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Gene Expression Profiling and Shared Promoter Motif for Cell Wall Modifying Proteins
Expressed in Soybean Cyst Nematode Infected Roots

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ABSTRACT

We hypothesized that soybean cyst nematode (SCN) co-opts part or all of one or more innate developmental process in soybean to establish its feeding structure, syncytium, in soybean roots. The syncytium is formed within the vascular bundle by partial degradation of cell walls and membranes between adjacent parenchyma cells. A mature syncytium incorporates as many as 200 cells into one large multinucleated cell. Gene expression patterns for several cell wall modifying proteins were compared in multiple tissues undergoing major shifts in cell wall integrity. These included SCN colonized roots, root tips where vascular differentiation occurs, flooded roots (aerenchyma), adventitious rooting in hypocotyls, and leaf abscission zones. A search in the 5’ upstream promoters of these genes identified a motif (SCNbox1 - WGCATGTG) common to several genes that were up-regulated in several different tissues. The polygalacturonase 11 promoters (GmPG11a/b) include the SCNbox1 motif. The expression pattern for GmPG11a was examined further in transgenic soybean containing a PG11a promoter fused to a β-glucuronidase (GUS) reporter gene. GUS expression was highest in cells undergoing radial expansion in the stele and/or cell wall dissolution. GUS staining was not observed in cortical cells where a lateral root tip or a growing nematode emerged through the root cortex.

Key words: abscission, adventitious roots, aerenchyma, cell wall, gene expression, GUS, Heterodera glycines, polygalacturonase, soybean cyst nematode, syncytium
INTRODUCTION

Soybean cyst nematode (SCN) forms a large multinucleated feeding cell (syncytium) within the vascular cylinder of the soybean root by inducing the degradation of cell walls and membranes between adjoining cells (Goverse et al., 2000). As many as 200 plant cells may be incorporated into a single syncytium (Jung and Wyss, 1999). Evidence indicates that the nematode secretes proteins that can alter plant cellular processes (Davis et al., 2008). The signals and plant processes involved in forming the feeding structure are of academic and practical importance. We hypothesized that SCN secreted proteins must co-opt one or more innate developmental processes in soybean to complete the necessary structural changes required for formation of the feeding structure. Since formation of the syncytium requires cell wall degradation between cells, one of the processes evoked by the nematode must include a coordinated expression of proteins that disassemble the cell wall.

The primary cell wall is comprised of many different polysaccharides (80%) and structural proteins (20%) (Carpita and Gibeaut, 1993). Nevertheless, the primary constituents of the wall are cellulose microfibrils and xyloglucan polymers, which crosslink and interlock the microfibrils into a semi-rigid framework (approximately 50% of the wall mass). This framework is intern embedded in a matrix of pectins (approximately 30%) made up primarily of polygalacturonic acid. The pectin matrix determines the porosity of the cell wall and may limit access of some enzymes to specific regions of the cellulo-xyloglucan framework. The middle lamella, which cements together the primary cell walls of adjacent cells, is mostly polygalacturonic acid (Carpita and Gibeaut, 1993). Thus, the complex nature of the cell wall requires coordinated expression of multiple enzymes to separate the cells and disassemble the wall matrix. The specific targets for the cell wall enzymes are generally unknown but are broadly classified by the type of substrate they degrade, e.g., carboxymethyl cellulose for cellulases and polygalacturonic acid for polygalacturonases, and their mechanism of bond cleavage, e.g., hydrolase, lyase, etc. Expansins are a particularly interesting class of cell wall protein that have no known enzymatic activity but generally aid in the loosening of cell walls in both cell expansion and cell wall degradation events (Li et al., 2003).

As a first step to understand the mechanism of regulation for the organized restructuring of cells to form the feeding structure, we cloned 30 transcripts for cell wall associated proteins (9 cellulases, 3 expansins, 14 polygalacturonases, 2 pectate lyases, and 2 xyloglucan...
endotransglucosylase/hydrolase) from SCN-infected roots and several were shown to be up-
regulated during SCN infection (Tucker et al., 2007). Several of the same genes were also
expressed in root tips where vascular development occurs and in leaf abscission where extensive
cell wall degradation leads to separation of the petiole from the stem (Tucker et al., 2007).

