderm is formed by the outwardly dividing meristematic phellogen layer that produces numerous layers of phellem cell derivatives that are also suberized and marked by the presence of SPP. The meristematically active phellogen also divides inwardly, producing a few layers of phelloderm cell derivatives. The phellem, phellogen, and phelloderm are three distinct types of cells that make up the wound periderm. After these distinct cells are in place and an adequate number of phellem cell layers have been produced, the phellogen cell layer becomes non-meristematic and no longer generates phellem cell derivatives; at this point wound periderm formation is

complete and wound-periderm maturation may ensue (Lulai and Freeman, 2001; Lulai, 2007a). During wound periderm maturation, the phellem (skin) becomes more tightly attached to the tuber and more resistant to excoriation re-injury (Sabba and Lulai, 2004). Sabba and Lulai (2002) observed that the characteristically thin and fragile cell walls of the phellogen thicken after cessation of meristematic activity. The thickening of the phellogen radial cell walls in wound periderm may provide the necessary strength and robustness to reduce susceptibility to re-injury of the wounds; this is an agriculturally important part of periderm maturation.

The wound-induced formation of the phellogen cell layer is the starting point for cell division and the development of wound periderm. Wound periderm formation involves the periclinal division of phellogen cells, generating organized rectangular files of phellem and phelloderm cells. Hence, the cell cycle will be induced upon injury and is active in phellogen cells during wound periderm formation. Horvath et al. (2006) identified a plant growth regulatory gene *StEBP1* encoding an epidermal growth factor binding protein. This regulatory gene is expressed in developing organs, promotes cell proliferation, and is required for the expression of *CDKB1:1* a form of cyclin-dependent kinase B (Horvath et al., 2006). Zhiponova et al. (2006) showed that *CDKB* was active in highly proliferating regions and in wounded tissues and proposed that the CDKB participates in a long-term wound response. The CDK regulatory subunit (*CKS1At*) was shown to be at peak transcript level when cell division was at maximum. Richard et al. (2001) showed that *CKS1At* was highly expressed in cell suspensions throughout

culture. Collectively, these studies suggest that *CKS1At* and *StCDKB* could be candidate genes for studying or marking cell cycle aspects in wound periderm development and maturation.

Certain cell wall proteins and pectins may have key roles in strengthening phellogen cell walls after meristematic inactivation, making them more resistant to fracture and the periderm more resistant to excoriation (Sabba and Lulai, 2002, 2004, 2005; Kloosterman et al., 2010; Ross et al., 2011). Extensins are a family of hydroxyproline-rich glycoproteins (HRGPs), which are major components in cell walls of dicots and are characterized by a pentapeptide motif of Ser-Hyp_4 which is repeated throughout the protein (Showalter, 1993). Extensin accumulation is tissue-specific and expression is elicited by both cellular and environmental factors (Ahn et al., 1998). According to Dey et al. (1997), extensins are associated with important physiological functions, such as secondary cell wall thickening. Also, Deepak et al. (2007) showed that there were higher levels of HRGP transcripts and associated protein in a cultivar of pearl millet resistant to *Sclerospora graminicola*, the causative agent of pearl millet downy mildew. Another protein involved in cell wall structure is pectin methyl esterase (PME); this gene product can increase the content of unesterified uronic acid residues allowing pectin to have higher Ca^{2+} binding efficiency, thereby increasing tissue firmness (Ross et al., 2011). Contiguous patterns of deesterification of homogalacturonan molecules by PME leads to what Siedlecka et al. (2008) described as egg-box structures, which contribute to pectin stabilization and cell wall stiffening. Cannon et al. (2008) proposed that extensin reacts with acidic pectin polymers forming

extensin pectate, which may be involved in the assembly of new cell walls. These studies indicate that extensin and PME have major roles in the development of a robust cell wall and that they may be linked to, or mark, wound-healing events.

Bown et al. (1993) screened a cDNA library for extensin-like (Ext-like) cDNAs and found that a separate Ext-like gene existed which is very similar to extensin. In *Arabidopsis,* a gene was found that did not exactly match the Ser-Pro_4 motifs of extensin, but shared strong similarities, hence *AtEPR1* was categorized as Ext-like (Dubreucq et al., 2000). Furthermore, Bown et al. (1993) found that Ext-like mRNA increased in tuber tissue after wounding. Another cell wall protein, a tyrosine- and lysine-rich protein (TLRP), appears to be involved in the final assembly and architecture of lignified secondary cell walls (Domingo et al. 1994). Domingo et al. (1994) determined that TLRP participates in cross-linking of proteins to cell walls and can be linked to aromatic side chains of lignin. This cell wall protein was also found to be expressed at higher levels in potato tubers within a population that displayed firmer cooked tuber texture (Ross et al., 2011). Collectively, these studies suggest that expression of extensin and TLRP genes may be linked to wound-healing events and wound-periderm maturation.

Despite the commercial importance of wound healing to the potato industry, the technologies and practices used to ensure complete wound healing are decades old, were derived empirically, and rely on coarse physical determination of the degree of healing. The development of improved methods to hasten and ensure proper wound healing will depend on the identification of robust biochemical/molecular markers to gauge the progress of this process under a

variety of storage conditions. The goals of this research were two-fold: 1. to determine the effects of wounding on a group of genes encoding proteins of known or presumed importance to the wound healing process, and 2. to determine the utility of these genes as coordinate indicators or molecular markers for wound healing progress and wound periderm maturation.

Materials and Methods

Plant material, storage conditions, and wound model system

First generation certified seed minitubers (*Solanum tuberosum* L., genotypes Russet Burbank (RB) and NDTX4271-5R (ND)) from 2008 and 2009 greenhouse harvests, (Valley Tissue Culture, Halstad, MN, USA) were used in this research. The russeted genotype RB and the red skinned genotype ND were selected for comparison because they are genetically diverse and it was previously shown that this russeted genotype and red skinned genotypes differ in periderm maturation (Lulai and Orr, 1993) and wound healing (Lulai and Corsini, 1998).

After harvest, the tubers were held in a controlled environment chamber in the dark for 14 d at 20 °C and 95% relative humidity (RH) to facilitate suberization/curing. Following the postharvest suberization/curing period, the tubers were stored in the dark at 3 °C and 95% RH to inhibit deterioration and sprouting. Three days before use, the tubers were gently hand washed and pre-equilibrated to experimental wound-healing conditions (dark, 20 °C, 95% RH).

Following equilibration, the tubers were cut in half from stem to bud end, and then placed wound-side up in a controlled environment chamber (dark, 20 °C and 95% RH) to wound-heal and to allow wound-periderm maturation. During the

wound-healing and wound-periderm maturation time course, tissues were sampled and analyzed for expression of the indicated target genes, phellogen radial cell wall thickness, and wound-phellem cell layer development as described below. All experiments were repeated for each of the harvests. Sampling for each time point was replicated and the data analyzed as described for each experiment.

Histochemical analysis of phellem cell development

The production of layers of wound phellem cells was determined and used as a measurable index for wound-periderm development and ensuing wound periderm maturation as phellem cell production ceased. The standard rating system for accumulation of SPP on closing layer cells and on developing layers of wound phellem cells was employed as described by Lulai et al. (2008). Tissue blocks (~ 2 mm x 1 mm x 0.3 mm) were excised from the cut surface of the wound-healing mini-tubers at the indicated time points and placed into Farmer's Fixative (3:1 v/v, absolute ethanol: glacial acetic acid). These blocks were sectioned (200 µm thick) with a Vibratome 1000 Plus Tissue Section System (The Vibratome Company, St. Louis, MO, USA). SPP ratings were determined via epifluorescence microscopy on three sections per block, two blocks from two different tubers per time point, with three ratings per section following methods described by Lulai and Corisini (1998) as modified by Lulai et al. (2008). A Zeiss Axioskop 50 microscope equipped with a white light source and an EXFO X-Cite 120 PC illumination unit as a UV source for epifluorescent illumination was used, as described by Sabba and Lulai (2002), to determine suberization ratings and capture digital images (Lulai and Corisini, 1998; Lulai et al., 2008). The rating system was designed such that

zero = no accumulation, 5 = complete accumulation around the first layer of existing cells (closing layer) and 7, 9, 11, 13, etc = accumulation of 2, 3, 4, 5, etc. additional phellem cell layers respectively (see appendix A) (Lulai et al., 2008). Average SPP ratings are presented as mean ± standard error (n=3) and the equivalent number of phellem cell layers are indicated.

Determination of phellogen radial cell wall thickness

Increases in phellogen radial cell wall thickness accompanying wound periderm development and cessation of meristematic activity within the wound periderm were used as indications of wound periderm development and maturation. Tissue blocks (~2 mm x 1 mm x 0.3 mm) were cut from the wound-healing/healed tuber surface at the indicated time points and prepared as described above. A Zeiss Axioskop 50 microscope, described above, was used to determine phellogen radial cell wall thickness and capture digital images (Sabba and Lulai, 2002). Following identification, digital images of the phellogen radial cell walls were captured with a Zeiss color AxioCam camera (Carl Zeiss Inc., Thornwood, NY, USA). The thickness of the phellogen radial cell walls was measured electronically via a stage micrometer that was embedded into the Zeiss AxioVision software for the AxioCam camera (Sabba and Lulai, 2002). Average thickness data are presented as mean ± standard error (n=3).

RNA extraction

Total RNA was extracted from cellular exudate collected at the wound site for qRT-PCR determination of gene expression. Two sets of four tuber halves were sampled per time point (0, 1, 5, 28, 42 and 56 d after wounding). Cellular

exudates were collected from the tuber halves for RNA extraction at 0 d and 1 d after wounding, by gently skimming exudate from the cut surfaces with a spatula, and at 5, 28, 42, and 56 d after wounding, by carefully pealing back the phellem from the healing surface and then using a spatula to gently skim exudate from the exposed phellogen layer on the tuber surface and phellem. Total RNA was extracted from the collected exudate according to the method of Narvaez-Vasquez and Ryan (2002) with modifications (Narvaez-Vasquez, personal communication; Destefano-Beltran et al., 2006). The collected cellular exudate from each set of tuber halves was transferred to a 1.5 mL centrifuge tube with 500 µL of 0.1 M Tris-HCL (pH 7.4) containing 1% (w/v) sodium sulfite. Buffer-saturated phenol (500 µL) (Invitrogen, Eugene, OR, USA) was added to the centrifuge tube and the mixture vortexed. The mixture was centrifuged (20,800 x g for 9 min at 4 °C) and the supernatant was re-extracted with an equal volume of acid-phenol/chloroform (5:1, v/v) (Ambion, Austin, TX, USA). The extract was treated with 50 µL of 3 M sodium acetate pH 5.2 and 500 µL of isopropanol and incubated for 10 min at room temperature to precipitate RNA. After 10 min, the RNA was centrifuged (10,600 x g for 10 min at 4 °C), washed with 70% ethanol, and re-centrifuged (6,800 x g for 5 min at 4 °C). The pellet was then dried and dissolved in 20 µl of molecular grade H_20 (Thermo Fisher, Waltham, MA, USA). RNA was quantified spectrophotometrically using a NanoDrop model ND-1000 Spectrophotometer set for RNA determination (NanoDrop Technologies, Wilmington, DE, USA). The quality of total RNA was examined by agarose (1%) gel electrophoresis in 1%

TAE, stained by ethidium bromide and visualized under UV light and ratio of absorbance at 280/260 nm.

qRT-PCR analysis

Quantitative RT-PCR (qRT-PCR) was used to determine the expression level of target genes during the experimental time course. Total RNA (2 µg) was first treated with DNA-Free (Ambion, Austin, TX, USA) to remove genomic DNA. Total RNA was reverse transcribed using a RETROscript kit (Ambion), with oligo dT_{18} primers using the procedures recommended by the manufacturer. After reverse transcription, the cDNA was diluted (1:6.5) to 150 µL total volume with molecular grade H_20 (Thermo Fisher, Waltham, MA, USA). The DNA Engine Opticon 2 (BioRad, Hercules, CA, USA) was used to amplify specific regions of targeted genes and for real-time detection of the resulting amplicons. Potato Gene Index Tentative Consensus (TC) numbers of the target genes were: *StExt*, TC203100, TC2224149, TC211436, TC211015, TC208933, TC203030, TC197747; *StEXT1*, TC203100; *StExtlk*, TC197382; *StCDKB*, TC201185; *StEBP*, TC197074; *StCKS1At*, TC209250; *StPME*, TC213276; *StPrePME*, TC197435 and *StTLRP*, TC208255. Primer pairs with corresponding sequences used for each gene are presented in Table 1.

The qRT-PCR reaction consisted of: 7 µL cDNA template, 2 µL 10x Hot Start Taq buffer (200 mM Tris-HCl (pH 8.3 at 25 °C), 200 mM KCl, 50 mM $(NH_4)_2SO_4$), 1.6 µL 25 mM $MgCl_2$, 1.6 µL 2.5 mM dNTPs, 1 µL DMSO, 1 µL 10x SYBR-green, and 0.2 µL (5 units/µL) Hot start Taq Polymerase (Thermo Fisher, Waltham, MA, USA). Primers were diluted in molecular grade H_20 (Thermo

Table 1. Primers Used for qRT-PCR Expression of Target Genes Involved in Wound Periderm Development and Maturation.

Gene/Accession#	Orientation	Sequences (5' – 3')
StExt, TC[b]	Forward	CGAAGTTCTTTTGTAGCTTC
	Reverse	GCAAACATACACAAAGACAT
StExt1, TC203100	Forward	GATCCACCACCCAAGAGGCC
	Reverse	GTGGTGGGGACTTGTAAATG
StExtlk, TC197382	Forward	GCTCACAATATGGCAACTAT
	Reverse	CGACCACGTCCACGTCCACA
StCDKB, TC201185	Forward	GTGACTGGCATGTTTATCCA
	Reverse	GCTGTCAAAGTATGGATGTT
StEBP, TC197074	Forward	GGTAGAAGAGGCATCTCAAG
	Reverse	GCTCAACGTGCCGAATCGAC
StCKS1At, TC209250	Forward	GTGGCGGGCAATTGGAGTT
	Reverse	GCAGAACTTGCTGAGTCTGG
StPME, TC213276	Forward	CAGTCGTCGACGGTGACGC
	Reverse	GTGCTGCCATCTTGCACATT
StPrePME, TC197435	Forward	GCATTCCAGGACACTCTCTA
	Reverse	GAACACAACTGCTGCTGTTAC
StTLRP, TC208255	Forward	CCCCCACATGGTGGATATAA
	Reverse	TCGTCATGTGGAGGGTTGTA
Efla, AB061263[a]	Forward	ATTGGAAACGGATATGCTCCA
	Reverse	TCCTTACCTGAACGCCTGTCA

[a] From Nicot et al. (2005).

[b] *StExt* primers were designed to amplify these homologs: TC203100, TC2224149, TC211436, TC 211015, TC208933, TC203030, and TC197747.

Fisher) to a concentration of 5 µM. The total volume of qRT-PCR reaction was 20 µL. The qRT-PCR reaction parameters were: 95 °C for 4 min, 56 °C for 1 min, 72 °C for 1 min; followed by 44 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 45 s, 75 °C for 3 s. A melting curve was determined at 65 °C to 90 °C, and the absorbance determined every 1 °C.

The qRT-PCR product for each target gene was separated on a 1% agarose gel, stained with ethidium bromide to verify amplification of a single product, and the amplicon compared with molecular standards to confirm that the fragment was of the correct size. The amplified fragment for each target gene was isolated from the agarose gel and sequenced (Northwoods DNA, Solway, MN). The sequence was compared (BLAST) with those of known genes in the Potato Gene Index to verify identity of the amplicon. qRT-PCR amplification of each target gene was then used to generate efficiency curves (Pfaffl, 2001). After efficiencies curves were established for each gene, all qRT-PCR reactions were conducted within the ranges of linearity established by the efficiency curves. Relative fold changes in expression for each target gene were calculated based on methods of Pfaffl (2001) using elongation factor 1-α (*EF1α*) as a reference (Nicot et al., 2005).

