Towards uncovering the roles of switchgrass peroxidases in plant processes

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INTRODUCTION

Perennial warm-season grasses such as switchgrass (Panicum virgatum L.), miscanthus (Miscanthus giganteus) and giant reed (Arundo donax L.) are expected to become major sources of renewable biomass for the biofuel sector (Lewandowski et al., 2003; Vogel et al., 2011; Kering et al., 2012). Switchgrass is a focus bioenergy crop for the central regions of the US and elsewhere based on its high yield potential and other useful characteristics (Vogel et al., 2011). Switchgrass can be sustainably grown as a biofuel crop on marginal croplands with limited inputs (Vogel et al., 2011). What is currently unknown is the extent to which these components might affect biomass quality and stress response in plants engineered for a specific end purpose. It can be expected that production of switchgrass on marginal lands will expose plants to diverse stresses, including herbivory by insects. Class III plant peroxidases have been implicated in many developmental responses such as lignification and in the adaptive responses of plants to insect feeding. Here, we have analyzed the class III peroxidases encoded by the switchgrass genome, and have mined available transcriptomic datasets to develop a first understanding of the expression profiles of the class III peroxidases in different plant tissues. Lastly, we have identified switchgrass peroxidases that appear to be orthologs of enzymes shown to play key roles in lignification and plant defense responses to hemipterans.

Keywords: switchgrass, peroxidases, lignin, Hemiptera, biotic stress, ROS

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CELLULAR ASPECTS OF THE BIOTIC INTERACTIONS OF THESE LOWER LIGNIN PLANTS

The basics of plant cell walls and their role in the biotic and abiotic stress (Almagro et al., 2009; Cosio and Dunand, 2011) have been investigated in a myriad of plant developmental processes and responses to the environment. LIGNIN, ETHANOL, AND PLANT FITNESS

Cell wall composition impacts resistance to pests and pathogens (Santiago and Malvar, 2010; Funnell-Harris et al., 2012), and is a critical component determining biomass quality (Sarath et al., 2008). Plant cell walls consist of three major polymers, namely cellulose, hemicellulose, and lignin (Boerjan et al., 2003). The polysaccharide polymers, cellulose and hemicellulose are the dominant sources of sugars for conversion into liquid fuels in biorefineries. Lignin is an aromatic polymer derived from cytoplasmically synthesized monolignols (Boerjan et al., 2003; Ralph et al., 2006). Monolignols are products of the phenylpropanoid pathway that is also the route for a range of other plant secondary metabolites associated with plant development and defense (Shadle et al., 2003; Zhao and Dixon, 2011). Polymerization of monolignols occurs in the cell wall apoplast after transport across the plasma membrane, catalyzed principally by wall-bound peroxidases and laccases (El Mansouri et al., 1999; Boerjan et al., 2003; Berthet et al., 2011; Cesarino et al., 2013). Lignin content and composition will be driven by the rate, amounts and types of monolignols that are transported to the apoplast. This transport process has not yet been fully elucidated, and previous hypothesis have suggested various mechanisms including passive diffusion, vesicle-mediated transport, facilitated diffusion through channels and active transport via transporters (Fagerstedt et al., 2010; labeling studies using [3H]phenylalanine in lodgepole pine have indicated Golgi vesicle-mediated transport is unlikely based on the finding that inhibition of protein synthesis decreased the Golgi label while inhibition of phenylpropanoid metabolism did not (Kaneda et al., 2008). More recently, investigations using isolated vesicles from Arabidopsis demonstrated that transport of monolignols was an ATP-dependent process for both vacuolar and plasma membrane vesicles (Miao and Liu, 2010). Also, using sodium orthovanadate and a variety of other inhibitors to inhibit ATP-binding cassette (ABC) transporter activity, it was shown that these inhibitors substantially reduced monolignol transport activity, indicating that ABC transporters were likely involved in the transport process. Disruptions to the membrane pH gradient or membrane potential did not exhibit the same degree of transport inhibition (Miao and Liu, 2010). Additionally, the Arabidopsis ABC transporter AtABCG29 was recently identified as a p-coumaryl alcohol transporter based on several lines of evidence (Alejandro et al., 2012). In this study, yeast strains-expressing AtABCG29 were highly sensitive to p-coumaryl alcohol and isolated yeast vesicles from AtABCG29-expressing yeast contained higher levels of p-coumaryl alcohol. Also, abcg29 knockout plant lines exhibited substantially reduced root length in media containing p-coumaryl alcohol, and lowered H, G, and S-lignin content based on thioacidolysis yields. RT-qPCR data showed AtABCG29 was upregulated in WT plants in response to p-coumaryl but not sinapyl or coniferyl alcohols (Alejandro et al., 2012). Taken together, this emerging evidence favors the active transport hypothesis and suggests that other monolignols are likely transported to the apoplast via monolignol-specific ABC (or possibly other) transporters that remain to be discovered.