In addition to gene expression in root tips and abscission that was examined previously
(Tucker et al., 2007), it was hypothesized that the gene expression profile for cell wall proteins in
SCN colonized roots might be similar to that for formation of aerenchyma -- increased
intercellular porosity and air channels that provide gas diffusion during flooded conditions. In
addition to examining gene expression in flooded roots, gene expression in hypocotyls induced
with auxin and ethylene to form adventitious roots was also examined. The induction of
adventitious rooting in hypocotyls elicits multiple cell wall degradation events, which includes
the formation of aerenchyma, but also cell wall loosening where the roots emerge through the
cortex of the hypocotyl. We hypothesized that a similar process of loosening of cells in the
cortex where roots emerge may also be evoked by the nematode when it expands through the
root cortex. Tucker et al., (2007) reported that a polygalacturonase (PG11) was up-regulated in
SCN infected roots but was not strongly expressed in root tips where cell division, cell
elongation and vascular differentiation occur. Thus, PG11 might be a candidate gene for up-
regulation during aerenchyma development and/or expression in the splitting of the cortex to
facilitate emergence of a lateral root initial or the emergence of a growing nematode. To
examine these possibilities we’ve fused a PG11 promoter to a GUS reporter gene and stably
transformed the construct into soybean.

The purpose for identifying innate developmental processes that might be co-opted by SCN
during formation of its feeding structure is to identify shared molecular signaling mechanism that
might be used in the above plant restructuring processes. Thus, the gene promoters for cell wall
modifying proteins whose transcripts were strongly up-regulated in SCN infected roots were
searched to identify motifs common to these genes.

In addition to information that relates to formation of the feeding structure for SCN,
observations of gene expression and morphological changes in the taproot and hypocotyls during
flooding are also of general interest. Bailey-Serres and Voesenek (2008) describe three forms of
aerenchyma: (a) lysigenous aerenchyma formed by cell death, (b) schizogenous aerenchyma in
which gas spaces develop through the separation of previously connected cells, and (c)
expansigenous aerenchyma, which is characterized by intercellular gas spaces that develop through cell division and cell enlargement, without cell separation or collapse/death. Lysigenous and schizogenous aerenchyma forming within the cortex have been termed primary aerenchyma (Thomas et al., 2005). Expansigenous aerenchyma may be similar to what has been termed secondary aerenchyma in adventitious rooting soybean (Thomas et al., 2005). Secondary aerenchyma arises from new cell divisions in the pericycle that give rise to a more spongy parenchymous tissue that displaces the cortex causing it to break away from the body of the plant (Thomas et al., 2005). In the current project there was evidence of primary aerenchyma, both lysigenous and schizogenous, in the flooded hypocotyls and possibly the beginning of the formation of secondary aerenchyma at the inner layer of the cortex.

RESULTS

QPCR and Morphology

Cell separation associated with aerenchyma development in soybean roots might have gene expression patterns similar to those associated with formation of the SCN syncytium, and the two processes may share regulatory signals. To test this hypothesis, the root systems of 14 day-old plants were submerged in water and whole root systems (WR) collected at 0, 1, 3, and 7 days and the RNA extracted. The hypoxic environment caused by the flooding elicits the formation of aerenchyma (Fukao and Bailey-Serres, 2004). Very few of the transcripts examined were up-regulated in these flooded roots (Fig. 1). Up-regulation of ADH gene expression was used in this study to confirm hypoxic conditions. The expression of three different ADH genes was examined, \( ADH1 \) (AF079058), \( ADH2 \) (AF079499), and \( ADH4 \) (putative ADH gene identified in genomic sequence, Supplement File S1). Only one, \( ADH2 \), markedly increased during flooding (Fig. 1). No lysigenous aerenchyma (air channels or well defined holes in the cortex) were seen in sections of the secondary roots (data not shown); however, after 7 days the primary taproot displayed a generalized separation of cortical cells (Fig. 2) that had the appearance of schizogenous aerenchyma (separated loosely packed cells) as described by Thomas et al. (2005).

Another tissue that undergoes considerable cell separation is hypocotyls induced with auxin and ethylene to form adventitious roots. A primary objective for the hypocotyl experiments was to examine the gene expression for cell wall proteins in the splitting of the cortex where the roots emerged. The loosening and splitting of the cortex is exaggerated in the rooting hypocotyls.
because so many roots emerge along a longitudinal split in the hypocotyl (Fig. 3e and 3j). In addition to splitting and root initiation, hypocotyls submerged in water for more than 4 days displayed a typical pattern of cell separation expected for primary lysigenous aerenchyma, appearing as holes in the transverse sections (Fig. 3f and 3g). After 8 days cells of the cortical layer outside the ring of phloem were loosely connected and easily shed with any mechanical contact to the extent that it made sectioning of this tissue difficult (Fig. 3k). The loose packing of the cells in the cortex looked similar to that in the taproots after 7 days of flooding and is presumed to be schizogenous aerenchyma formed by separation and enlargement of mature cortical cells (Thomas et al., 2005; Bailey-Serres and Voesenek, 2008). The pattern of gene expression in the rooting hypocotyls shares similarity with that for the flooded roots, which suggested that a large part of the change in gene expression for cell wall modifying proteins was more closely associated with formation of aerenchyma than initiation of adventitious roots (Fig. 1).