Results

Determination of closing layer and phellem cell layer formation via SPP ratings

The SPP rating system was used to assay the accumulation of SPP on outer tangential, radial, and inner tangential cell walls of the developing closing layer and associated wound phellem cells. Importantly, this approach also provides: (1) an index for quantification of phellem cell generation (see

supplemental Table S1) and (2) determination of the time line for subsequent cessation of wound phellem cell development and initiation of ensuing wound periderm maturation. In this analysis, the SPP ratings indicated that wound phellem cell development was distinctly different in the two genotypes (ND vs. RB) for both the 2008 and 2009 harvests. There was no SPP accumulation directly upon wounding, since the walls of undamaged cortical and parenchyma cells of the tuber contain no SPP. However, by 1 d after wounding, both genotypes had SPP ratings of ~2 (Fig. 3), indicating that SPP had accumulated on the majority of outer tangential cell walls at the wound surface, and the initiation of closing layer formation. At 5 d after wounding, a rating of 5 to 6 was determined (Fig. 3, 4A and C), indicating SPP accumulation on one to one and half-cell layers for both genotypes and harvests. Past 5 d after wounding, a significant difference in SPP ratings was observed between genotypes. ND tuber tissues increased in SPP ratings which reached a maximum by 28 d to 42 d after wounding; these are the time points for cessation of wound phellem cell layer development, which is then followed by wound periderm maturation (Fig. 3). SPP laminated a total of 5 cell layers throughout the wound-healing time course for ND tubers from both harvests (Fig. 3 and 4B). RB tuber tissue experienced a dramatic increase in SPP laminated cell layers from 5 d through 28 d after wounding, but also leveled off on or before 28 d after wounding. Here again, these time points indicate cessation of wound phellem cell layer development, ensued by wound periderm maturation. At 14 d after wounding RB tissues had SPP ratings of 14 to 16, whereas ND only had ratings of 8 to 9 (Fig. 3). These results are significant since SPP ratings of 14

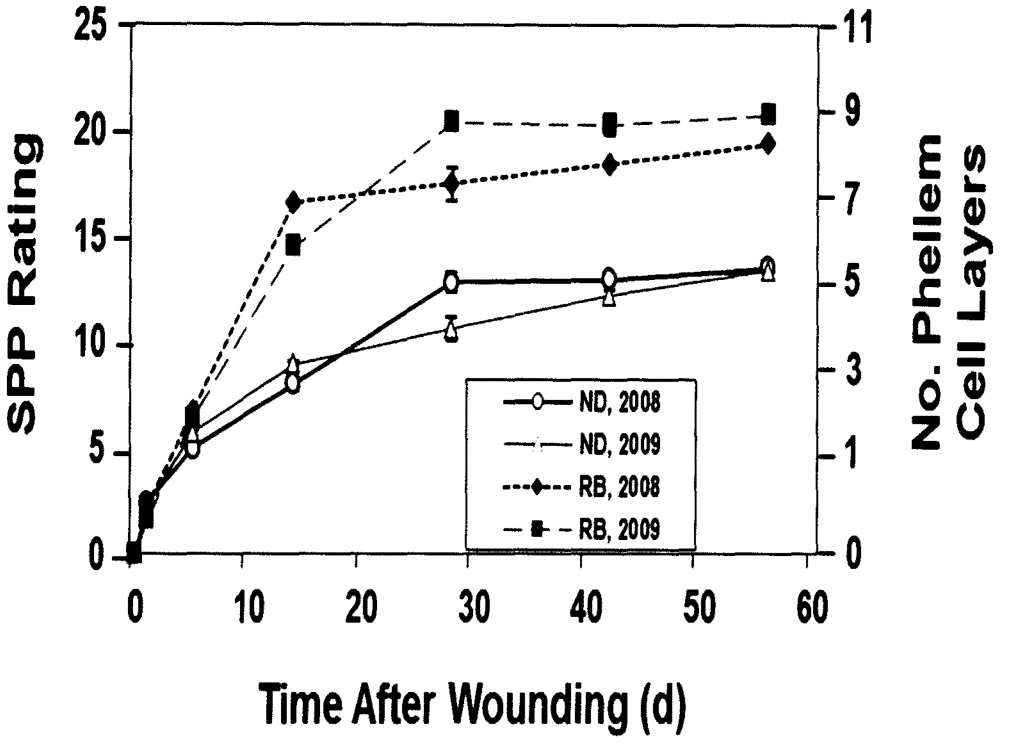

Fig. 3. The change in suberin poly(phenolics) (SPP) ratings during wound healing of tubers from the genotypes NDTX4271-5R and Russet Burbank (2008 and 2009 harvests). SPP ratings are indicative of the number of tangential and radial cell walls impregnated with SPP and reflect the number of wound phellem cell layers generated by meristematic action of the wound phellogen; this data is an effective index for progress and cessation of wound-induced cell division during wound periderm development. SPP ratings were determined on three sections per disc with three ratings per section. Bars represent the standard error of the means at each time point. Note that a rating of 5 = 1 complete layer of cells, 7 = 2 complete layers of cells, 9 = 3 complete layers of cells, etc., and 19 = 8 complete layers of cells (See Appendix A). There were no significant differences in SPP ratings, i.e. the number of phellem cell layers present, after 28 d based on one-way analysis of variance (ANOVA) $p \leq 0.05$.

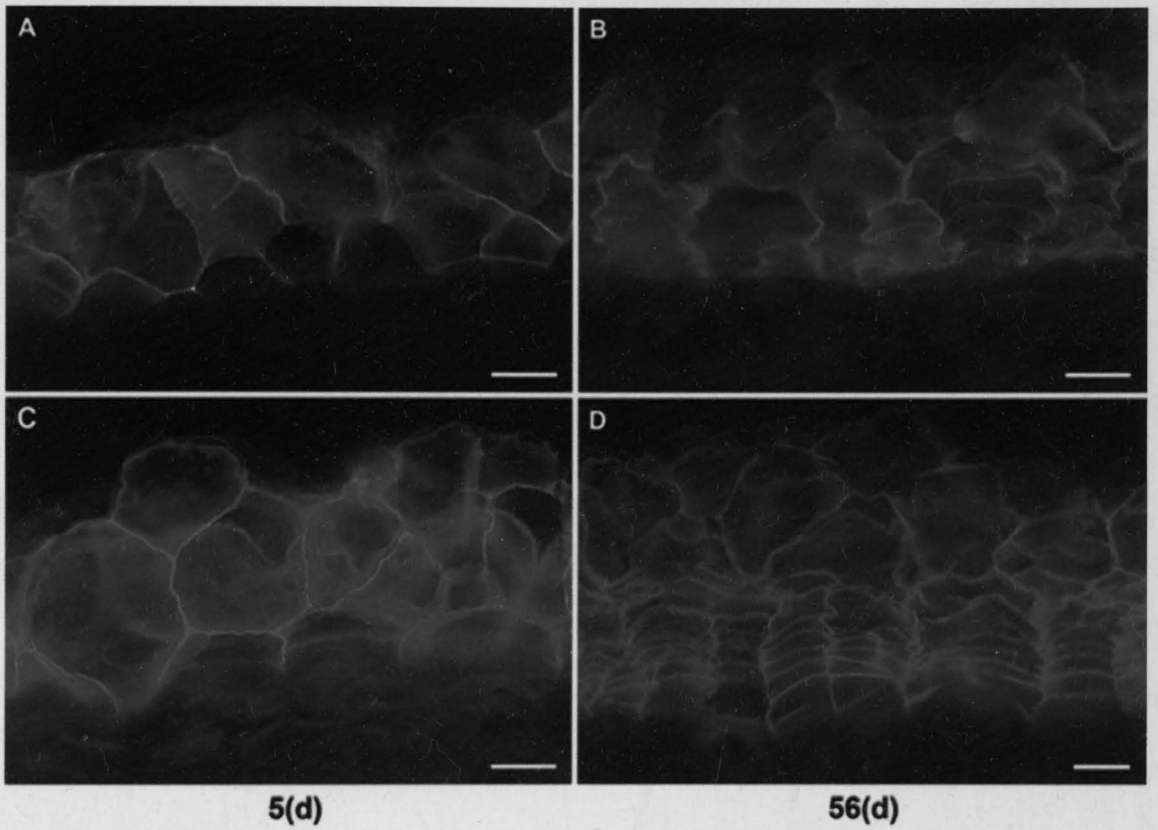

Fig. 4. Effect of wound-healing and genotype on autofluorescent suberin poly(phenolic) (SPP) accumulations. The period through 5 d includes closing layer formation, and the 56 d time point includes closing layer formation and accumulation of layers of phellem cells distinguishable by their organized files of rectangular cells: (A) Genotype ND tuber tissue wound healed 5 d (B) Genotype ND tuber tissue wound healed 56 d. (C) Genotype RB tuber tissue wound healed 5 d (D) Genotype RB tuber tissue wound healed 56 d. Bar = 5 μm. SPP accumulation in the cell walls of these tissues was visualized using autofluorescence microscopy as a means of determining closing layer formation illustrated in "A" and "C" and closing layer plus phellem cell layer development illustrated in "B" and "D." Note the differences between 5 d (A and B) and 56 d (C and D) for each genotype. Also note the differences in accumulation of SPP at 56 d (B and D) between the genotypes.

to 16 equal 5.5 to 6.5 cell layers compared to 2.5 to 3 cell layers (SPP ratings of 8 to 9) for ND tubers. SPP ratings for RB tissues reached a maximum at approximately the same time as those of ND, but had SPP ratings of ~ 18 to 20 at 56 d after wounding for a total accumulation of ~7.5 to 8.5 layers of phellem cells

(Fig. 3 and 4D). In both genotypes, the most rapid development of phellem cells occurred during the initial 14 d after wounding. By 28 d after wounding, and throughout the remainder of the time course, there was no significant difference ($p \leq 0.05$) in phellem cell development. Twenty eight days after wounding was determined to be the time frame for cessation of phellem cell production and associated meristematic activity, and the time for onset of ensuing wound periderm maturation.

Phellogen radial cell wall measurements of wound periderm

The wound periderm is composed of phellem, phellogen, and phelloderm tissues (Lulai, 2007a) and the formation and alteration of these cell types were monitored throughout the wound-healing and wound periderm maturation time course. Of these cell layers, the phellogen has been shown to be the critical layer in susceptibility to excoriation. Under the conditions used in these studies, the phellogen layer became visible by 5 d after wounding. An increase in the phellogen radial cell wall thickness was histochemicially detectable throughout the time course for wound responding tissues from both ND and RB tubers in both years (Fig. 5 and 6). ND is a slow maturing genotype and these tubers produced phellogen radial cell walls that measured 0.38 µM at 28 d (cessation of wound periderm development and initiation of periderm maturation) to 0.48 µM at 56 d after wounding for both 2008 and 2009 harvests (Fig. 5 and 6B). For tubers from the 2008 harvest of RB, phellogen radial cell walls measured 0.39 µM at 28 d (cessation of wound periderm development and initiation of periderm maturation) and 0.56 µM at 56 d after wounding (Fig. 5 and 6D). Phellogen cell wall

measurements of wound-healing tuber tissues from the 2009 crop of RB had a similar but more amplified trend-line, compared to ND (2008 and 2009) and RB 2008. Wound-healing tuber tissues from the RB 2009 harvest had phellogen radial cell walls that measured 0.58 µM at 28 d and 0.72 µM at 56 d after wounding (Fig. 5 and 6D). Overall, phellogen radial cell walls were 110% thicker at 56 d, compared to the first measurement of nascent cell walls at 5 d after wounding for ND tubers from both harvests. Similarly, phellogen cell walls of RB tubers were 120% and 100% thicker at 56 d compared to 5 d for the 2008 and 2009 harvest, respectively. However, it is important to note that aside from the percent increase

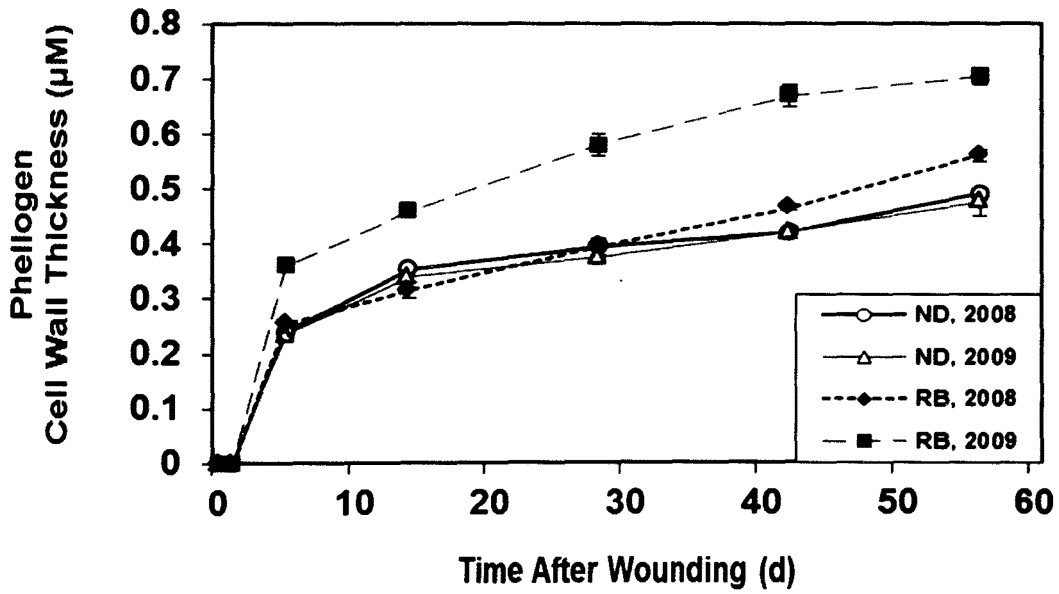

Fig. 5. Changes in phellogen radial cell wall thickness during wound periderm development and maturation for the genotypes NDTX4271-5R and Russet Burbank (2008 and 2009 harvests). The phellogen radial cell wall thickness was measured via digital microscopy. Each measurement was determined in triplicate. Bars represent the standard error of the means.

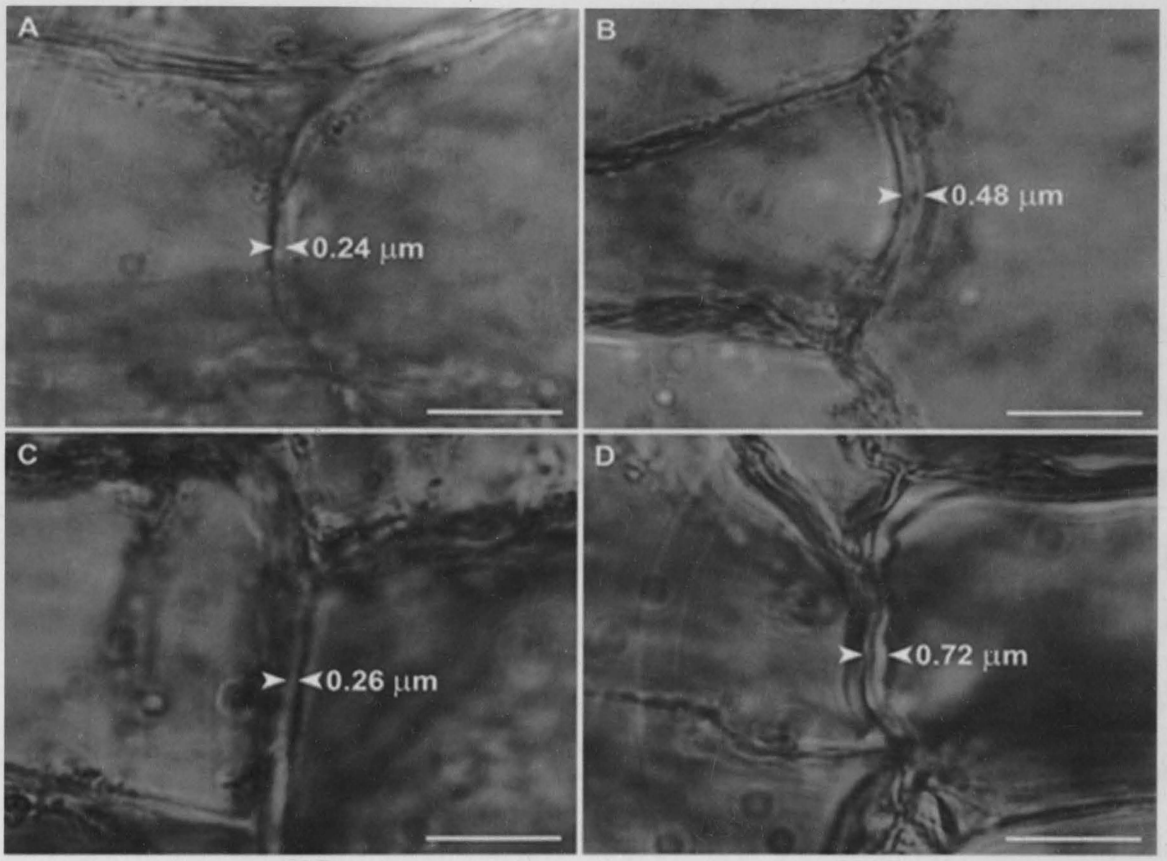

Fig. 6. Light micrographs illustrating differences in radial cell walls of nascent active phellogen (5 d after wounding) and inactive phellogen from mature tuber wound periderm (56 d after wounding) for the genotypes ND4271-5R (2009) and Russet Burbank (2009): (A) Immature thin radial cell wall from nascent, meristematically active phellogen 5 d after wounding, an immature wound periderm from the genotype ND. (B) Thickened radial cell wall from inactive phellogen 56 d after wounding, a mature wound periderm from the genotype ND. (C) Thin radial cell wall from nascent, meristematically active phellogen 5 d after wounding, an immature wound periderm from the genotype RB (D) Thickened radial cell wall from inactive phellogen 56 d after wounding, a mature wound periderm from the genotype RB. Bar = 5 µm. The area between the arrows indicates phellogen radial cell wall thicknesses in µm. Note the thicker phellogen radial cell wall at 56 d for both genotypes.

in radial wall thickness, wounded RB tubers from the 2009 harvest produced phellogen radial cell walls that were consistently 25% to 30% thicker throughout the time course (Fig. 5).