Once transport to the apoplast is complete, monomers are polymerized into developing or new polymers, primarily via radical-coupling mechanisms initiated by multiple classes of apoplastic enzymes including peroxidases, laccases, and oxidases (Boerjan et al., 2003). The basic radical coupling mechanism has long been postulated (Freudenberg, 1959). In general, the polymerization of lignin has appeared to proceed under chemical control rather than the more confining and controlled nature of biochemical control that governs most other plant processes. The proposed mechanism involves dehydrogenation and subsequent polymerization of the radicals; each coupling requires the generation of two radicals and polymerization may proceed through a radical transfer or redox shuttle mechanism since the growing lignin polymer and monolignols such as sinapyl alcohol are not easily oxidized (Boerjan et al., 2003; Liu, 2012).

Within the plant cell walls, lignin forms a physical barrier against the entry of pests and pathogens (Boerjan et al., 2003; Sarler and Funnell-Harris, 2013), acts as an antifeedant for herbivorous insects (Ralph et al., 2006; Deng et al., 2013) and is the major factor impeding the conversion of herbaceous biomass to ethanol (Dunn et al., 2009; Fu et al., 2011; Saathoff et al., 2011; Sarath et al., 2011). The negative impact of lignin in the biochemical conversion of herbaceous feedstocks to liquid fuels has led to intense efforts to develop a range of biofuel feedstocks with lowered lignin content (Carroll and Somerville, 2009; Steder et al., 2011; Robbins et al., 2012). Lowering lignin generally negatively impacts plant fitness, especially in perennial grasses (Casler et al., 2002; Pedersen et al., 2005), although many of molecular and cellular aspects of the biotic interactions of these lower lignin plants remain to be explored.