To further support that the observed changes in gene expression in the rooting hypocotyls were more closely associated with the stem rather than the adventitious roots, the root tips were trimmed off the 8 day hypocotyls and RNA extracted from both the root tips and trimmed hypocotyls. Although gene expression in these separated tissues was not markedly different, there were a few differences of interest (Fig. 1). $PG1$, $PG5$, $PG11a$, and $PG12a/b$ transcripts were more abundant in the trimmed hypocotyls than the adventitious roots. This same group of genes was also of lesser abundance in the first 12 mm of the root tips (Fig. 1), which suggests that their primary role is not in vascular development in the root tip but some other function. Other transcripts, e.g., $Cel7a$ and $PG16a$, were higher in the root tips than the trimmed hypocotyls and they were also strongly expressed in the root tips collected from the root system (Fig. 1). Moreover, $Cel7a$ and $PG16a$ were not up-regulated during flooding.

PG11a::GUS Expression

The $PG11a$ gene was selected for more detailed study because it was observed to be strongly up-regulated in SCN infected root pieces and abscission zones but not as strongly expressed in root tips or leaves. It was hypothesized that $PG11a$ might be expressed during syncytium development, aerenchyma formation and/or the splitting of the cortex where the lateral root and nematode emerge. To further examine the tissue and cell-specific expression of
the *PG11a* gene (Supplement File S1) the *PG11a* promoter was fused to a GUS reporter gene and stably transformed into soybean. Histochemical GUS staining indicated that expression increased first in the stele of the root at the site of SCN infection (Fig. 4a). A transverse section of a lateral root with a young female nematode attached stained darkly for GUS activity in the stele (Fig. 4a). Cross sections of lateral roots that were not infected with SCN also showed GUS staining in the stele but staining was less intense than where a nematode was attached (result not shown). The stele immediately below a young adult nematode and either side of the nematode is generally darker blue and enlarged in comparison to a root piece without an attached nematode (Fig. 4a). At this stage of nematode development the syncytium immediately below the nematode is clearly evident and the cell walls that separated cells incorporated into the syncytium are mostly degraded (Fig. 4b and 4c). As the nematode and syncytium mature, the GUS stain disappears near the nematode but continues to be prevalent at short distances from the nematode (Fig. 4e). A thin section prepared from a root piece with a more mature adult female nematode attached shows the greatest amount of GUS stain at the ends of the expanding syncytium (Fig. 4g). In some sections of lateral roots with more mature nematodes attached GUS staining could also be seen in cortical cells near the ends of the growing syncytium (result not shown). GUS staining, however was not observed in the split in the cortex where the nematode emerged through the cortex.

PG11a::GUS expression also increased in hypocotyls induced to form adventitious roots (Fig. 3). The rooting hypocotyls provide several different developmental processes and cell types that could be examined for cell-specific expression of the *PG11a* promoter, i.e., root initiation and attending vascular development, formation of aerenchyma, and cortical cell separation immediately above the growing root initial. In hypocotyls stained for GUS activity prior to treatment with auxin and ethylene (0 days) the stain was distributed fairly evenly across the entire hypocotyl (Fig. 3d). At 4 days post treatment, GUS staining increased and was more evident around the sclerenchyma, thick walled dead cells at the border of the phloem and cortex (Fig. 3h and 3i). The cortex and pith were also stained at 4 days (Fig. 3i), although less than around the sclerenchyma. After 8 days, GUS staining was mostly concentrated around the sclerenchyma cells (Fig. 3m and 3n). GUS staining was great enough at 8 days to be visible in thinner 15 μm sections (Fig. 3l). In 15 um sections GUS stain was greatest in cells immediately inside and between the sclerenchyma bundles.
GUS expression was also detected in abscission zones, petioles and leaves (Fig. 4). QPCR indicated that \textit{PG11a} expression was strongly up-regulated during abscission and that this increase was primarily restricted to the abscission zone (Fig. 4). Although histochemical staining for GUS activity indicated enhanced staining at the separation layer 2 days after induction of abscission (Fig. 4g), there was also staining throughout the petiole (results not shown). Moreover, where QPCR indicated a low level of \textit{PG11a} expression in leaves, GUS activity was relatively high in leaf tissue (Fig 4h). The reason for these discrepancies in \textit{PG11a} transcript accumulation and GUS activity might be due to missing regulatory elements in the 1951 bp \textit{PG11a} promoter used for the PG11::GUS construct, or elements in the nearby 35S CaMV promoter, which controls the expression of the selectable herbicide resistance marker, may have influenced the expression of the GUS transcript (Yoo et al., 2005).