The effect of wounding on cell cycle, cell wall protein, and cell wall pectin methyl esterase gene expression

Wound-induced changes in the relative expression (fold change) of selected genes putatively involved in tuber wound-healing and wound periderm maturation (i.e. *StEBP, StCDKB, StCKS1At, StExt, StExt1, StExtlk, StTLRP, StPME,* and *StPrePME*) were determined using qRT-PCR. Changes in expression were evident in tubers of both potato genotypes (i.e. RB and ND) after zero time (i.e. basal level of expression in non-wounded tissue) through 56 d after wounding (Fig. 7, 8, and 9). Because the quantitative effects of wounding on gene expression varied between harvest years, both sets of data for each genotype are presented. Although all of these genes were wound-inducible and expression changed throughout the measured wound response time course, there was no discernable difference in gene expression patterns between these two diverse potato genotypes for the two harvests. The basal levels of expression for these genes was low, but detectable within 45 cycles of qRT-PCR; these basal Ct values represent initial baselines of expression and are essential for calculations of fold change in gene expression. Wounding induced increased expression of the three cell cycle genes examined. The cell cycle gene *StEBP* was relatively weakly induced and appeared to be most highly expressed at 1 d after wounding, exhibiting a ~3 to 4 fold increase, and then decreased throughout the time course (Fig. 7A). The maximum fold increase in expression for *StEBP* was much lower than that of *StCDKB* (~90 to 190 at 5 d) and *StCKS1At* (~6 to 22 at 1 d and ~8 to 21 at 5 d). Although in the 2008 harvest of RB tubers the highest fold increase in

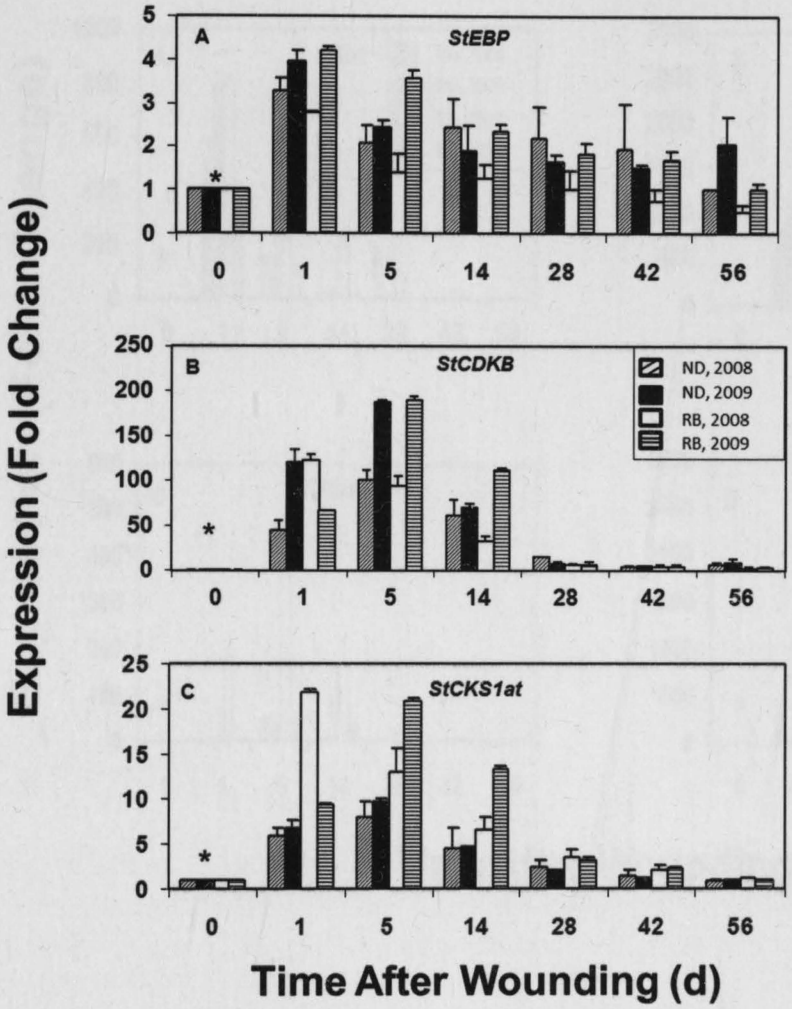

Fig. 7. Wound-induced changes in the expression of genes encoding cell cycle related proteins in tuber tissue for the genotypes NDTX4271-5R and Russet Burbank (2008 and 2009 harvests): (A) *StEBP*, (B) *StCDKB*, (C) *StCKS1At*. Gene expression was determined in triplicate by qRT-PCR using *EF1α* as a reference gene. * Basal levels of gene expression, i.e. zero days after wounding, were normalized to a value of one based on the method of Pfaffl (2001) for relative quantification in real-time RT-PCR. See Table 1 for primer pairs. Bars represent the standard error of the means.

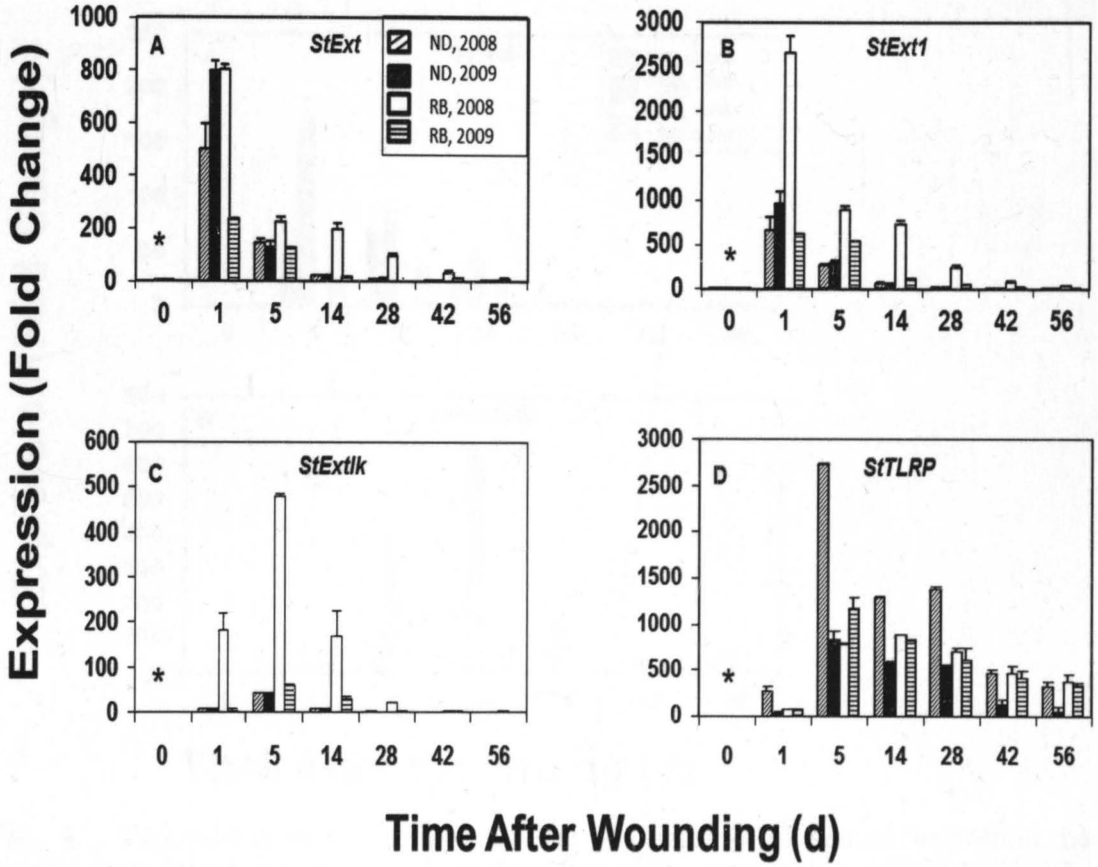

Fig. 8. Wound-induced changes in the expression of genes encoding the indicated structural cell wall proteins in tuber tissue for the genotypes NDTX4271-5R and Russet Burbank (2008 and 2009 harvests): (A) *StExt,* (B) *StExt1*, (C) *StExtlk*, (D) *StTLRP*. Gene expression was determined in triplicate by qRT-PCR using *EF1α* as a reference gene. * Basal levels of gene expression, i.e. zero days after wounding, were normalized to a value of one based on the method of Pfaffl (2001) for relative quantification in real-time RT-PCR. See Table 1 for primer pairs. Bars represent the standard error of the means

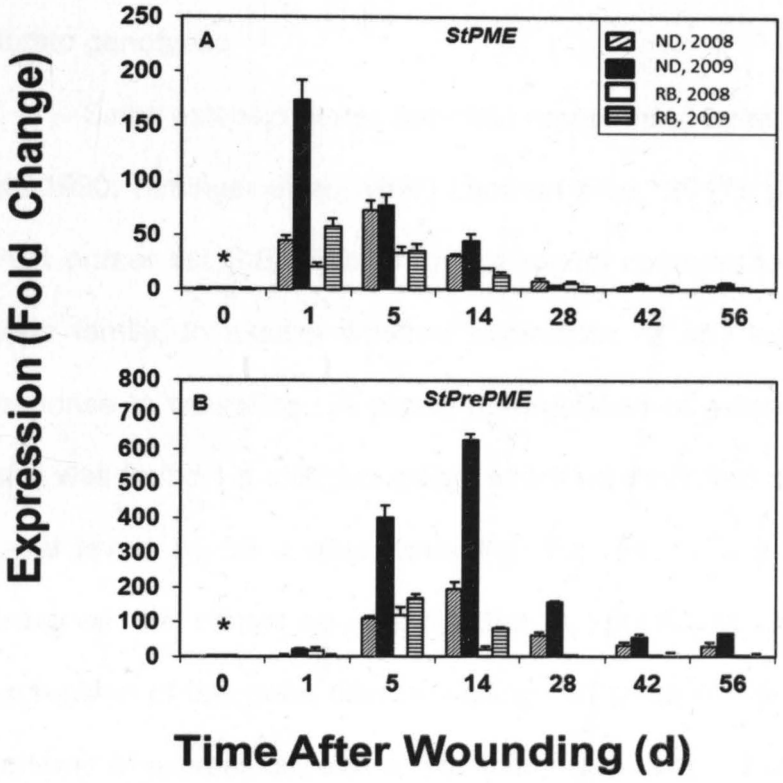

Fig. 9. Wound-induced changes in the expression of genes encoding pectin methyl esterase proteins in tuber tissue for the genotypes NDTX4271-5R and Russet Burbank (2008 and 2009 harvests): (A) *StPME* and (B) *StPrePME*. Gene expression was determined in triplicate by qRT-PCR using *EF1α* as a reference gene. * Basal levels of gene expression, i.e. zero days after wounding, were normalized to a value of one based on the method of Pfaffl (2001) for relative quantification in real-time RT-PCR. See Table 1 for primer pairs. Bars represent the standard error of the means.

expression of *StCKS1At* was ~22 at 1 d after wounding (Fig. 5C), the maximum increase in *StCDKB* and *StCKS1At* expression in RB tubers from the 2009 harvest and ND tubers from the 2008 and 2009 harvests appeared to be delayed, and most highly expressed by 5 d after wounding with a ~8 to 190 fold increase followed by a gradual decrease to near basal levels. Although harvest year influenced the fold change of wound-induced expression, there was no discernable

difference in the overall pattern of gene expression between these two diverse potato genotypes.

Since extensin genes are often represented by multigene families (Evans et al., 1990; Hirsinger et al., 1997; Lamport et al., 2011), we first designed the qRT-PCR primer set *StExt* (Table 1) in a region common to several members of this gene family, to assess whether expression of any extensin is up-regulated in response to wounding. A strong up-regulation of extensin transcript (up to ~800 fold) was found 1 d after wounding, which was followed by a gradual return to near basal levels by 56 d after wounding (Fig. 8A). To investigate this further, we designed the primer set *StExt1* that is specific to *StExtensin1* to interrogate expression of this gene after wounding. In general, *StExt* and *StExt1* had similar patterns of expression, with a dramatic increase at 1 d after wounding followed by a gradual return to near basal levels by, or before, 56 d after wounding (Fig. 8A and 8B), suggesting that *StExt1* represents most of the extensin transcript quantified with the general extensin primer set *StExt*. Overall, wound-induced expression of *StExt1* and *StExtlk* appeared to be higher in tubers from the 2008 harvest of Russet Burbank than in those from ND (2008 and 2009) and RB (2009). With the exception of the 2008 RB tubers, maximum increases in expression of the related gene, *StExtlk*, were lower (~50 fold or less) than *StExt1* (~230 to 2700 fold) and remained near basal levels at 1 d after wounding for both genotypes. *StExtlk* then exhibited its greatest measured increase in expression by 5 d after wounding with a ~50 fold increase (~470 fold for 2008 Russet Burbank) followed by a gradual decline (Fig. 8C). Although RB tubers from the 2008 harvest overall had higher

wound-induced expressions for *StExt1* and *StExtlk*, the expression patterns were similar to those obtained in tuber tissue from ND (2008 & 2009) and RB (2009). *StTLRP* expression was distinct, exhibiting a ~40 to 270 fold increase by 1 d after wounding that was followed by a much larger ~800-2700 fold increase at 5 d after wounding (Fig. 8D). However, unlike the genes coding for hydroxyproline-rich glycoproteins (*StExt*, *StExt1,* and *StExtlk*), *StTLRP* expression remained high throughout the time course with relatively small decreases through 56 d after wounding. Also unlike the extensin related genes, *StTLRP* expression did not return to basal levels by 56 d after wounding (Fig. 8D). Although harvest year influenced the fold change of wound-induced expression, there was no discernable difference in the overall pattern of gene expression between these two diverse potato genotypes.

Wounding induced increases in expression of both genes (*StPME* and *StPrePME*) involved in tuber pectin demethylation in both potato genotypes and harvests (2008 and 2009) (Fig. 9A and 9B). However, the expression patterns of these two PME genes differed. Expression of *StPME* increased significantly after wounding; relative expression levels were highest by 1 d and 5 d after wounding with increases ranging from ~20 to 170 fold during this time. These increases were followed by decreases, returning expression to near initial levels from 28 d through 56 d after wounding (Fig. 9A). Unlike *StPME*, expression of *StPrePME* remained relatively low at 1 d compared to 5 d and 14 d after wounding, at which times the overall increase in relative expression was highest, ranging from a ~100 to 600 fold, followed by a decrease in expression often to near original levels (Fig.

9B). Tubers from the 2009 harvest of ND had larger overall fold changes than that for the 2008 harvest or that of RB (2008 and 2009) for both *StPME* and *StPrePME* (Fig. 9A and 9B). In general, wounding induced increases in *StPME* expression by 1 d and 5 d after wounding; this was followed by decreases to near basal level for both genotypes from both harvests (2008 and 2009). Whereas a major increase in *StPrePME* expression did not occur until later, by 5 d and 14 d after wounding, and then declined. There was no discernable genotypic difference in the wound-induced expression profiles for either of these pectin methyl esterase related genes.

Discussion

A wide range of genes are induced after tuber excoriation and other wounding injuries. These genes encode proteins that play key roles in closing layer and wound periderm development. Closing layer formation followed by generation of the wound periderm provides the necessary barrier to resist pathogen invasion and water loss, thereby maintaining tuber quality and reducing loss of fresh weight. The genes examined in this research encode proteins that are either targeted to cell wall polymers, including cell wall proteins and pectins, or are part of meristematic activity involved in the cell cycle/divisional process of wound-healing. An understanding of how the induction of these genes coordinates with the biological processes of wound healing could be of benefit to agriculture and consumers in the development of approaches to address costly wound related losses and quality deterioration. In this study, histochemical techniques and qRT-PCR analysis were used to provide a better understanding of the induction profile

of genes that appear to play leading roles in wound-induced cell wall biosynthesis and associated cell division during closing layer and wound periderm development and maturation.

Wounding induces an avalanche of cellular responses. Perhaps the most important wound-induced process is development of the suberized closing layer followed by formation of a wound periderm (Lulai, 2007a). SPP constitute the first suberin biopolymer to accumulate on cell walls during wound-healing, and are essential for resistance to bacterial pathogens (Lulai and Corsini, 1998). SPP accumulation was monitored throughout the time course to assess closing layer development and to quantify phellem cell production as an index of cell division. Closing layer development was complete by 5 d after wounding for both genotypes, although RB was slightly more advanced (Fig. 3 and Fig. 4). The appearance of a nascent phellogen by 5 d after wounding gave indication that phellem development was about to occur (Fig. 5 and Fig. 6). Phellem accumulation occurred most rapidly from 5 d through 14 d after wounding, indicating a period of rapid cell division. ND, a red, slower maturing potato genotype, had accumulated 2.5 to 3 phellem cell layers compared to 5.5 to 6.5 phellem cell layers at 14 d for RB (Fig 3). This difference in the number of phellem cell layers is indicative of differences in wound-healing. RB SPP accumulation leveled off at 28 d after reaching a total of 8.5 phellem cell layers, which were 3.5 more cell layers compared to ND (Fig. 3). We suggest that more layers of phellem cell accumulation equates to a more robust phellem in mature periderm, but this does not directly relate to resistance to excoriation type of re-injury which is

determined by phellogen radial cell wall strength/thickness. By 28 d after wounding, the number of phellem cell layers was no longer increasing, indicating cessation of cell division and inactivation of the phellogen. These results indicate that the phellogen radial cell wall thickness reached before the average inflection point at 28 d was linked to wound periderm development and could be considered immature; thickening obtained past the inflection point at 28 d, i.e. after cessation of cell division, would then be part of periderm maturation because wound periderm development had ceased (Fig. 3, Fig. 5 and Fig. 6).