CLASS III PEROXIDASES AND LIGNIN BIOGENESIS

Class III peroxidases are ubiquitous plant enzymes that are coded by a large number of related genes within a plant genome (Passardi et al., 2004, 2007; Tobias et al., 2008), and have been implicated in a myriad of plant developmental processes and responses to biotic and abiotic stress (Almagro et al., 2009; Cosio and Dunand, 2011).
Some peroxidases appear to have a specialized role in lignification. In tobacco, overexpression of a chimeric anionic peroxidase resulted in plants containing higher basal levels of lignin compared to control plants (Lagrimini, 1991). Wounding of these plants also appeared to result in higher polymerization of phenolic acids, particularly in pith tissue which had much higher levels of peroxidase activity than control plants, although the transgenic lines exhibited transgene throughout the plant due to the use of the cauliflower mosaic virus 35S promoter (Lagrimini, 1991). Additional work in tobacco demonstrated that other peroxidases may also be involved in lignification. Antiense gene silencing of the cationic peroxidase TPrx6 (NiPrx60) in tobacco resulted in several plant lines that exhibited lower lignin content based on thioacidolysis, acetyl bromide and nitrobenzene determinations (Blee et al., 2003). Later work showed some of the generated T1 plants had abnormal phenotypes including discoloration, altered leaf morphologies, and poorly developed xylem in one of the lines (Kavousi et al., 2010). In poplar, a cationic cell wall-bound peroxidase, dubbed CNPO-C, was found to preferentially oxidize sinapyl alcohol monomers as well as sinapyl alcohol polymers (Sasaki et al., 2004). This finding indicated that the suggested radical transfer or redox shuttle mechanisms for lignin polymerization may be unnecessary in at least some circumstances. Other work in tomato demonstrated that overexpression of a tomato basic peroxidase, tpxt, resulted in higher cell wall peroxidase activity and higher leaf lignin levels (El Mansouri et al., 1999). A basic peroxidase from Zinnia elegans was found to be composed of two isoforms, ZePrx34.70 and ZePrx33.44 that were studied in detail (Gaboldón et al., 2005). Here, the peroxidases were shown to have high affinity for sinapyl alcohol and carried out polymerization of this substrate, suggesting a role in polymerization of S-lignin during growth (Gaboldón et al., 2005). Similar results were found for the anionic peroxidases Ppx3, Ppx4, and Ppp5 which were isolated from poplar xylem (Christensen et al., 1998), and several peroxidases from silver birch (Betula pendula) and Norway spruce (Picea abies) were shown to have activity on monolignol substrates (Marjamaa et al., 2003). In aspen, GUS staining revealed that the anionic peroxidase prxA3a was predominately expressed in lignifying stem tissue, particularly xylem (Li et al., 2003). Furthermore, down regulation of this gene using an antisense construct resulted in plants with lower total peroxidase activity and a lowered lignin content that, depending on the transgenic line, approached 20% (Li et al., 2003).

Several peroxidases have been identified in Arabidopsis that appear to have roles in lignification. Arabidopsis ATP A2 (APxS3), a cationic peroxidase, was found to localize to lignified tissues and transgenic plants exhibited differential phenolics staining compared to WT plants; unfortunately, lignin levels were not reported (Ustergaard et al., 2005). Modeling based on the ATP A2 crystal structure indicated monomeric substrates could dock in the active site (Ustergaard et al., 2000), although class III peroxidases are known to be capable of oxidizing a wide variety of phenolic compounds (Marjamaa et al., 2003). Recently, Arabidopsis ATPx37 was found to be highly expressed in roots as well as flower stems and mature leaves (Pedreira et al., 2011). Overexpression of this gene fused to a GUS reporter gene showed localization in vascular tissue; mutant lines exhibited shorter roots, delayed development and dwarfism, which led to the hypothesis that overexpression of AtPPrx37 led to higher cell wall cross-linking (Pedreira et al., 2011). A microarray study in Arabidopsis identified eight peroxidases and several laccases with expression profiles that clustered with monolignol synthesis (AtPrx2, AtPrx17, AtPrx37, AtPrx9, and AtPrx35) were peroxidases that were noted to show the strongest co-expression patterns (Ehlting et al., 2005). Gravitistimulation was used in one study to alter the mechanical forces acting on stem region of the inflorescence, which was then excised into apical, middle, and basal parts (Yokoyama and Nishitani, 2006). Expression profiling using a microarray showed upregulation of AtPrx42, AtPrx64, and AtPrx71 in basal stem regions compared to middle and apical regions; however, the statistical significance of the expression change was not reported (Yokoyama and Nishitani, 2006). More recently, AtPPrx 5, 52, 49, and 72 were suggested to have roles in lignification based on homology to ZePPrx and in silico characterization of other properties including surface charge, mRNA stability, and amino acid positions (Herrero et al., 2013). Identification of peroxidases that have a primary, or even secondary, role in lignification will likely remain challenging.