13 \textbf{Conserved Sequence Motif}
14
15 Many of the transcripts that increased in abundance in the SCN infected roots also increased in root tips, rooting hypocotyls and abscission zones. As a first step in identification of common regulatory mechanisms, the promoters of these up-regulated genes (Supplemental File S1) were searched with MEME (Bailey and Elkan, 1994) to identify shared sequence motifs that might act as regulatory cis-acting elements. A MEME search identified a seven nucleotide sequence (GCATGTG) conserved in many of these gene promoters (Table I). Subsequent searches with AlignACE (Roth et al., 1998) and Weeder (Pavesi et al., 2004) identified the same motif in the SCN up-regulated genes. The seven nt sequence was subsequently extended to eight nt to include a conserved A/T (W) at the 5´ end. The WGCATGTG motif was named SCNbox1. Many of the genes that included the SCNbox1 motif were also expressed in root tips, rooting hypocotyls and abscission zones (Table I). Figure 5 displays a more detailed expression profile for one of each class of cell wall modifying proteins that also included at least one copy of the SCNbox1 motif in their gene promoter. Although not identical, the gene expression profile is similar for each of the genes that include the SCNbox1 motif (Fig. 5).

\textbf{DISCUSSION}

The experiments described herein had two broad objectives, (1) identify changes in gene expression common to SCN infection and other plant processes that involve cell wall loosening
and (2) search their gene promoters for shared sequence motifs. Together, this information could then be used to link already identified mechanisms of gene regulation to other less well characterized developmental processes and identify potentially new regulatory mechanisms.

Formation of the SCN induced syncytium is a complex process requiring a delimited degradation of existing cell walls and membranes between adjoining cells, and also the restructuring and synthesis of new cell wall material that strengthen the syncytium boundary as well as strengthen the many membranous protrusions into the syncytium that function to increase the surface area of the syncytium for nutrient uptake (Goverse et al., 2000). In regards to synthesis of new cell wall material, Cel12a/b are in a family of KORRIGAN type membrane cellulases that are most closely associated with cellulose synthesis in elongating cells (Molhoj et al., 2002). QPCR results indicate that transcripts for Cel12a/b were in all the tissues examined but were at their highest concentration 2-7 mm behind the apex of the root tip where cell elongation is greatest (Tucker et al., 2007). Cel12a/b were not up-regulated in flooded roots, rooting hypocotyls or abscission, but slightly up-regulated in SCN infected root pieces where cell wall synthesis might be expected. The expression and localization of KORRIGAN type cellulases in the syncytium has also been demonstrated by others (Goellner et al., 2001; Wang et al., 2007). Interestingly, the expression pattern for PG10a/b is very similar to Cel12a/b (Fig. 1). PG10a/b may also play a role in synthesis of new wall material rather than degradation of the cell walls.

Of all the developmental and environmental responses examined, the gene expression profile in root tips was most like that in SCN-infected root pieces (Fig. 1). However, there were a few notable exceptions. Transcripts for PG1, PG5, PG11a and PG12a/b were up-regulated in SCN infected root pieces but not particularly abundant in the root tips suggesting that these gene products are not involved in cell division, cell elongation, or vascular development but some other process. One process that might be expected to involve the expression of cell wall modifying proteins and would not be prominent in root tips is the formation of aerenchyma (Bailey-Serres and Voesenek, 2008). Aerenchyma formation is induced in mature parts of the soybean root system within 7 days of flooding (Thomas et al., 2005). The loose packing of cells in the taproot of flooded plants suggests that schizogenous aerenchyma, cell separation without cell death, formed within the 7 days of treatment; however, lysigenous aerenchyma, small air channels in the cortex formed by cell death, were not observed in the taproots (Fig. 2). However,
hypocotyls submerged in water with $10^{-5}$ M IBA and exposed to 1 $\mu$L L$^{-1}$ of ethylene produced
what appeared to be lysigenous aerenchyma after 4 days and schizogenous aerenchyma after 8
days (Fig. 3). Ethylene was proposed to be necessary for induction of cell death in lysigenous
aerenchyma (Drew et al., 2000) and the addition of exogenous ethylene to the hypocotyl
treatment but not the flooded roots may have enhanced formation of what appeared to be
lysigenous aerenchyma in the hypocotyls but not the roots.