Phellogen radial cell wall thickness was measured throughout the time course to determine its relationship with wound periderm development and maturation. Radial cell walls were histologically visible by 5 d after wounding (Fig. 6). Lulai and Freeman (2001) showed that native periderm excoriation occurred as a result of the fracture of fragile phellogen radial cell walls and separation of the phellem from the tuber. Also, Sabba and Lulai (2002) showed that the phellogen cell walls thicken during native periderm maturation. All of these data, including verification by light and electron microscopy, show that the fracture occurs at the phellogen layer and that this layer is responsible for excoriation related wound injuries.

As illustrated in Fig 6, an active phellogen, present in immature tuber periderm, has relatively thin cell walls that are easily fractured. After wound-induced formation of the phellogen and to ca. 28 d after wounding, the phellogen layer was active; this suggests the wound phellem is susceptible to skinning type injuries. As the wound periderm formed, the radial cell walls of the phellogen

thickened (Fig. 5 and Fig. 6). After the phellogen became non-meristematic at around 28 d after wounding, the phellogen cell walls continued to thicken suggesting increased strength and resistance to fracture.

After wounding and during closing layer development, a phellogen layer is formed wherein the cell cycle and accompanying cell division occurs to form wound periderm cells. The expression profiles of candidate genes, selected to assess cell cycle activity, were monitored throughout the wound-healing time course. Horvath et al. (2006) discovered that *StEBP1* promotes cell proliferation and is required for the expression of *CDKB1:1* in developing tubers. Our results show that *StEBP* is wound-inducible and most highly expressed at 1 d (Fig. 7A), while the trend for maximum *StCDKB* and *StCKS1At* expression occurred after 5 d (Fig. 7B and 7C). These results are consistent with those of Horvath et al. (2006) who showed that *EBP1* is a dose dependent regulator of certain cell cycle genes. Although *StEBP* was up-regulated, it was not as highly induced as *StCDKB* and *StCKS1At*, suggesting that the putative regulatory role of EBP1 in tuber wound-healing involves a sensitive dose response. *StCDKB* was highly expressed after wounding and during the period of most rapid cell division up through 14 d after wounding and was significantly down-regulated upon cessation of meristematic generation of phellem cells by 28 d after wounding (Fig. 7B). The wound-induced expression profile for *StCDKB* is consistent with established precepts in developing plant tissues and organs, where CDKB transcription patterns and kinase activity reach a maximum during mitosis and are down-regulated upon the onset of endoduplication (Inze and Veylder 2006). The profile of *StCDKB* up-

regulation is consistent with a role in wound-induced cell division during wound-healing and its expression may serve as an indicator of induction and cessation of wound-induced cell division. Richard et al. (2001) showed that the CDK regulatory subunit *CKS1At* was at peak transcript level when cell division was near maximum. Like *StCDKB*, *StCKS1At* was rapidly up-regulated after wounding and its expression remained high until 28 d after wounding (Fig. 7B and 7C), the approximate time range for cessation of phellem cell accumulation and associated cell division. Although the wound-induced up-regulation of *StCKS1At* expression was not as great as that of *StCDKB*, down-regulation of both genes (Fig. 7B and 7C) mirrored the indices for cell division (Fig. 3); also suggesting a role in the formation of the wound periderm. Our results show that upon wounding, these two cell cycle genes are coordinately up- and then down-regulated in concert with phellem cell production, linking them to initiation and cessation of wound periderm formation.

Extensin biopolymers are structural hydroxyproline-rich glycoproteins that undergo self-assembly forming cell wall scaffolding that is strengthened by cross-linking (Cannon et al., 2008; Lamport et al., 2011). The extensin gene *StExt1* was rapidly up-regulated by 1 d after wounding followed by a steady decrease throughout the time course returning to basal levels (Fig. 8A and B). The rapid induction of *StExt1* by 1 d after wounding suggests a role in primary cell wall modification occurring as the primary cell walls at the wound surface become impregnated with SPP; these results indicate that the closing layer suberization processes are acting in concert with expression of *StExt1* (Fig. 3, Fig. 4A and 4C,

and Fig. 8A and 8B)). A nascent phellogen is detectable histologically by 5 d after wounding (Fig. 6). Importantly, our results suggest that extensin is involved in closing layer formation at a time when there is no or little primary wall formation. The continued up-regulation of *StExt1* at 5 d and later suggests that they are also involved in wound periderm formation. These results are consistent with those of Ahn et al. (1998) who showed that extensin transcripts were induced within 1 d of wounding in soybean roots. Present observations provide evidence that in potato, extensin is needed soon after wounding, putatively to facilitate cell-wall scaffold formation as part of self-assembly and subsequent cross-linking as suberization of the closing layer and wound-periderm development progress.

Related extensin-like gene transcripts were most abundant by 5 d after wounding (Fig. 8C), suggesting that these transcripts are also required for cell wall strengthening, but at a slightly later time during modification of closing layer cells and construction of phellem cell walls. Recently, Finkers-Tomczak et al. (2011) demonstrated that there are several copies of genes encoding extensin-like proteins at the *H1* locus in potato, which is known for the resistance to potato cyst nematode infections. Also, extensin-like genes were up-regulated by infection and wounds, and may have a role in strengthening the cell wall for defense (Finkers-Tomczak et al., 2011).

TLRPs are cell wall proteins that contain nine tyrosine residues, which account for 14% of the total amino acid residues of the protein; this is a major characteristic of structural cell wall proteins (Domingo et al., 1994). TLRPs are localized in cell walls during and after lignification, indicating a role in secondary

cell wall formation. Our results show that *StTLRP* expression was rapidly up-regulated by 5 d after wounding and remained highly expressed through 56 d (Fig. 8D). *StTLRP* participation in formation and completion of secondary cell wall construction may explain the high levels of expression through 56 d after wounding. Paralleling the evidence of Domingo et al., (1994 and 1999) for TLRP involvement with lignification, TLRP in tubers may be associated with the wound-induced deposition of lignin-like SPP in cell walls particularly during phellem cell development. This is an important point because in wound-healing tuber secondary cell walls do not contain true lignin, but instead are impregnated with SPP which have biochemical and structural similarities to lignin (Bernards, 2002; Lulai, 2007a). Tyrosine may also participate in the cross-linking of proteins to cell walls and may be linked to aromatic side chains of lignin or the lignin-like SPP material. Proteins with high tyrosine content could be used as potential nucleation sites aiding the deposition of lignin or lignin-like SPP material during secondary cell wall formation (Domingo et al., 1999). Importantly, the elevated *StTLRP* expression after 28 d suggests a role for this gene in phellogen cell wall thickening and strengthening after cessation of meristematic activity. This hypothesis is consistent with the results of Ross et al. (2011) and Kloosterman et al. (2010) who showed that *StTLRP* was more highly expressed in firm tuber tissues.

Esterified pectins are produced in the golgi and then transported to the cell wall where some de-esterification takes place by PME enzymes (Micheli, 2001). Negatively charged pectin reacts with the extensin-based cell wall scaffolding to provide a structural complex that becomes a template for further deposition of cell

wall materials (Cannon et al., 2008). Unesterified pectin produced by PME, could increase the rigidity of cell walls via calcium bridges (Thakur et al., 1997). Unesterified pectin content increased in the phellogen cell walls during native periderm maturation (Sabba and Lulai, 2005). Epitopes of monoclonal antibodies of relatively unesterified (JIM5) and esterified (JIM7) pectin were found to sparsely label wound periderm in potato tubers (Sabba and Lulai, 2004). The epitope for these antibodies may have been blocked or not present, thereby explaining the disparity between the weak antibody labeling reported by Sabba and Lulai (2004) and the increased expression of *StPME* from 1 d to 5 d (Fig. 9A) and *StPrePME* between 5 d to 14 d (Fig. 9B) in this study. The wound-induced expression of these PME genes provides evidence that PME activity could be present in the phellogen cell layer and be available for de-esterification and ultimately formation of calcium bridging and pectate gel formation. *StPME* was up-regulated by 1 d and through 14 d suggesting a role in closing layer and wound periderm development. The later up-regulation of *StPrePME* suggests that its role in de-esterification of esterified pectins is further along in the wound-heal time course coinciding with wound periderm development. Both PME genes were down-regulated after cessation of phellogen meristematic activity and the generation of phellem derivatives.

In conclusion, these cell wall and cell cycle genes were up-regulated during the wound-healing time course for both harvests and genotypes. Although no clear genotypic difference in the pattern of expression for these genes was observed, the biological variation inherent with different harvests did influence the

amplitude of wound induced expression. Several of the genes were highly expressed by 1 d after wounding, this coordinately suggested involvement with closing layer formation and formation of the phellogen cell layer. After cessation of phellogen meristematic activity, the expression of *StEBP, StCDKB, StCKS1At, StExt1, StExtIk, StPME and StPrePME* declined, while *StTLRP* remained up-regulated. These results suggest that the up-regulation of cell wall genes and cell cycle genes is coordinated with closing layer development, phellogen formation and phellem cell development, while down regulation of these genes coordinated with cessation of wound phellem cell development as an indicator of meristematic inactivation of mother phellogen cells. Our results also showed that once phellem cell generation had ceased and expression of most of the genes monitored returned to basal levels, the phellogen radial cell walls continued to thicken. This research provides insight into the coordinate induction of these genes in marked relationships to the biological responses of wound healing including closing layer formation, phellogen formation and phellem generation during wound periderm formation. Importantly, the continued up-regulation of TLRP after cessation of meristematic activity suggest this gene merits future research as a determinate for phellogen cell wall thickening and resistance to re-injury by phellem excoriation.

References

Ahn JH, Choi Y, Kim SG, Kwon YM, Choi YD, Lee JS. Expression of a soybean hydroxyproline-rich glycoprotein gene is correlated with maturation of roots. Plant Physiol 1998;116:671–79.

Bernards MA. Demystifying suberin. Can J Bot 2002;81:227-40.

Bown DP, Bolwell GP, Gatehouse JA. Characterization of potato *(Solanum tuberosum* L.) extensins: a novel extensin-like cDNA from dormant tubers. Gene 1993;134:229-33.

Brook RC. Bruising leaves mark on potato industry. Brook RC, editor. Potato Bruising. How and Why Emphasizing Black Spot Bruise. Running Water Publishing, Haslett, Michigan,1996. p.3-7.

Cannon MC, Terneus K, Hall Q, Tan L, Wang Y, Wegenhart B. Self-assembly of the plant cell wall requires an extensin scaffold. PNAS 2008;105:2226-31.

Deepak S, Shailasree S, Kini RK, Hause B, Shetty SH, Mithofer A. Role of hydroxyproline-rich glycoproteins in resistance of pearl millet against downy mildew pathogen *Sclerospora graminicola*. Planta 2007;226:323-33.

Dey PM, Brownleader MD, Pantelides AT, Trevan M, Smith JJ, Saddler G. Extensin from suspension-cultured potato cells: a hydroxyproline-rich glycoprotein devoid of agglutinin activity. Planta 1997;202:179-87.

Destefano-Beltran, L, Knauber D, Huckle L, Suttle J. Chemically forced dormancy termination mimics natural dormancy progression in potato tuber meristems by reducing ABA content and modifying expression of genes involved in regulating ABA synthesis and metabolism. J Exp Bot 2006;57:2879–86.

Domingo C, Gomez MD, Caíias L, Hernandez-Yago J, Conejero V, Vera P. A novel extracellular matrix protein from tomato associated with lignified secondary cell walls. Plant Cell 1994;6:1035-47.

Domingo C, Sauri A, Mansilla E, Conejero V, Vera P. Identification of a novel peptide motif that mediates cross-linking of proteins to cell walls. Plant J 1999;20:563-70.

Dubreucq B, Berger N, Vincent E, Boisson M, Pelletier G, Caboche M, et al. The *Arabidopsis AtEPR1* extensin-like gene is specifically expressed in endosperm during seed germination. Plant J 2000;23:643-52.

Evans IM, Gatehouse LN, Gatehouse JA, Yarwood JN, Boulter D, Croy RRD. The extensin gene family in oilseed rape (*Brassica napus* L.): characterization of sequences of representative members of the family. Mol Genet 1990;223:273-87.

Finkers-Tomczak A, Bakker E, Boer J, Van der Vossen E, Achenbachen U, Golas T, et al. Comparative sequence analysis of the potato cyst nematode resistance locus *H1* reveals a major lack of co-linearity between three haplotypes in potato (*Solanum tuberosum* ssp.). Theor Appl Genet 2011;122:595-08.

Ginzberg I, Barel G, Ophir R, Tzin E, Tanami Z, Muddarangappa T, de Jong W, Fogelman E. Transcriptomic profiling of heat-stress responses in potato periderm. J Exp Bot 2009;60:4411-21.

Hirsinger C, Parmentier Y, Durr A, Fleck J, Jamet E. Characterization of a tobacco extensin gene and regulation of its gene family in healthy plants and under various stress conditions. Plant Mol Biol 1997;33:279-89.

Horvath BM, Magyar Z, Zhang Y, Hamburger AW, Bako L, Visser RG, Bachem CW, Bogre L. EBP1 regulates organ size through cell growth and proliferation in plants. EMBO J 2006;25:4909–20.

Inze D, Veylder LD. Cell cycle regulation in plant development. Ann Rev Genet 2006;40:77-105.

Kloosterman B, Oortwijn M, uitdeWilligen J, America T, de Vos R. Visser RGF, et al. From QTL to candidate gene: genetical genomics of simple and complex traits in potato using a pooling strategy. BMC Genomics 2010;11:158-73.

Lamport DTA, Kieliszewski MJ, Chen Y, Cannon MC. Update on the extensin superfamily. Role of the extensin superfamily in primary cell wall architecture. Plant Physiol 2011;156:11-19.

Lulai EC. Skin-set, wound healing, and related defects. *In* Vreugdenhil D. editor. Potato Biology and Biotechnology: Advances and Perspectives. Amsterdam. The Netherlands: Elsevier Limited: 2007a. p. 471-96.

Lulai EC. The Canon of Potato Science: Skin-set and wound-healing/suberization. Potato Res 2007b;50:387-90.

Lulai EC, Corsini DL. Differential deposition of suberin phenolic and aliphatic domains and their roles in resistance to infection during potato tuber (*Solanum tuberosum L.*) wound-healing. Physiol Mol Plant Path 1998;53:209-22.

Lulai EC, Freeman TP. The importance of phellogen cells and their structural characteristics in susceptibility and resistance to excoriation in immature

and mature potato tuber (*Solanum tuberosum* L.) periderm. Ann Bot 2001;88:555-61.

Lulai EC, Orr PH. Determining the feasibility of measuring genotypic differences in skin-set. Am Potato J 1993;70:599-09.

Lulai EC, Suttle JC, Pederson SM. Regulatory involvement of abscisic acid in potato tuber wound-healing. J Exp Bot 2008:59;1175-86.

Micheli F. Pectin methylesterases: cell wall enzymes with important roles in plant physiology. TRENDs in Plant Science 2001;6:414-18.

Nicot N, Hausman JF, Hoffmann L, Evers D. Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. J Exp Bot 2005;56:2907-14.

Narvaez-Vasquez, J. and C.A. Ryan, Jr. 2002. The systemin precursor gene regulates both defensive and developmental genes in *Solanum tuberosum*. Proc Natl Acad Sci USA 2002;99:15818-21.

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:2002-7.

Richard C, Granier C, Inze D, Veylder LD. Analysis of cell division parameters and cell cycle gene expression during the cultivation of *Arabidopsis thaliana* cell suspensions. J Exp Bot 2001;52:1625-33.

Ross HA, Wright KM, McDougall GJ, Roberts AG, Chapman SN, Morris WL. Potato tuber pectin structure is influenced by pectin methyl esterase activity and impacts on cooked potato texture. J Exp Bot 2011;62:371-81.

Sabba RP, Lulai EC. Histological analysis of the maturation of native and wound periderm in potato (*Solanum tuberosum* L.) tuber. Ann of Bot 2002;90:1-10.

Sabba RP, Lulai EC. Immunocytological comparison of native and wound periderm maturation in potato tuber. Am Potato J 2004;81:119-24.

Sabba RP, Lulai EC. Immunocytological analysis of potato tuber periderm and changes in pectin and extensin epitopes associated with periderm maturation. J Hort Sci 2005;130:936-42.

Showalter AM. Structure and function of plant cell wall proteins. Plant Cell 1993;5:9-23.