The fact that a pectate binding site has been identified in an anionic peroxidase from zucchini which suggested possible involvement in lignification (Carpin et al., 2001), further research indicated that the physiological role of the protein involved auxin oxidation in termination of hypocotyl elongation (Cosio and Dunand, 2009). Some characteristic features of syringyl peroxidases have been found. These have included a VSCAD motif compared to a VS add motif in G peroxidases as well as the finding that S peroxidases lack helix D’ (Gómez Ros et al., 2007). These changes were postulated to result in conformational changes in the peroxidase active site that allowed sinapyl alcohol to more successfully dock, and thus undergo oxidation (Gómez Ros et al., 2007). In general, basic and neutral peroxidases do not efficiently oxidize sinapyl alcohol owing to steric hindrance of the substrate with the active site (Ustergaard et al., 2000). In contrast, acidic peroxidases can oxidize sinapyl alcohol (Sasaki et al., 2004; Gabaldón et al., 2005), indicating that both basic and acidic peroxidases have complementary roles in lignification.

BIOTIC STRESS, ROS, AND PEROXIDASES

An essential function for class III peroxidases is to protect the cellular membranes against oxidative damage. More specifically, class III secreted peroxidases are players in both reactive oxygen species (ROS) removal and ROS generation (Passardi et al., 2005). Although high levels of ROS are deadly, sub-lethal levels of ROS can serve as signals, prompting cells to prepare for sustained oxidative stress (Miller et al., 2009; Torres, 2010). Due to the potential exposures of deadly ROS, higher plants have evolved enzymes to detoxify these molecules (Mitchel et al., 1999). Catalases, peroxidases and superoxide dismutase have all been documented as ROS scavengers in plants stressed by insects and pathogens (Felton et al., 1994b; Heng-Moss et al., 2004; Franzen et al., 2007; Kusnierczyk et al., 2008).

Plants have also evolved complex signaling networks intended to detect specific pathogens in order to trigger the appropriate

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defense responses. A growing body of evidence suggests that plants have evolved intricate mechanisms to exert control over pathogen-induced defense pathways. The hypersensitive response (HR) is a complex early defense response that causes necrosis and cell death that can restrict the growth and spread of a pathogen. This interaction leads to a change in the membrane potential and ion permeability of the host cell plasma membrane resulting in localized cell death (Health, 2000). One of the first biological responses of the HR is an oxidative burst which includes the generation of ROS including hydrogen radicals (−OH), nitric oxide (NO), hydrogen peroxide (H2O2), and superoxide (Apostol et al., 1989). Left unchecked, these ROS may cause protein, lipid, and nucleic acid damage (Baldwin-Arjona and Ariza, 2009; Sharma et al., 2012).

PEROXIDASES AND DEFENSE AGAINST INSECTS

Insect infestation and herbivory has often been linked to changes in cellular ROS and peroxidase activity (Hiraga et al., 2001; Ni et al., 2001; Kawanos, 2003; Heng-Moss et al., 2004; Pasandl et al., 2005; Torres, 2010; O'Brien et al., 2012; War et al., 2012). Plant peroxidase levels in response to hemipterans have been particularly well-studied (Hildebrand et al., 1986; Felton et al., 1994; Stout et al., 1999; Ni et al., 2001; Heng-Moss et al., 2004). It has been observed that peroxidase levels increase following chewing bug and aphid feeding in tolerant buffalograsses, sorghum, and barley (Heng-Moss et al., 2004; Franzen et al., 2007; Gulsen et al., 2007, 2010). Recently, studies have shown that in wheat, rice, switchgrass and tomato class III peroxidases transcripts were upregulated in response to insect herbivory (Dowd and Johnson, 2009; Liu et al., 2010; Suzuki et al., 2012; War et al., 2012).

It is likely that the abundance of several class III peroxidases identified in switchgrass (see below) will also play significant roles in tolerances against insects. Previous global analysis in rice and wheat challenged with Hessian fly attack identified 34 class III peroxidases that were upregulated in resistant plants versus 22 peroxidases in susceptible plants (Liu et al., 2010). In Arabidopsis, two peroxidases, At5g64120 (AtPrx71) and At5g05340 (AtPrx52), were found to be induced by Pieris brassicae eggs were also induced by Pieris rapae herbivory (Little et al., 2007). For a better understanding of the roles of individual peroxidases in switchgrass against hemipterans, experiments using both resistant and susceptible cultivars challenged with a variety of these potential pests should be conducted. It is likely that class III peroxidases will display similar roles in switchgrass when challenged with hemipterans as well. Currently, roles of most of these proteins in plant defense or resistance in switchgrass are not known, but appear to be important areas of future research.