Siedlecka A, Wiklund S, Péronne M-A, Micheli F, Leśniewska J, Sethson I, et al. Pectin methyl esterase inhibits intrusive and symplastic cell growth in developing wood cells of *Populus*. Plant Physiol 2008;146:554-65.

Soler M, Serra O, Fluch S, Molinas M, Figueras M. A potato skin SSH library yields new candidate genes for suberin biosynthesis and periderm formation. Planta 2011;233:933-45.

Thakur BR, Rakesh KS, Avtar KH. Chemistry and uses of pectin-A review. Critical Rev Food Sci Nutr 1997;37:47-73.

Zhiponova M, Pettkkó-Szandtner A, Stelkovics É, Neer Z, Bottk S, Krenács T, et al. Mitosis-specific promoter of alfalfa cyclin-dependent kinase gene (*Medsa;CDKB2;1)* is activated by wounding and ethylene in a non-cell division-dependent manner. Plant Physiol 2006;140:693-03.

PAPER 2. THE USE OF MOLECULAR TECHNIQUES TO MONITOR THE CHANGES OCCURING IN THE PROCESS OF NATIVE PERIDERM MATURATION IN POTATO (*Solanum tuberosum* L.) TUBERS

Abstract

Potato (*Solanum tuberosum* L.) tubers interact with many different types of abiotic and biotic stresses throughout their life cycle. A key defense mechanism for potato tubers is the robust skin. Many biological processes are involved in the formation of this stout tissue; however, little is known about induction of genes that may be associated with this process. In this study, native periderm maturation events along with native periderm maturation expression profiles of selected cell cycle, cell wall protein, and pectin methyl esterase genes were determined in two diverse potato genotypes (NDTX4271-5R (ND) and Russet Burbank (RB) tubers; 2008 and 2009 crops at four harvest maturities), to assess their involvement in the process of native periderm maturation. After the second harvest, the generation of phellem cell layers had plateaued indicating cessation of cell division by the phellogen. Phellogen radial cell walls continued to thicken throughout all of the harvests for both genotypes and crops. In the ND genotype, no significant changes in expression were detected for cell cycle genes encoding epidermal growth factor binding protein (*StEBP*), cyclin-dependent kinase B (*StCDKB*) and cyclin-dependent kinase regulatory subunit (*StCKS1At)* by the second harvest. These cell cycle genes had similar expression patterns for RB, except for *StCDKB* whereby it was down-regulated after the first harvest. Genes encoding the structural cell wall proteins extensin (*StExt1*) for ND and extensin-like (*StExtlk)* for

RB and ND remained up-regulated by the second harvest, suggesting involvement with phellem cell layer formation. The coordinate induction and expression profile of *StTLRP,* a gene encoding a cell wall strengthening "tyrosine-and lysine-rich protein," suggested a role in the phellem cell generation and cell wall thickening in non-meristematically active phellogen cells in ND. Unlike RB were *StTLRP* expression was around initial levels by the second harvest and was down-regulated thereafter. *StPrePME* expression began to down-regulate by the second harvest for ND and by the third harvest for RB, indicating possible reduction in its possible role in the phellogen cell layer compared to *StPME.* Collectively, these results suggest that the expressions of cell wall genes and cell cycle genes are coordinated with phellem cell development and the on-set of native periderm maturation.

Introduction

The native periderm of potato (Solanum *tuberosum* L.) tubers undergo many biological processes to protect the tuber from abiotic and biotic stresses. Although some of these processes have been studied, little research has been directed towards the molecular and regulatory mechanisms involved in the formation, development, and maturation of the native periderm. Research that determines genes involved in these processes could be used to decrease financial losses due to tuber periderm injury during harvest, handling, and storage operations. Consequently, the biological processes in native periderm maturation are agriculturally and economically important.

Tuber periderm formation begins by divisions of both the epidermal and subepidermal cells (Artschwager, 1924; Reeve et al., 1969; Barel and Ginzberg, 2008). After the phellogen layer is formed and becomes meristematically active, it produces numerous protective layers of phellem cells, i.e. skin, by outwardly dividing and inwardly dividing to produce the phelloderm. These three cell layers, i.e. phellem, phellogen, and phelloderm, make up the tuber periderm, which is essential for the survival of the tuber. Prior to periderm maturation, the "immature" periderm is susceptible to skinning injury, whereby the protective layer of skin is excoriated. Upon periderm maturation, the periderm becomes resistant to excoriation. In addition, the tuber periderm is enriched with plant defense components, further illustrating the importance of the process of periderm maturation (Barel and Ginzberg, 2008).

Few techniques have been developed to determine resistance to skinning; none have been established as a standard. The most common experimental technique of measuring skin-set involves the tangential or torsional force required to mechanically shear the phellem (skin) from the tuber (Lulai, 2002). By using this technique to assess skin-set development, physiological factors associated with resistance to skinning injury can be addressed.

The suberin barrier is essential for resistance to invasion by fungi and bacteria that are living amongst the tubers, the walls of phellem cells are impregnated and laminated with the suberin biopolymers to provide resistance to intruders (Lulai, 2007). Suberin poly(phenolics) (SPP) are the first suberin biopolymers to accumulate on the walls of these cells followed by suberin

poly(aliphatics) and glycerol (Lulai and Corsini, 1998; Bernards, 2002; Lulai, 2007). The presence of SPP in tuber periderm can be detected by autofluorescence microscopy and has been used to monitor the number of phellem cell layers of immature vs. mature wound periderm (Neubauer et al. 2012). Within the native periderm, (i.e. phellem, phellogen and phelloderm), the layers of phellem are the only cells that accumulate SPP. After these distinct cells are in place and an adequate number of phellem cell layers have been produced, the phellogen cell layer becomes non-meristematic and no longer generates phellem cell derivatives; at this point native periderm maturation may ensue (Lulai and Freeman, 2001; Lulai, 2007). During this process, the phellem (skin) becomes more tightly attached to the tuber and more resistant to excoriation. Importantly, it was determined that immature phellogen cells had thin walls and fractured easily. In contrast the phellogen cell walls that are strengthened and thickened considerably in tubers are fully resistant to skinning (Lulai and Freeman, 2001). These results illustrate how important the phellogen layer is in the protection of the potato tuber and how it is an agriculturally important part of periderm maturation.

The cell cycle must be active during the process of native periderm formation, as phellogen cells divide, possibly from cell to cell signaling, produce phellem and phelloderm cells as their progeny (Barel and Ginzberg, 2008). Horvath et al. (2006) identified a plant growth regulatory gene, *StEBP1,* which is expressed in developing organs. *StEBP1* promotes cell proliferation and is required for the expression of *CDKB1:1*, a form of cyclin-dependent kinase B (*CDKB*). Boudolf et al. (2004) stated that *CDKB* was highly expressed in the

actively dividing cells of *Arabidopsis*. Also a *CDK* regulatory subunit (*CKS1At*) was shown to be involved with cell division control (De Veylder et al., 2001). Collectively, these studies suggest that monitoring certain aspects of the cell cycle in native periderm development and maturation could provide insight by marking key events during these crucial processes.

After the phellogen layer becomes meristematically inactive, certain cell wall proteins and pectins likely play key roles in the resistance to fracture (Sabba and Lulai, 2002, 2004, 2005). Extensins are a family of hydroxyproline-rich glycoproteins (HRGPs) that are a highly abundant component in cell walls of dicots. In dicots, these proteins are characterized by having a high content of hydroxyproline and serine, with some combination of valine, tyrosine, lysine and histidine (Showalter, 1993). After extensin is secreted into the cell wall, it is rapidly insolubilized by formation of intra- and intermolecular cross-linkages such as isodityrosine (IDT) bridges. This phenomenon can occur quickly by an oxidizing reaction involving peroxidase and hydrogen peroxide (Deepak et al., 2007). Additionally, cross-linking provides added resistance to cell wall degrading enzymes that are secreted by pathogens (Deepak et al., 2010). Another structural component, pectin methyl esterase (PME), produces unesterified pectin which could be responsible for increase rigidity of cell walls via calcium bridges (Sabba and Lulai, 2005). Pectins also combine with hemicelluloses to constitute the matrix in which cellulose microfibrils are embedded. This integrated combination creates the rigid yet dynamic and pliable properties of the cell wall (Harholt et al., 2010). These studies indicate that extensin and PME have major roles in the development

of a robust cell wall and could be involved in the process of native periderm maturation.

Dubreucq et al. (2000) found an extensin-like protein in *Arabidopsis thaliana* AtEPR1. *AtEPR1* was expressed during seed germination and has been hypothesized to be involved in morphogenic control of cell-wall architecture during the process of cellular differentiation (Dubreucq et al., 2000). Bucher et al. (2002) suggested that LeExt1, an extensin-like protein in tomato, had direct correlation with cellular tip growth and is expressed at later stages of epidermis development. Domingo et al. (1994) determined that TLRP, another structural cell wall protein, appears to participate in cross-linking of proteins to cell walls and is involved in the final assembly and architecture of lignified secondary cell walls. Also, these proteins are thought to make other proteins insoluble by crosslinking to cell walls (Domingo et al., 1999). Kloosterman et al. (2010) described a TLRP gene that appears to be involved in firmness of potato tubers. Evidence from these studies suggest that expression of extensin and TLRP genes could mark, or be involved in, important steps of native periderm maturation.

As new technologies are developed, potato breeding programs may incorporate these scientific tools to increase genetic gain. Identification of biochemical/molecular markers to gauge the progress of native periderm maturation is crucial for cultivar improvement and the success of the potato industry. The goals of this research were 1. to determine if the gene expression results from Neubauer et al. (2012) involving wound periderm are similar to the gene expression profiles during the process of native periderm maturation, and 2.

to determine if these expression profiles could also be coordinate indicators or molecular markers for monitoring the progress of native periderm maturation.

Materials and Methods

Native model system plant material

Certified seed minitubers (*Solanum tuberosum* L., genotypes Russet Burbank (RB) and NDTX4271-5R (ND), Valley Tissue Culture, Halstad, MN) were planted and grown (2008 and 2009 crops) under controlled conditions in a plant growth chamber. Growth chamber conditions were initially set for plant growth (14 h light (day) at 21 °C and 10 h dark (night) at 19 °C) for the first 90 days. After 90 days, the growth chamber conditions were re-set to further encourage tuber growth (10 h light (day) at 20 °C and 14 h dark (night) at 18 °C). Relative humidity was set at 90% for the entire experiment. The tubers were harvested at the time point of 90 to 100 d after planting to provide the first/early harvest (green vines and immature periderm), and every 2 w thereafter to provide the second and third intermediate harvests (tubers varying in intermediate levels of maturity) and a fourth/late harvest (senesced vines and mature periderm). This labeling strategy to distinguish harvests and associated maturity will be condensed in the text to first, second, third and fourth harvest. Tuber sizes ranged from three to five cm in diameter. Vine maturity was determined at each harvest as described below. After harvesting, the tubers were immediately measured for resistance to excoriation, i.e. skinning injury/skin-set, (Lulai and Orr, 1993) and sampled for total RNA (Narvaez-Vasquez and Ryan, 2002).

Vine maturity index

Potato vine maturity was determined on all harvested tubers using an index that quantified vine senescence (Dennis Corsini, personal communication and Corsini et al., 1999). Vine maturity was measured on all harvested plants. The vine maturity index range was from 0 (no sign of senescence) to 9 (greater than 90% senescence) in increments of zero to ten. The index was used as a reference for assessing the physiological age/maturity of the plants. This guide aided in determining harvest intervals for the native periderm maturation process.

Skin-set measurements

Directly following harvest of the growth chamber grown tubers, the resistance to tuber skinning injury/excoriation, i.e. skin-set, was determined as outlined by Lulai and Orr (1993). A Snap-on "Torqometer" (registered trade mark name), model TQSO50FUA (0-96oz in/0-678 mNm range), attached with a Halderson skin-set testing device, as modified by Lulai and Orr (1993) (Mayo Manufacturing, East Grand Forks, MN), was used to determine resistance to excoriation (Lulai and Orr, 1993). The skin-set measurements were obtained by the maximum torque reading reached in the excoriation of the skin. This reading was recorded as a skin-set value (1 in oz. = 7.061 mNm) to determine the maturity of the native periderm. If the periderm had matured and the phellem/skin could not be delaminated by the device, the tuber was considered to be fully set (FS) and beyond measurement.

Histological determination of phellem cell development and phellogen radial cell wall thickness

The production of periderm phellem cell layers was determined to provide a measurable index of the meristematic status of the phellogen and periderm development. Cessation of phellem cell production was used to indicate termination of cell division within the native periderm and approximate timing for ensuing periderm maturation. The number of phellem cell layers was determined using autofluorescence microscopy as outlined by (Lulai et al., 2008). Tissue blocks (~ 2 mm x 1 mm x 0.3 mm) were excised from the tubers directly upon harvest from the growth chamber at each of the indicated harvest periods and then placed into Farmer's Fixative (3:1 v/v, absolute ethanol: glacial acetic acid). Tissue blocks were sectioned (200 µm thick) with a Vibratome 1000 Plus Tissue Section System (The Vibratome Company, St. Louis, MO, USA). Autofluorescence of suberin polyphenolics located within the phellem cell walls was used to visualize and count the number of periclinal layers of phellem cells within the periderm. The number of layers of phellem cells was determined from three tissue sections per block, using two blocks from two different tubers per harvest; three determinations were made per section following the microscopy methods described by Lulai and Corisini (1998) as modified by Lulai et al. (2008). A Zeiss Axioskop 50 microscope equipped with the conventional Zeiss white light source, an EXFO X-Cite 120 PC illumination unit as a UV source for epifluorescent illumination and a Zeiss color AxioCam camera was used to detect, determine and capture digital images of layers of phellem cells (Sabba and Lulai, 2002; Lulai and

Corisini 1998). The number of layers of phellem cells is presented as the mean ± standard error (n=3).

Increases in phellogen radial cell wall thickness accompanying cessation of meristematic activity within the periderm were also used as an index of native periderm maturation. Microscopy methodology, similar to that described above, was used to determine phellogen radial cell wall thickness. Once again a Zeiss Axioskop 50 microscope was used to visualize and identify the phellogen cell layer. Following identification, digital images of the phellogen radial cell walls were captured with a Zeiss color AxioCam camera (Carl Zeiss Inc., Thornwood, NY, USA). The thickness of the phellogen radial cell walls was measured electronically via a stage micrometer that was embedded into the Zeiss AxioVision software for the AxioCam camera. Average thickness data are presented as the mean ± standard error (n=3).

RNA extraction

Total RNA was extracted from cellular exudates collected from tubers after harvest. Four to six tubers were sampled at each interval (first/early harvest (immature), second and third/intermediate harvest, fourth/late harvest (mature)). Cellular exudate was collected from the tubers directly after harvest by peeling back the phellem from the tuber and then using a spatula to skim exudate from the exposed phellogen layer on the exposed tuber surface and phellem. Total RNA was extracted from the collected exudate according to the method of Narvaez-Vasquez and Ryan (2002) with modifications (Narvaez-Vasquez, personal communication; Destefano-Beltran et al., 2006) and briefly outlined in the

following. The collected cellular exudate from the harvested tubers was transferred to a 2mL centrifuge tube with 700 µL of 0.1 M Tris-HCL (pH 7.4) containing 1% (w/v) sodium sulfite. Buffer-saturated phenol (700 µL) (Invitrogen, Eugene, OR, USA) was added to the centrifuge tube and the mixture vortexed. The mixture was centrifuged (20,800 x g for 9 minutes at 4 °C). The supernatant 600 µL was re-extracted with an equal volume of acid-phenol/chloroform (5:1, v/v) (Ambion, Austin, TX, USA). The extract was treated with 50 µL of 3 M sodium acetate pH 5.2 and 500 µL of isopropanol and incubated for 10 minutes at room temperature. After 10 min, the RNA was centrifuged (10,600 x g for 10 min at 4 °C), the pellet was washed with 70% ethanol, and the washed RNA centrifuged (6,800 x g for 5 min at 4 °C). RNA was quantified spectrophotometrically using a NanoDrop model ND-1000 Spectrophotometer set for RNA determination (NanoDrop Technologies, Wilmington, DE, USA). The quality of total RNA was assessed by agarose gel electrophoresis (1%) in 1% TAE, stained by ethidium bromide and visualized under UV light and by ratio of absorbance at 280/260 nm.

qRT-PCR analysis

Quantitative RT-PCR was used to assess the level of expression of target genes during the time course of native periderm maturation. Total RNA (2 µg) was initially treated with DNA-Free (Ambion, Austin, TX, USA) to remove genomic DNA. Following the DNA-Free treatment, oligo dT_{18} primers were used to reverse transcribe the total RNA using a RETROscript kit (Ambion) following manufacturer's recommendations. The cDNA was diluted 1:6.5 with molecular grade water (Thermo Fisher, Waltham, MA, USA). The DNA Engine Opticon 2

(BioRad, Hercules, CA, USA) was used to amplify specific regions of targeted genes and for real-time detection of the resulting amplicons. Primers for selected genes were designed based on Potato Gene Index Tentative Census Sequences (Table 2).

The qRT-PCR protocol consisted of: 7 µL cDNA template, 2 µL 10x Hot Start Taq buffer (200 mM Tris-HCl (pH 8.3 at 25 °C), 200 mM KCl, 50mM $(NH_4)_2SO_4$), 1.6 µL 25 mM $MgCl_2$, 1.6 µL 2.5 mM dNTPs, 1 µL DMSO, 1 µL 10x SYBR-green, and 0.2 µL (5 units/µL) Hot start Taq Polymerase (Thermo Fisher, Waltham, MA, USA). Primers were diluted in molecular grade H_20 (Thermo Fisher) to a concentration of 5 µM. The total volume of qRT-PCR reaction was 20 µL. The qRT-PCR reaction parameters were: 95 °C 4 minutes, 56 °C 1 minute, 72 °C 1 minute, followed by 94 °C 30 seconds, 56 °C 30 seconds, 72 °C 45 seconds, 75 °C 3 seconds, plate read, and the absorbance determined with each of the 44 cycles and a melting curve determined at 65°C to 90°C, and absorbance determined every 1°C.

The qRT-PCR product for each target gene was separated on a 1% agarose gel, stained with ethidium bromide to verify amplification of a single product, followed by isolation from the agarose gel for sequencing (Northwoods DNA, Solway, MN). The sequence was compared (BLAST) with those of known genes in the Potato Gene Index to verify identity of the amplicon. These efficiencies curves were established for each gene, all qRT-PCR reactions were conducted within the ranges of linearity established by the efficiency curves.

Table 2. Primers Used for qRT-PCR Expression of Target Genes Involved in Native Periderm Maturation.

Gene/Accession#	Orientation	Sequences (5' – 3')
StExt, TC[b]	Forward	CGAAGTTCTTTTGTAGCTTC
	Reverse	GCAAACATACACAAAGACAT
StExt1, TC203100	Forward	GATCCACCACCCAAGAGGCC
	Reverse	GTGGTGGGGACTTGTAAATG
StExtlk, TC197382	Forward	GCTCACAATATGGCAACTAT
	Reverse	CGACCACGTCCACGTCCACA
StCDKB, TC201185	Forward	GTGACTGGCATGTTTATCCA
	Reverse	GCTGTCAAAGTATGGATGTT
StEBP, TC197074	Forward	GGTAGAAGAGGCATCTCAAG
	Reverse	GCTCAACGTGCCGAATCGAC
StCKS1At, TC209250	Forward	GTGGCGGGCAATTGGAGTT
	Reverse	GCAGAACTTGCTGAGTCTGG
StPME, TC213276	Forward	CAGTCGTCGACGGTGACGC
	Reverse	GTGCTGCCATCTTGCACATT
StPrePME, TC197435	Forward	GCATTCCAGGACACTCTCTA
	Reverse	GAACACAACTGCTGCTGTTAC
StTLRP, TC208255	Forward	CCCCCACATGGTGGATATAA
	Reverse	TCGTCATGTGGAGGGTTGTA
Efla, AB061263[a]	Forward	ATTGGAAACGGATATGCTCCA
	Reverse	TCCTTACCTGAACGCCTGTCA

[a] From Nicot et al. (2005).
[b] *StExt* primers were designed to amplify these homologs: TC203100, TC2224149, TC211436, TC 211015, TC208933, TC203030, and TC197747.

Relative fold changes in expression for each of the indicated genes were calculated based on methods of Pfaffl (2001) using elongation factor 1-α (ef1α) as a reference (Nicot et al., 2005).

Results

Vine maturity and skin-set

Skin-set measurements were obtained for ND and RB tubers using vine maturity index ratings as a guide for harvest intervals. The potato vines for both genotypes ranged from green (early harvest) to fully senesced (late harvest). As for vine maturity, ND 2009 crop never became FS, unlike ND 2008 and RB. The data is presented below in Table 3.

Table 3. Vine Maturity and Skin-set Measurements of NDTX4271-5R and Russet Burbank genotypes (2008 and 2009 crops) Throughout Four Harvests Spaced 2w Apart.
*Vine maturity 1=10% senesced to 9=90% senesced
*FS=Fully Set/Skin-set advanced not measurable

		NDTX		
	2008 Crop		2009 Crop	
Tuber Sampling	Vine Maturity	Skin-set (mNm)	Vine Maturity	Skin-set (mNm)
1st	0.5 ± 0.33	247.1 ± 9.9	0	254.2 ± 12
2nd	4.7 ± 0.88	310.7 ± 12.7	6.7 ± 1.5	296.6 ± 7.061
3rd	9 ± 0	331.9± 7.1	9 ± 0	324.8 ± 4.9
4th	9 ± 0	F.S.	9 ± 0	310.7 ± 3.5

		Russet Burbank		
	2008 Crop		2009 Crop	
Tuber Sampling	Vine Maturity	Skin-set (mNm)	Vine Maturity	Skin-set (mNm)
1st	0	261.3 ± 17	0	283.9 ± 10.6
2nd	1 ± 0	254.2 ± 11.3	9 ± 0	324.8 ± 0/F.S.
3rd	7 ± 0	331.9 ± 22.6	9 ± 0	F.S.
4th	9 ± 0	F.S.	9 ± 0	F.S.

Histological determination of phellem cell layers

The number of native phellem cell layers was distinctly different in the two genotypes for the 2008 and 2009 crops. ND had accumulated 5 phellem cell layers in 2008 and 6 phellem cell layers in 2009 in the first harvest, and then leveled off during second harvest through fourth harvest for both crop years. A total accumulation of 7 phellem cell layers was determined for both crop years of ND (Fig. 10). RB had similar results by the second harvest where the total number of phellem cell layers leveled off by or before the fourth harvest. The 2008 crop of RB had accumulated 5.5 phellem cell layers by the first harvest, and increase to 10 by the second harvest followed by leveling off. The 2008 crop of RB had 11.5 phellem cell layers at the fourth harvest (Fig 10). As for the 2009 crop of RB, 11.5 phellem cell layers were present at the first harvest when the vines were not senescing, followed by a slight increase by the second harvest. Following the second harvest, 13 phellem cell layers occurred at the fourth harvest (Fig. 10). By the fourth harvest, ND had 7 phellem cell layers for both crop years whereas RB had 11 (2008 crop) and 13 phellem cell layers (2009 crop) respectively (Fig. 10). These results showed a total of 7 phellem cell layers for ND, but a total of 11.5-13 phellem cell layers for RB were observed (Fig. 10 and 11). Past the second harvest for both genotypes and crops, there was no significant difference ($p \leq 0.05$) in number of phellem cell layers suggesting a cessation of phellem cell layer development, ensued by native periderm maturation.

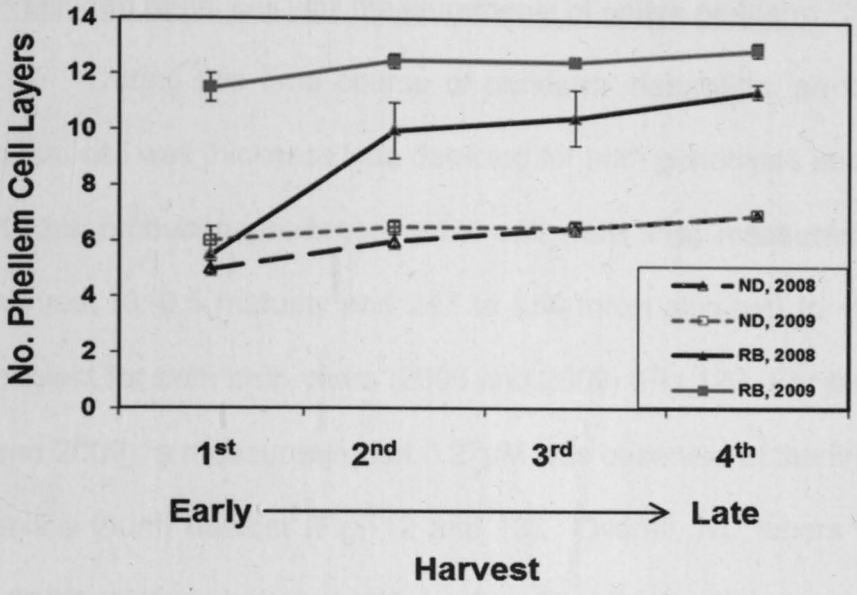

Fig. 10. The accumulation of phellem cell layers during native periderm maturation of tubers from the genotypes NDTX4271-5R and Russet Burbank (2008 and 2009 crops). The number of phellem cell layers was determined on three tissue sections per disc with three ratings per section. Bars represent the standard error of the means at each harvest. There were no significant differences in the number of phellem cell layers after the second harvest based on one-way analysis of variance (ANOVA) $p \leq 0.05$.

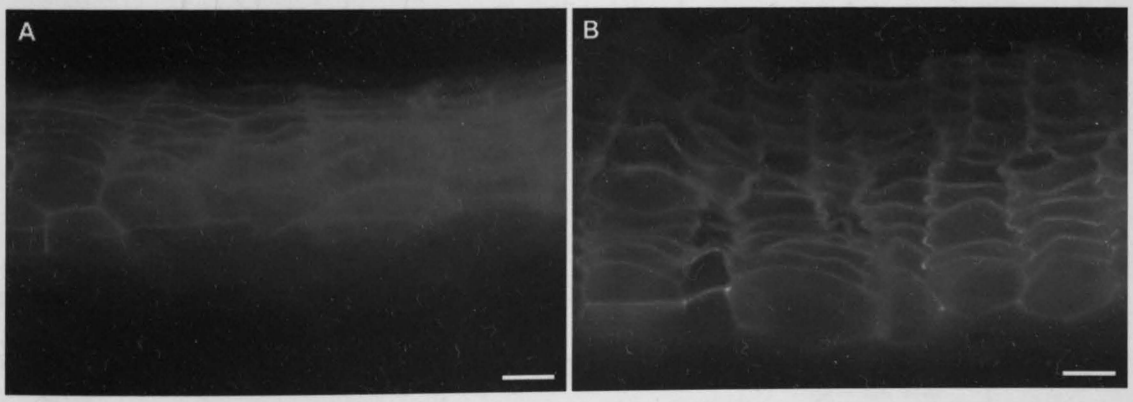

Fig. 11. Determination of phellem cell layer accumulations during the process of native periderm maturation. During this process, the accumulation of layers of phellem cells is distinguishable by their organized files of rectangular cells: (A) Genotype ND (2009 crop) mature native periderm tuber tissue and (B) Genotype RB (2009 crop) mature native periderm tuber tissue. Bar = 5 µm. Phellem cell walls of these tissues were visualized using autofluorescence microscopy as a means of determining phellem cell layer development illustrated in "A" and "B". Note the differences in accumulation of phellem cell layers for genotype "A" versus "B".

Phellogen radial cell wall measurements of native periderm

During this time course of periderm maturation, an increase in phellogen radial cell wall thickness was detected for both genotypes and crops (Fig. 12). ND tubers produced phellogen radial cell walls that measured 0.27µM at the first harvest (0.-0.5 maturity and 247 to 254 mNm skin-set) to ~0.51µM at the fourth harvest for both crop years (2008 and 2009) (Fig 12.) For the tubers of RB (2008 and 2009), a measurement of 0.27µM was observed at the first harvest to~0.57µM at the fourth harvest (Fig. 12 and 13). Overall, ND tubers from both crops had ~110% thicker phellogen radial cell walls at the fourth harvest compared to the first harvest. RB tubers had ~130% thicker phellogen radial cell walls at the fourth harvest compared to the first harvest for both crops. Even though both genotypes had similar radial wall thicknesses at the first harvest and similar trends for radial wall thickening, RB overall had approximately 14% thicker phellogen radial cell walls by the fourth harvest (Fig. 13).

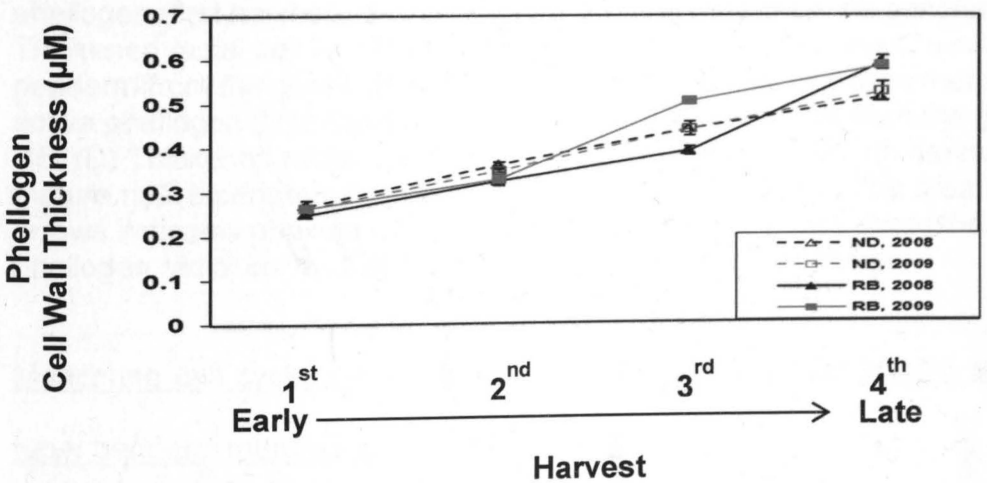

Fig. 12. Changes in phellogen radial cell wall thickness during native periderm development and maturation for the genotypes NDTX4271-5R and Russet Burbank (2008 and 2009 crops). The phellogen radial cell wall thickness was measured via digital microscopy. Each measurement was determined in triplicate. Bars represent the standard error of the means.

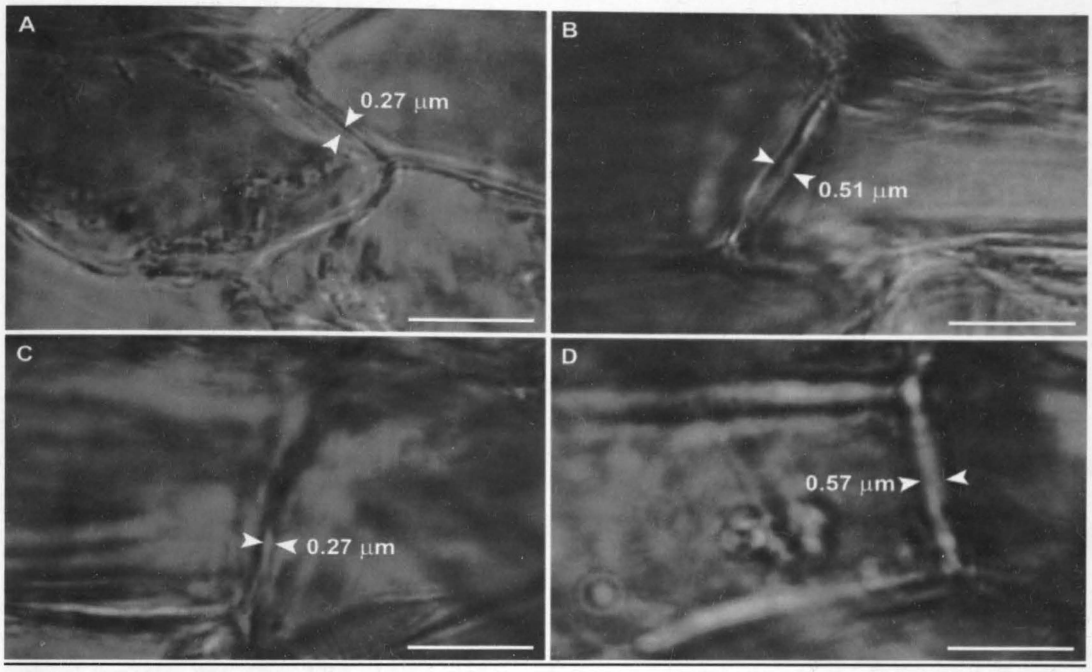

Fig. 13. Light micrographs illustrating differences in radial cell walls of active phellogen (first harvest) and inactive phellogen from mature tuber native periderm (fourth harvest) for the genotypes NDTX4271-5R (2009 crop) and Russet Burbank (2009 crop): (A) Immature thin radial cell wall from meristematically active phellogen (first harvest), an immature native periderm from the genotype ND; (B) Thickened radial cell wall from inactive phellogen (fourth harvest), a mature native periderm from the genotype ND and (C) Thin radial cell wall from meristematically active phellogen (first harvest), an immature native periderm from the genotype RB; (D) Thickened radial cell wall from inactive phellogen (fourth harvest), a mature native periderm from the genotype RB. Bar = 5 µm. The area between the arrows indicates phellogen radial cell wall thicknesses in µm. Note the thicker phellogen radial cell wall at the fourth harvest for both genotypes.

Monitoring cell cycle, cell wall protein, and cell wall pectin related genes during tuber periderm maturation

Changes in expression were evident in tubers of both potato genotypes after early harvest (first harvest, immature periderm) through the fourth harvest with mature periderm (Fig. 14, 15, and 16). Although all of these genes were

differentially expressed during periderm maturation, there was no consistent discernable difference in gene expression profiles between these two diverse potato genotypes. However, the 2008 crop for the ND genotype frequently had increases in gene expression. The reference levels of expression for these genes at early harvest was low, but detectable within 45 cycles of qRT-PCR; these basal Ct values represent initial baselines of expression (first harvest) and are essential for calculations of fold change in gene expression.