ENERGY CROP–INSECT INTERACTIONS

One of our long-term research goals is to develop a molecular understanding of switchgrass responses to hemipterans, utilizing a selection of tetraploid switchgrasses. The incidence of arthropod pests is likely to increase in switchgrass systems due to an anticipated shift to monoculture-based biomass production systems (Vogel et al., 2011). Additionally, due to plant-breeding efforts to reduce traits that interfere with biofuel processing, some plant defense mechanisms may be negatively impacted (Nabity et al., 2012). While switchgrass may be one of the better studied warm-season native grasses, most research has focused on agro-ecological qualities and disease issues. Few studies have examined the arthropod communities associated with switchgrass (Boerner and Harris, 1991; Gottwald and Adam, 1998; Kindler and Daly-rymple, 1999; McIntyre and Thompson, 2003; Schaefter et al., 2011). This basic information is required to identify and define the organisms that may cause reduced yields. Although much uncertainty exists in predicting which insects will be the key pests of this new market-use of switchgrass, recent research has provided clear evidence that biomass crops are susceptible to a number of key pests of other important crop plants (Prasifka and Gray, 2012). In switchgrass, potential pests have included stem-boring insects (Prasifka et al., 2010, 2011b), defoliators (Prasifka et al., 2009, 2011a) and piercing-sucking insects (Schaefter et al., 2011; Burd et al., 2012). Some of these insects have broad host ranges with multiple biotypes (Prasifka et al., 2009), while others are apparently very specific to switchgrass (Adamski et al., 2010; Prasifka et al., 2010).

Importantly, our development of switchgrass as a biofuel feedstock is, in part, a response to global climate change (Energy Independence and Security Act of 2007, 42 U.S.C. §17001), to which some desirable insect species (Pelini et al., 2010) will not adapt and some important pest species will overcome (Davis et al., 2006). Aphids (winged aphids in particular) transmit viruses with their mouthparts and are the most predominant vector for plant viruses (Hall, 2001). Depending on the virus, they can remain on the aphid’s mouthparts (specifically, their stylets) or circulate throughout the vector (some viruses replicate within the aphid) prior to transmission. Aphids are well-known to sample plant tissues (i.e., probing behavior) with their stylets to determine host acceptability. Additionally, some aphid species alternate between plant species as a function of their seasonal life cycle. This probing and host-alternation behavior of aphids is highly conducive to the introduction of new plant–virus relationships. For the above reasons, we have begun to explore the potential for targeting peroxidases in switchgrass genotypes for focused plant-breeding efforts.

PHYLOGENETIC RELATIONSHIPS OF SWITCHGRASS PEROXIDASES

Peroxidases are grouped into one of two superfamilies. One superfamily, the peroxidase-cyclooxygenase superfamily, generally consists of animal peroxidases that are structurally unrelated and important in the innate immune system (Soderhall, 1999). The other superfamily, the peroxidase–catalase superfamily, includes plant, fungal, and bacterial peroxidases. The catalase–peroxidase superfamily is further divided into three
distantly related structural classes (Welinder, 1992). Plant peroxidases fall into the first and third classes and are heme-containing enzymes that play key roles in important biological processes such as biosynthesis of lignin, degradation pathways and host-defense mechanisms. Class I peroxidases are intracellular peroxidases without signal peptides, calcium ions, or disulfide bridges. They show moderate substrate specificity of ascorbic acid and are located in the chloroplasts, mitochondria, peroxisomes, and the cytosol (Almagro et al., 2009). Extracellular secretory fungal peroxidases comprise class II peroxidases (Reddy, 1995) that include lignin-modifying peroxidases and manganese peroxidases. Class III peroxidases are glycoproteins that are located in vacuoles and cell walls and further divided into eight distinct groups based on sequence (Passardi et al., 2004). These peroxidases are involved in cell elongation, cell wall construction, and responses to various abiotic stresses and biotic plant pathogens (Intapruk et al., 1994; Klotz et al., 1998; González-Rábade et al., 2012).