Changes in expression were detected among cell cycle genes during the process of native periderm maturation. The cell cycle gene *StEBP* was slightly down-regulated throughout the harvests for the ND 2008 crop and remained at basal levels for the ND 2009 crop by the second harvest, followed by a small down-regulation thereafter (Fig. 14A). In the ND 2008 and 2009 crops, *StCDKB* expression was around initial levels at the second harvest, and then decreased throughout (Fig. 14B). As for *StCKS1At*, there were no significant changes in expression after the first harvest for ND 2008 and 2009 crop years (Fig. 14C). All three cycle cell genes monitored were down-regulated after the first harvest for RB 2008 and 2009 crop years (Fig. 14). As summarized in Neubauer et al. (2012), *StExt* represents several members of the extensin family whereas *StExt1* is specific to *StExtensin1*. The ND 2008 crop was the only crop that a fold or more increase occurred for both *StExt* and *StExt1*, with an increase of ~2.6 to 5.5 fold by the second harvest, followed by a sharp decrease thereafter (Fig. 15). As for the ND 2009 and RB 2008 and 2009 crops, a clear reduction in transcripts was observed for *StExt* and *StExt1* after the first harvest (Fig. 15A and 15B). The

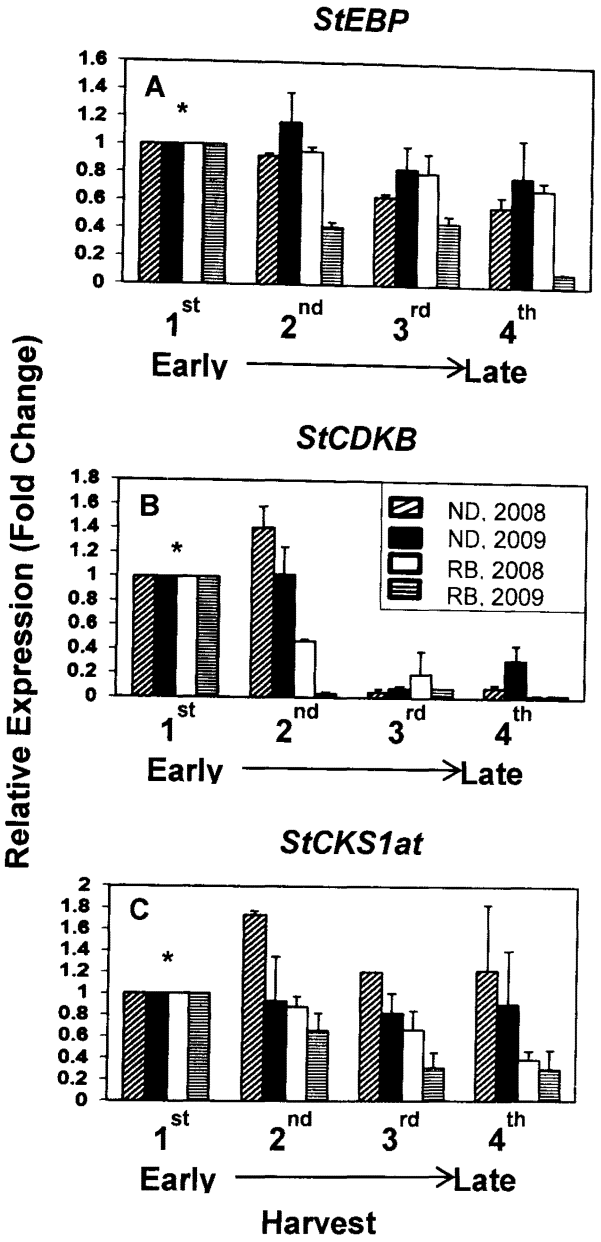

Fig. 14. Changes in the expression of indicated cell cycle genes during the process of native periderm maturation. The genes (A) *StEBP*, (B) *StCDKB*, (C) *StCKS1At* encode cell cycle related proteins in tuber tissue for the genotypes NDTX4271-5R and Russet Burbank (2008 and 2009 crops): Gene expression was determined in triplicate by qRT-PCR using *EF1α* as a reference gene. * Initial levels of gene expression, i.e. first Harvest, were normalized to a value of one based on the method of Pfaffl (2001) for relative quantification in real-time RT-PCR. See Table 1 for primer pairs. Bars represent the standard error of the means.

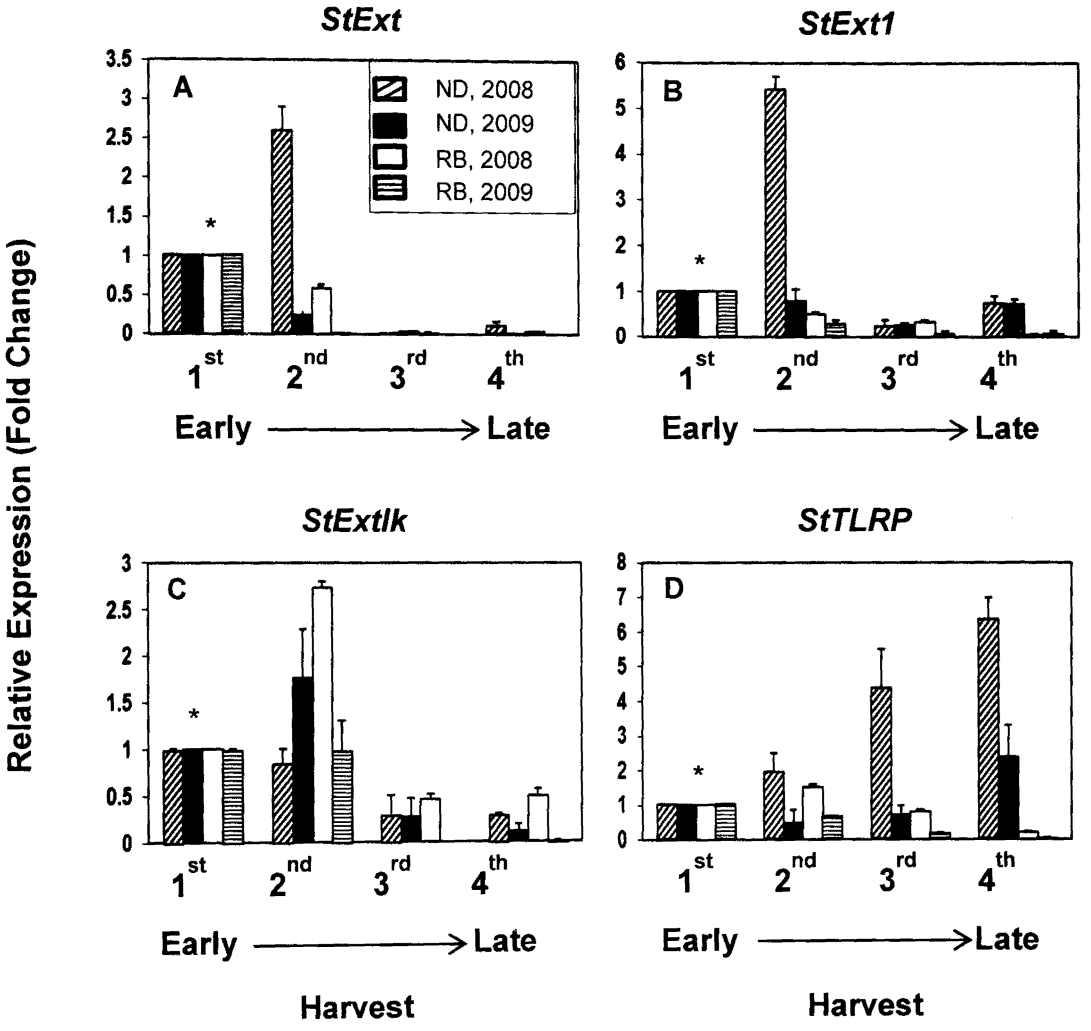

Fig. 15. Changes in the expression of indicated cell wall genes during the process of native periderm maturation. The genes expressed in tuber tissue for the genotypes NDTX4271-5R and Russet Burbank (2008 and 2009 crops): (A) *StExt*, (B) *StExt1*, (C) *StExtlk*, (D) *StTLRP*. Gene expression was determined in triplicate by qRT-PCR using *EF1α* as a reference gene. * Initial levels of gene expression, i.e. first Harvest, were normalized to a value of one based on the method of Pfaffl (2001) for relative quantification in real-time RT-PCR. See Table 1 for primer pairs. Bars represent the standard error of the means.

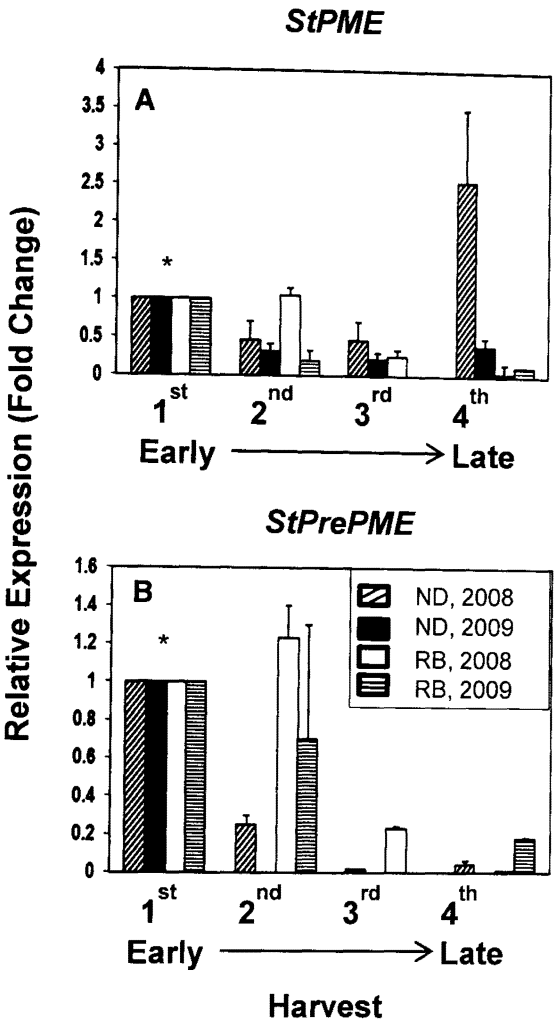

Fig. 16. Changes in the expression of pectin methyl esterase genes during the process of native periderm maturation in tuber tissue for the genotypes NDTX4271-5R and Russet Burbank (2008 and 2009 crops): (A) *StPME* and (B) *StPrePME*. Gene expression was determined in triplicate by qRT-PCR using *EF1α* as a reference gene. * Initial levels of gene expression, i.e. first Harvest, were normalized to a value of one based on the method of Pfaffl (2001) for relative quantification in real-time RT-PCR. See Table 1 for primer pairs. Bars represent the standard error of the means.

related gene *StExtlk* had a different expression pattern for the ND 2009 crop and the RB 2008 crop whereby up-regulation occurred by the second harvest ~2 to 2.7 fold increase followed by a decrease thereafter (Fig. 15C). These results are

unlike those of the ND 2008 crop and RB 2009 crop where no increase in expression of *StExtlk* was observed (Fig. 15C). The ND 2008 crop had a unique expression pattern for the *StTLRP* gene; up-regulation occurred throughout the harvests ending with a ~6.3 fold increase at the fourth harvest (Fig. 15D). In the 2009 crop of ND, the *StTLRP* gene was down-regulated at the second and third harvest, but then was up-regulated to ~2.3 fold by the fourth harvest (Fig. 15D). The *StTLRP* gene for RB 2008 crop, had no significant changes by the second harvest and then down-regulated thereafter (Fig. 15D). As for the RB 2009 crop, the *StTLRP* gene was down-regulated after the first harvest (Fig. 15D). There were no consistent differences in gene expression between these genotypes although crop years influenced the pattern of gene expression.

During the process of periderm maturation process, variable increases occurred for *StPME* and *StPrePME* genes in both potato genotypes and crops (Fig. 16). Expression of *StPME* for the ND 2008 crop increased only at the fourth harvest by ~2.5 fold. In the RB 2008 crop, no significant change occurred at second harvest, followed by down-regulation throughout the rest of the harvests for *StPME* (Fig. 16A). The 2009 crop of ND and RB were both down-regulated after the first harvest for the *StPME* gene (Fig. 16A). Unlike the *StPME* gene, expression of *StPrePME* remained at initial levels for the RB 2008 crop by the second harvest, followed by down-regulation. The ND both crop years and RB 2009, were down-regulated after the first harvest for *StPrePME* (Fig. 16B). In general, *StPME* and *StPrePME* showed no consistent differences in gene expression for both genotypes that was not more highly influenced by crop year.

Discussion

It is difficult to overstate the agricultural importance of the biological processes involved in tuber periderm maturation. Prior to periderm maturation, the immature periderm is susceptible to costly skinning injury. As the periderm matures, it becomes resistant to excoriation, thus maintaining food quality and reducing crop loss. The genes examined in this research were thought to be involved in processes associated with periderm maturation including the cell cycle/divisional processes of meristematically active phellogen cells and those encoding proteins that are targeted to cell wall polymers in the periderm, i.e. cell wall proteins and pectins. Monitoring and understanding the roles of these genes could be of great benefit to farmers and end users. This research uses histochemical techniques in conjunction with qRT-PCR analysis to provide a better understanding of the expression profiles of genes that appear to play prominent roles in the process of native periderm development and maturation.

The vine maturity index revealed a threshold level relative to skin-set measurements; i.e. after a rating of 9 (>90% senesced) was observed, a FS (skin-set advanced/not measurable) was recorded from the torqometer. Skin-set was measured at four harvest intervals to determine periderm maturity. ND, the slower maturing genotype, (Neubauer et al., 2012), took longer to reach full maturity (FS) than RB (Table 3). A FS measurement indicates that the tuber periderm has matured; this maturation occurred after phellem cell layer accumulation had level off as a result of cessation of cell division and inactivation of the phellogen layer. As the tubers from ND 2008, RB 2008 and 2009 crops became FS, the phellogen radial cell walls continued to thicken. These results suggest that as the periderm

becomes mature, the phellogen radial cell walls continue to thicken increasing robustness of the skin (Table 3 and Fig. 12).

Many events take place during periderm formation; perhaps the most important is layering of suberin on the outside of the phellem cell walls. This suberized phellem cell layer is crucial in the defense against bacterial and fungal pathogens (Lulai and Corsini, 1998). Therefore, the native periderm was monitored throughout the harvests to assess the accumulation of phellem cell layers as an index of cell division. The total number of phellem cell layers was steady throughout the harvests, except for RB 2008 where an increase in phellem cell layers occurred from the first to the second harvest (Fig. 10). After the second harvest, the number of phellem cell layers no longer increased, indicating cessation of cell division. As for the total amount of phellem cell layers, ND had ~7 cell layers, whereas RB had ~13 cell layers. These results are consistent with Neubauer et al. (2012), whereby it was suggested that a more robust phellem equates to more layers of phellem cells. With the exception of ND 2009 crops skin-set measurements showed that by or before the fourth harvest, these genotypes had become fully set. Collectively, these results indicate that after the second harvest, the process of periderm maturation could advance.

Throughout these harvests, phellogen radial cell wall thickness was measured to determine its relationship with native periderm development and maturation. Lulai and Freeman (2001) showed that as the tuber became fully resistant to skinning, the phellogen cell walls strengthened and thickened considerably. At the time of the first harvest, the active phellogen had thin and

fragile cell walls (Fig. 13). During the process of native periderm maturation the phellogen radial cell walls continued to thicken for both genotypes (Fig. 12 and Fig. 13). An increase in radial cell wall thickness occurred by the second harvest for both genotypes, providing evidence that after phellem cell layer accumulation ceased, the radial cell walls of the phellogen continued to thicken. These results are consistent with those of Sabba and Lulai (2002) who showed that the phellogen cell walls had thickened in mature periderm. Neubauer et al. (2012) also showed that there was continued thickening of phellogen radial cell walls in wound periderm after the production of phellem cell layers had ceased, suggesting increased strength towards the resistance of re-injury.

During cell cycle activity and the cell divisional process, the inwardly and outwardly dividing phellogen layer forms the native periderm. Certain gene expression profiles were used to monitor and assess this cellular activity. The cell cycle genes *StEBP, StCDKB,* and *StCKS1at* had no significant changes in expression by the second harvest (Fig. 14). The expression of *StEBP* possibly occurred by or before the first harvest, which is consistent with the results of Horvath et al. (2006) who showed that *EBP1* is a dose dependent regulator of certain cell cycle genes. *CDKB* expression was confined to actively dividing tissues in *Arabidopsis* (Dewitte and Murray, 2003). Results in this study agree with Dewitte and Murray, since *StCDKB* was down-regulated when meristematic activity had ceased (Fig. 10 and 14B). Boruc et al. (2010) showed that two CDK regulatory subunits (CKS proteins) had identical localization patterns in dividing cells. In this study, *StCKS1At* expression for ND tubers remained at initial levels

while the phellogen was meristematically active and then began to slowly down-regulate, but remained near first harvest levels upon cessation of meristematic activity. Conversely *StCKS1At* was slowly down-regulated throughout all harvests in RB tubers (Fig. 14C). Taken together these results suggest that these cell cycle genes are linked to the development and cessation of native periderm formation.

Extensins are a family of HRGPs which are highly abundant and are major protein components in cell walls of dicots (Showalter, 1993). A group of extensin genes collectively encoded in *StExt* for ND 2008 were rapidly up-regulated by second harvest and down-regulated throughout the harvests for ND (2009) and RB (2008 and 2009) tubers (Fig. 15A and 2.B). Therefore, we designed the primer set *StExt1* that is specific to *StExtensin1*. Results were similar to that found in wound periderm (Neubauer et al., 2012) whereby both *StExt* and StExt1 had similar patterns of expression for both genotypes. The induction pattern of *StExt1* by the second harvest for ND (2008) suggests a role in primary cell wall modification during the production of new phellem cells from the meristematically active phellogen. After these phellem cells are produced, the primary cell walls are rapidly impregnated with SPP (Lulai, 2007). This suggests that the suberization processes are acting in conjunction with expression of *StExt1* (Fig. 15A, 15B and Fig. 11A, 11C). These results provide evidence that in potato, extensin is needed during native periderm maturation, putatively to facilitate cell wall scaffold formation and subsequent cross-linking as suberization of the phellem cells occurs (Cannon et al., 2008; Lamport et al., 2011).

The related extensin-like gene transcript was most abundant by second harvest, suggesting a similar role to that of extensin (Fig. 15C). Dubreucq et al. (2000) suggested that the extensin like gene *AtEpr1* is involved in morphogenic control of cell-wall architecture during the process of cellular differentiation. These results provide more evidence that extensin-like gene transcripts are involved during the process of native periderm maturation and phellogen cell wall thickening.

Tyrosine, a structural component of TLRP, is proposed to participate in the cross-linking of proteins to cell walls and can be linked to aromatic side chains of lignin (Domingo et al., 1994 and 1999). This cross-linking facilitates integration of TLRPs with other components of the cell walls (Domingo et al., 1999). *StTLRP* expression was up-regulated in the slower maturing ND genotype and remained expressed throughout the harvests (Fig. 15D). *StTLRP* participation in formation and completion of secondary cell wall construction may explain the expression through all four harvests. Domingo et al. (1999) suggested that proteins with high tyrosine content could be used as potential nucleation sites that may help the deposition of lignin. Likewise, such proteins may facilitate in deposition of lignin-like SPP material, during secondary cell wall formation. This data suggests that the extended period of *StTLRP* expression is related to the process of native periderm maturation. Importantly, the elevated *StTLRP* expression after the second harvest suggests a role for this gene in phellogen cell wall thickening and strengthening after cessation of meristematic activity. This role is consistent with the results obtained with wound periderm Neubauer et al. (2012) and is supported

by the results of Ross et al. (2011) and Kloosterman et al. (2010) who showed that *StTLRP* was more highly expressed in firm tuber tissues.

Pectin accounts for ~35% of the dry weight in dicot cell walls (Micheli, 2001). Pectins combined with hemicelluloses constitute the matrix in which cellulose microfibrils are embedded. This integrated combination creates the rigid yet dynamic properties of the cell wall (Harholt et al., 2010). Sabba and Lulai (2004) used monoclonal antibodies with specificities for relatively unesterified (JIM5) and esterified (JIM7) pectin to determine the presence of pectin epitopes associated with these different degrees of esterification. The expression of *StPME* at the fourth harvest (ND 2008) (Fig. 16A) is consistent with the results of Sabba and Lulai (2004) who observed that the phellogen layer of immature native periderm had weak labeling, but was strongly labeled in mature native periderm. Importantly the expression pattern of *StPME* suggests that its role in de-esterification of esterified pectins is later in native periderm development for ND 2008. *StPrePME* began to down-regulate by the second harvest for ND tubers and by the third harvest for RB tubers (Fig. 16B), indicating possible reduction in the phellogen cell layer compared to *StPME* and its possible role in de-esterification and ultimately formation of calcium bridging and pectate gel formation. In this study the PME genes had different expression profiles, but results suggest important roles in the process of periderm maturation.

In conclusion, the selected cell wall genes were up-regulated during the process of native periderm formation i.e. up through the first harvest when the vines were not senesced and in general down-regulated during periderm

maturation for later harvests and both genotypes. No clear genotypic difference in the pattern of expression for these genes was observed because of the biological variation inherent with different crop years and the influence on the amplitude of the expression. Some of the genes were up-regulated at or before the second harvest, suggesting roles in the phellogen layer and the generation phellem cells. After the phellogen became inactive, the expressions of *StEBP, StCDKB, StCKS1At, StExt1, StExtIk* and *StPrePME* were down-regulated, while *StPME* and *StTLRP* expression remained at detectable levels. These results suggest that the expressions of cell wall genes and cell cycle genes are coordinated with phellem cell development and the on-set of native periderm maturation while down regulation of these genes coordinated with the inactivation of mother phellogen cells and cessation of phellem cell production. This research provides insight into the possible involvement of these genes in native periderm development and native periderm maturation.

Potato tuber periderm is an important topic for future research. These data suggest that *StTLRP* and other genes have important roles in continued phellogen cell wall thickening leading to resistance to excoriation. Importantly, the continued expression of *StTLRP* after cessation of meristematic activity suggests that future research should be conducted regarding the role of this gene in phellogen cell wall thickening and resistance to excoriation.

References

Artschwager E. Studies on the potato tuber. J Agr Res 1924;27:809-35.

Barel, G, Ginzberg, I. Potato skin proteome is enriched with plant defense components. J Exp Bot 2008;59:3347-3357.

Bernards MA. Demystifying suberin. Can J Bot 2002;81:227-40.

Bown DP, Bolwell GP, Gatehouse JA. Characterization of potato *(Solanum tuberosum* L.) extensins: a novel extensin-like cDNA from dormant tubers. Gene 1993;134:229-33.

Boruc J, Mylle E, Duda M, Clercq RD, Rombauts S, Geelen D, et al. Systematic localization of the Arabidopsis core cell cycle proteins reveals novel cell division complexes. Plant Physiol 2010;152:553-65.

Boudolf V, Vlieghe K, Beemster GTS, Magyar Z, Torres Acosta JA, Maes S, et al. The plant specific cyclin-dependent kinase CDKB1;1 and transcription factor E2Fa-DPa control the balance of mitotically dividing and endoreduplicating cells in Arabidopsis. Plant Cell 2004;16:2683-92.

Bucher M, Brunner S, Zimmerman P, Zardi G, Amrhein N, Willmitzer L. The expression of an extensin-like protein correlates with cellular tip growth in tomato. Plant Physiol 2002;128:911-23.

Cannon MC, Terneus K, Hall Q, Tan L, Wang Y, Wegenhart B. Self-assembly of the plant cell wall requires an extensin scaffold. PNAS 2008;105:6.

Corsini D, Stark J, Thornton M. Factors contribution to the blackspot bruise potential of Idaho potato fields. Am J Potato Res 1999;76:221-26.

Deepak S, Shailasree S, Kini RK, Hause B, Shetty SH, Mithofer A. Role of hydroxyproline-rich glycoproteins in resistance of pearl millet against downy mildew pathogen *Sclerospora graminicola*. Planta 2007;226:323-33.

Destefano-Beltran, L, Knauber D, Huckle L, Suttle J. Chemically forced dormancy termination mimics natural dormancy progression in potato tuber meristems by reducing ABA content and modifying expression of genes involved in regulating ABA synthesis and metabolism. J Exp Bot 2006;57:2879–86.

De Veylder L, Beeckman T, Beemster GTS, Drois L, Terras F, Landrieu I, et al. Functional analysis of cyclin-dependent kinase inhibitors of Arabidopsis. Plant Cell 2001;13:1653-67.

Dewitte W, Murray JAH. The plant cell cycle. Ann Rev Plant Biol 2003;54:235–64.

Domingo C, Gomez MD, Caíias L, Hernandez-Yago J, Conejero V, Vera P. A novel extracellular matrix protein from tomato associated with lignified secondary cell walls. Plant Cell 1994;6:1035-47.

Domingo C, Sauri A, Mansilla E, Conejero V, Vera P. Identification of a novel peptide motif that mediates cross-linking of proteins to cell walls. Plant J 1999;20:563-70.

Dubreucq B, Berger N, Vincent E, Boisson M, Pelletier G, Caboche M, et al. The *Arabidopsis AtEPR1* extensin-like gene is specifically expressed in endosperm during seed germination. Plant J 2000;23:643-52.

Harholt J, Suttangkakul A, Vibe Scheller H. Biosynthesis of pectin. Plant Physiol 2010;153:384-95.

Horvath BM, Magyar Z, Zhang Y, Hamburger AW, Bako L, Visser RG, Bachem CW, Bogre L. EBP1 regulates organ size through cell growth and proliferation in plants. EMBO J 2006;25:4909–20.

Kloosterman B, Oortwijn M, UitdeWilligen J, America T, de Vos R. Visser RGF, Bachem CWB. From QTL to candidate gene: Genetical genomics of simple and complex traits in potato using a pooling strategy. BMC Genomics 2010:11; 158-73.

Lamport DTA, Kieliszewski MJ, Chen Y, Cannon MC. Update on the extensin superfamily. Role of the extensin superfamily in primary cell wall architecture. Plant Physiol 2011:156;11-19.

Lulai EC. The roles of phellem (skin) tensile-related fractures and phellogen shear-related fractures in susceptibility to tuber-skinning injury and skin-set development. Am Potato J 2002;79:241-48.

Lulai EC. Skin-set, wound healing, and related defects *In* Vreugdenhil D. editor. Potato Biology and Biotechnology: Advances and Perspectives. Amsterdam. The Netherlands: Elsevier Limited: 2007. p. 471-96.

Lulai EC, Corsini. Differential deposition of suberin phenolic and aliphatic domains and their roles in resistance to infection during potato tuber (*Solanum Tuberosum L.*) wound-healing. Physiological and Molecular Plant Path 1998:53;209-22.

Lulai EC, Freeman TP. The importance of phellogen cells and their structural characteristics in susceptibility and resistance to excoriation in immature and mature potato tuber (*Solanum tuberosum* L.) Periderm. Ann Bot 2001;88:555-61.

Lulai EC, Orr PH. Determining the feasibility of measuring genotypic differences in skin-set. Am Potato J 1993;70:599-09.

Lulai EC, Suttle JC, Pederson SM. Regulatory involvement of abscisic acid in potato tuber wound-healing. J Exp Bot 2008:59;1175-86.

Magliano TMA, Casal JJ. I*n vitro* cross-linking of extensin precursors by mustard extracellular isoforms of peroxidase that respond either to phytochrome or to wounding. J Exp Bot 1998;49:1491-99.

Micheli F. Pectin methylesterases: cell wall enzymes with important roles in plant physiology. TRENDs in Plant Science 2001;6:414-18.

Narvaez-Vasquez, J. and C.A. Ryan, Jr. 2002. The systemin precursor gene regulates both defensive and developmental genes in *Solanum tuberosum.* Proc Natl Acad Sci USA 2002;99:15818-21.

Neubauer JD, Lulai EC, Thompson AL, Suttle JC, Bolton MD. Wounding coordinately induces cell wall protein, cell cycle and pectin methyl esterase genes involved in tuber closing layer and wound periderm development. 2011. J Plant Physiol (submitted 2011).

Nicot N, Hausman JF, Hoffmann L, Evers D. Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. J Exp Bot 2005;56:2907-14.

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:2002-7.

Reeve RM, Hautala E, Weaver ML. Anatomy and compositional variation within potatoes. 1. Developmental histology of the tuber. Am Potato J 1969;46:361-73.

Ross HA, Wright KM, McDougall GJ, Roberts AG, Chapman SN, Morris WL. Potato tuber pectin structure is influenced by pectin methyl esterase activity and impacts on cooked potato texture. J Exp Bot 2011;62:371-81.

Sabba RP, Lulai EC. Histological analysis of the maturation of native and wound periderm in potato (*Solanum tuberosum* L.) tuber. Ann of Bot 2002;90:1-10.

Sabba RP, Lulai EC. Immunocytological comparison of native and wound periderm maturation in potato tuber. Am Potato J 2004;81:119-24.

Sabba RP, Lulai EC. Immunocytological analysis of potato tuber periderm and changes in pectin and extensin epitopes associated with periderm maturation. J Hort Sci 2005;130:936-42.

Showalter AM. Structure and function of plant cell wall proteins. Plant Cell 1993;5:9-23.

OVERALL CONCLUSION

Overall cell wall and cell cycle genes were up-regulated during the wound-healing time course for both harvests and genotypes. Although no clear genotypic difference in the pattern of expression for these genes was observed, the biological variation inherent with different harvests did influence the amplitude of wound induced expression. Several of the genes were highly expressed by 1 d after wounding, this suggested coordinate involvement with closing layer formation and formation of the phellogen cell layer. After cessation of phellogen meristematic activity, the expression of *StEBP, StCDKB, StCKS1At, StExt1, StExtlk, StPME and StPrePME* declined, while *StTLRP* remained up-regulated. These results suggest that the up-regulation of cell wall genes and cell cycle genes is coordinated with closing layer development, phellogen formation and phellem cell development while down regulation of these genes coordinated with cessation of wound phellem cell development as an indicator of meristematic inactivation of mother phellogen cells. Our results also showed that after phellem cell generation had ceased and expression of most of the genes monitored returned to basal levels, the phellogen radial cell walls continued to thicken.

In the native periderm model, these cell wall and cell cycle genes were up-regulated during the process of native periderm formation i.e. up-regulated through the first harvest when the vines were not senesced and in general down-regulated during periderm maturation for later harvests and both genotypes. No consistent° genotypic difference in the pattern of genes expression was observed between these genotypes because of the biological variation inherent with different crop

years and the influence on the amplitude of the expression. Also, most of the genes were up-regulated by or before the second harvest, suggesting roles in the phellogen layer and the generation phellem cells. After the phellogen became meristematically inactive, the expressions of *StEBP, StCDKB, StCKS1At, StExt1, StExtIk* and *StPrePME* were down-regulated, while *StPME* and *StTLRP* expression remained at detectable levels (ND crop). These results suggest that the expressions of cell wall genes and cell cycle genes are coordinated with phellem cell development, and the on-set of native periderm maturation while down regulation of these genes coordinated with the inactivation of mother phellogen cells and cessation of phellem cell production.

Potato tuber periderm maturation is an important topic for future research since these data suggest that *StTLRP* and other genes have important roles in continued phellogen cell wall thickening leading to resistance to excoriation or re-injury by phellem excoriation. Importantly, the continued expression of *StTLRP* after cessation of meristematic activity suggests that future research should be conducted regarding the role of this gene in phellogen cell wall thickening and resistance to excoriation and resistance to re-injury. *In-situ* and gene silencing techniques could possibly show how this gene is involved in these important processes.

This research provides insight into the coordinate induction of these genes in marked relationships to the biological responses of wound healing including closing layer formation, phellogen formation and phellem generation during wound periderm formation. Furthermore, this research provides insight into the possible

involvement of these genes in native periderm development and native periderm maturation.

APPENDIX A. RATING SYSTEM FOR THE ACCUMULATION OF AUTOFLUORESCENT SUBERIN POLY(PHENOLICS) (SPP) ON SUBERIZING CELL WALLS DURING WOUND-HEALING[1].

AF or BB/SPP Rating	Fluorescence/Accumulation of the Suberin Poly(phenolic) Domain[2]
0	None
1	Some suberin poly(phenolic) accumulations detected on wound surface.
2	Majority of wound surface outer tangential cell walls possess suberin poly(phenolic) accumulations.
3	All of the wound surface outer tangential cell walls have accumulated suberin poly(phenolics) forming a contiguous barrier.
4	Suberin poly(phenolic) accumulations extend to radial walls.
5	One complete cell layer (out tangential, radial and inner tangential cell walls) possesses suberin poly(phenolic) accumulations.
6	1 ½ cell layers possess suberin poly(phenolic) accumulations.
7	2 cell layers possess suberin poly(phenolic) accumulations.
8	2 ½ or more cell layers possess suberin poly(phenolic) accumulations.
9	3 cell layers possess suberin poly(phenolic) accumulations.

Additional accumulations are rated using the same increments as above.

[1] Adapted from **Lulai EC, Corsini DL.** Differential deposition of suberin phenolic and aliphatic domains and their roles in resistance to infection during potato tuber (*Solanum tuberosum* L.) wound-healing. Physiol Mol Plant Path 1998;53:209-22. Also, listed as supplement table S1 in **Lulai EC, Suttle JC, Pederson SM.** Regulatory involvement of abscisic acid in potato tuber wound-healing. J Exp Bot 2008; 59:1175-86.

[2] Autofluorescence (AF) or staining with the fluorochrome berberine (BB) may be used to detect accumulation of the suberin poly(phenolic) domain in wound-healing tuber tissue.