In the PeroxiBase database (http://peroxibase.toulouse.inra.fr/), a total of 8,695 peroxidases have been collected. Of these, 5,430 (approximately 61%) are class III peroxidases which have been identified (March, 2013) from multiple plant species. For example, the genomes of Brachypodium distachyon, Arabidopsis thaliana, and Oryza sativa appear to code for 143, 73, and 155 class III peroxidases, respectively (Welinder et al., 1996; Passardi et al., 2004; Vogel et al., 2008). Comparisons of the peroxidase families between rice and Arabidopsis led to a better understanding of the evolution of monotocots and dicots that diverged from a common ancestor 150 million years ago (Wikström et al., 2001). Monocotyledon peroxidases differ slightly in intron/exon site and structure from eudicotyledons, but the majority of class III peroxidases are highly conserved.

In switchgrass, the preliminary classification of sequences obtained from a cDNA library showed class III peroxidases were extremely well-represented, with approximately 400 ESTs identified (Tobias et al., 2008). Using representative proteins belonging to different peroxidase groups in rice, all putative matches were identified in the switchgrass genome using the Blastp algorithm (Altschul et al., 1990, 1997) and an e-value threshold of 1 × 10−5. Putative matches and representative rice protein sequences were aligned using FastTree (Price et al., 2009) and visualized using Dendroscope version 2 (Huson et al., 2007). Two major peroxidase subfamilies, the heme and thiol peroxidases were separated. The thiol peroxidases were further subdivided into glutaredoxins, peroxiredoxins and glutathione peroxidases. The heme-containing switchgrass peroxidases were separated into class III and ascorbate (Class I) peroxidases (Figure 1). The class III peroxidases were further analyzed by reclustering and seeded with rice proteins belonging to different evolutionary groups of class III peroxidases (Passardi et al., 2004). Based on these analyses, the ancestral switchgrass peroxidase genes were identified as Pavirv00010935m, Pavirv00050429m, Pavirv00055443m, and Pavirv006000322m. The monocot-specific clade Group V.1 contained 33 members (Figure 2).

A comparison of switchgrass peroxidases with two rice class III peroxidases induced during gall midge attack (Lin et al., 2010) found the most similar switchgrass gene for Ochoterenaea zeae was Pavirv00015572m and for Os06g0347400 was Pavirv00059991m.

In rice, these two genes were significantly elevated in expression at 12 h post attack and eventually decreased in expression after 72 h post. It is possible that these two class III peroxidases in switchgrass may be potential sources for ROS production and defense during an insect attack as well. Two other class III peroxidases associated with insect defense from Arabidopsis (Cosio and Dunand, 2009) share similarity with switchgrass peroxidases. The Arabidopsis protein PRX52 (At5g64120) shares similarities with Pavirv000138711m and PRX71 (At5g64120) is homologous to Pavirv00606707m. These findings shed light on possible peroxidase targets to evaluate during insect attack.

GLOBAL ANALYSIS OF PEROXIDASE TRANSCRIPTS IN SWITCHGRASS TISSUES

To probe the profiles of class III peroxidase expression in different switchgrass populations and plant organs, we queried transcriptomic datasets obtained using NGS platforms (Roche 454 Life Sciences instrument; Table A1 in Appendix). Publically available NGS datasets from two contrasting ecotypes of switchgrass (lowland cv. Alamo and upland cv. Summer) were mined to obtain class III peroxidase expression profiles in different tissues (Figure 3). There appear to be significant differences in the profiles of class III peroxidase genes expressed in the different tissues based on plant developmental stage and ecotypes. Clusters of class III peroxidases were strongly represented in roots and shoots harvested from plants at early vegetative (EV), stem elongation (SE), and reproductive (RP). At each of these stages in Summer, there appeared to be strong transcriptional control of the expression of specific sets of class III peroxidase genes. As an example, many peroxidases were upregulated in roots during the SE stage of harvest (Figure 3), possibly related to greater root growth at this stage of plant development. Transcripts for many of these genes were less abundant at the RP stage, although expression of a different cluster of genes in the roots of Summer plants was apparent. In the shoots of Summer plants, greatest abundance in peroxidase transcripts apparently occurred during stages of active tiller elongation (EV and SE; Figure 3), suggestive of roles in cell wall formation.

![Figure 1] Phylegetic clustering of heme- and thiol peroxidases (Prx) present in the switchgrass genome. Heme peroxidases (oval) consist of the class I ascorbate peroxidases (green), and class II peroxidases (red). Thiol peroxidases (circle) consist of the glutathione peroxidase (purple), peroxiredoxin (blue) and glutaredoxin (cyan).
A small cluster of peroxidases were upregulated in Alamo flow-
er. Similar to what was observed from these NGS datasets,
and active tiller growth processes. There was enrichment in trans-
cripts for a small number of potentially organ-specific peroxidases
in flowers. In the lowland cultivar Alamo, there appeared to be
notable differences in class III peroxidase gene expression patterns
relative to Summer plants. Maximal transcript abundances for
roots were observed at the EV stage of growth, and a majority of
these transcripts were found in lower abundances at later har-
est dates. However, the physiological significance of these initial
observations is unclear. For Alamo shoots, a similar pattern to
those described for roots were seen, except that highest apparent
transcript abundance was seen at the SE stage of growth. Based on these data, it is apparent that different peroxi-
dases become active in switchgrass crown and rhizome tissue as
the plant transitions from active growth through flowering and
into dormancy, and that the study of class III peroxidases is likely
to yield significant insights into switchgrass developmental pro-
cesses and the interactions of the plant with biotic and abiotic
stress.

An analysis of crown and rhizome transcriptomes for expres-
sion profiles of class III peroxidases obtained from field-grown
plants is shown in Figure 4. Tissues were harvested from plants
over the course of a growing season as described earlier (Palmer
et al., 2011). Since these tissues are critical for perenniality of the
plants, knowledge of the molecular mechanisms that might impact
perenniality will be useful both from a biological and breeding per-
spective. Below ground herbivory and attack from other pathogens
can result in reduced shoot biomass, negatively impact plant sur-
vival and overall system sustainability (for example corn root
worm, nematodes, etc.). Peroxidases can serve as effective markers
for plant stress (reviewed above) and understanding expres-
sion profiles will provide insights into the cellular state of these
tissues.

As observed for other switchgrass tissues (see Figure 3), there
were clusters of peroxidases overexpressed at specific harvest dates
(Figure 4). These field harvest dates coincided approximately to
spring emergence (green-up; May), late vegetative (June), flower-
ing (July), late seed set (August), and senescence of aerial tissues
after a killing frost (October). The largest number of strongly
upregulated peroxidases appeared to occur in May and June, coin-
ciding with a time of rapid vegetative growth, somewhat similar to
the patterns seen in the Alamo datasets. The total numbers of peroxidase transcripts exhibiting greater expression declined at
the last three harvest dates, with relatively few genes overex-
pressed at the August and October harvests (Figure 4). Some of
these genes appear to be specific for a given harvest date, and are probably reflective of the developmental stage of the
plants. These highly expressed genes were separable into six clus-
ters (C1–C6, Figure 4). Peroxidase distribution in these clusters
based on their phylogenetic classification (Figure 2) suggested
members of different groups became active at various times
throughout the growing season. In May, highly expressed per-
oxidases clustered mainly into C4, with some upregulation in
C3 and C2 apparent. In C4, the highest numbers of peroxi-
dases came from Group I and Group IV, with lower amounts
in other groups. In June, most peroxidase upregulation clas-
tered into C3, with some upregulation in C1. In the larger C3
cluster, upregulated peroxidases were found again in Groups I
and IV, with the latter containing double the number of upreg-
ulated members when compared to May. In July, upregulated peroxidases clustered into two groups (C1 and C2); cluster C1
had highly expressed members at approximately equal levels from
Groups III, IV, V, and VI while cluster C2 contained a slightly
different breakdown: Group I, V, and VI contained most of the
apparently upregulated peroxidases. August and October peroxi-
dase expression mainly clustered into C3 and C6, respectively,
which showed upregulation primarily in Groups I and IV in both
cases. Based on these data, it is apparent that different peroxi-
dases become active in switchgrass crown and rhizome tissue as
the plant transitions from active growth through flowering and
into dormancy, and that the study of class III peroxidases is likely
to yield significant insights into switchgrass developmental pro-
cesses and the interactions of the plant with biotic and abiotic
stress.

![Phylogenetic relationships and numbers within each evolutionary clade, as defined for rice, for switchgrass class III peroxidases. The distribution of peroxidases relative to the out-group sequences (blue lines) are shown in the circular phylogram. The rice and ancient peroxidase protein sequences used as representatives for these analyses, and the numbers of switchgrass members in each clade are indicated. The peroxidase sequences include loci from both the A and B genomes.](image-url)
CONCLUSION

Sustainable production of switchgrass and other bioenergy grasses will require effective management against biotic stressors. The need to raise these crops on marginal land with lowered inputs will necessitate developing cultivars with enhanced tolerance to a range of biotic and abiotic stresses. We are only now beginning to probe the genotypic diversity that exists in switchgrass populations to potential insect pests. Identification of potential insect pests and detailed characterization of the plant–insect interaction will better enable us to address emergent insect pests in switchgrass production fields. Additionally, it is unclear how manipulation of plants for quality traits (for example lower/higher lignin) will affect plant resistance to insect herbivory and other endogenous mechanisms that confer resistance. However, based on extensive scientific literature, it can be safely predicted that the class III peroxidases are going to play a key role in the defensive mechanisms of switchgrass plants to insect herbivory, specifically to insects containing piercing-sucking mouthparts. The combination of genomic resources and improved phenotyping methods are likely to help decipher these molecular circuits, and provide guidance for the continued improvements of switchgrass as a bioenergy feedstock.

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### APPENDIX

**Table A1 | Switchgrass NGS datasets.**

| Cultivar | Time          | Tissue         | # Reads   | SRA         |
|----------|---------------|----------------|-----------|-------------|
| Summer   | May           | Crown          | 445,432   | SRX257007   |
| Summer   | June          | Crown          | 379,264   | SRX257030   |
| Summer   | July          | Crown          | 317,557   | SRX257031   |
| Summer   | August        | Crown          | 930,114   | SRX102934   |
| Summer   | October       | Crown          | 389,555   | SRX257032   |
| Summer   | Early vegetative stage | Root | 265,190 | SRX026147 |
| Summer   | Early vegetative stage | Shoot | 211,124 | SRX026148 |
| Summer   | Stem elongation stage | Root | 187,263 | SRX026150 |
| Summer   | Stem elongation stage | Shoot | 210,071 | SRX026149 |
| Summer   | Reproductive stage | Root | 240,166 | SRX026153c |
| Summer   | Reproductive stage | Shoot | 228,101 | SRX026151c |
| Summer   | Reproductive stage | Flower | 240,696 | SRX026155c |
| Alamo    | Early vegetative stage | Root | 1,317,713 | SRX057831 |
| Alamo    | Early vegetative stage | Shoot | 1,298,485 | SRX057830 |
| Alamo    | Stem elongation stage | Root | 1,113,868 | SRX057829 |
| Alamo    | Stem elongation stage | Shoot | 1,407,916 | SRX057828 |
| Alamo    | Reproductive stage | Root | 1,032,727 | SRX057826 |
| Alamo    | Reproductive stage | Shoot | 52,757 | SRX057827 |
| Alamo    | Reproductive stage | Flower | 1,143,746 | SRX057834 |