The loose association of cells in the cortex in the tap roots of flooded plants (Fig. 2) and the
submerged hypocotyls (Fig. 3) resembles schizogenous aerenchyma. The marked change in cell
wall structure suggests an increase in cell wall modifying proteins. However, in the flooded
roots none of the genes examined displayed a large increase in expression. Nevertheless, Cel9,
PG1, and PG6a displayed a slight increase in expression during flooding of roots and in the
submerged rooting hypocotyls, and may, therefore, be associated with formation of schizogenous
aerenchyma. Expression of PG1 in SCN infected roots and hypocotyls was reported previously
(Mahalingam et al., 1999), but its association with flooding was not examined. It is likely that
additional genes for cell wall modifying proteins not included in this study increase during
flooding; nevertheless, the fact that the gene expression profile in the SCN colonized root pieces
is not similar to the profile for flooded roots indicates dissimilar mechanisms for regulating gene
expression in these two processes.

Transcript for $PG11a$ did not increase in the flooded roots but did increase in submerged
hypocotyls treated with auxin and ethylene (Fig. 1 and 5). Moreover, the increase in transcript
expression for $PG11a$ was restricted primarily to the stem rather than the adventitious roots (Fig.
5). Histochemical GUS staining for the PG::GUS construct was observed fairly uniformly across
the hypocotyl before submergence and treatment with auxin and ethylene (0 day) and began to
disappear from the cortex as lysigenous aerenchyma became apparent at 4 days (Fig. 3). Blue
stain was almost completely gone from the cortex at 8 days when schizogenous aerenchyma,
loose packing of cells, was prominent. The most intense increase in stain was associated with
cells nearer the phloem. In the 8 days of treatment the hypocotyls do not elongate but swell in
diameter (Kemmerer and Tucker, 1994). It is possible that cell division occurred in this region
near the pericycle; however, it seems more likely that existing cells were expanding radial.
Thus, expression of PG::GUS correlates with the swelling of the hypocotyl but does not appear
to be correlated with formation of primary aerenchyma in the cortex. Of interest in this regard is
that PG11a gene expression was not strong in the first centimeter of the root where cell elongation is prominent but was greater farther away from the root apex where radial expansion is greater (Fig. 1). We propose that PG11a expression is best linked to radial expansion of cells and/or dissolution of the cell wall as would occur in abscission and syncytium development and not formation of aerenchyma.

In addition to all the processes that would normally occur in hypocotyls during induction of adventitious roots (e.g., aerenchyma, cell division, vascular development, etc.), the hypocotyls also display an exaggerated separation of cells (splitting) where the roots emerge from the stem (Fig. 3). The loosening of cell walls immediately above an emerging lateral root is another process that would not occur in the root tip but would be prominent in other parts of the root system. Enzymatic cell wall separation has been reported for the loosening of cells above an emerging lateral root (Roberts et al., 2002), and more recently Lee et al (2011) demonstrated using a GUS reporter gene that a PG gene that is up-regulated at the site of nematode (H. schachtii) infection of Arabidopsis roots is also expressed in non-infected roots in a layer of cortical cells immediately above an emerging lateral root. Thus, this Arabidopsis PG may be linked to the loosening of cortical cells where the lateral root emerges. In regard to SCN infection of soybean roots, splitting of the roots is clearly evident where the female nematode begins to protrude from the root and the split continues to enlarge as the nematode expands through the split. Enzymatic separation of cells in the root cortex where the nematode emerges may be necessary for the nematode to expand through the cortex. Expression of the PG11a polygalacturonase was thought to be a possible candidate for this process because it is not strongly expressed in root tips (Fig. 1 and Fig. 4). However, no GUS staining was observed in the splits where nematodes emerged or in the splits in the rooting hypocotyls. Although other polygalacturonases may be associated with this splitting event, PG11a is not.

The expression of the PG11a promoter (GUS) in parenchyma cells surrounding the vascular system in the hypocotyl may suggest a regulatory mechanism in common with SCN-induced syncytium since the syncytium also forms from vascular parenchyma cells (Golinowski et al., 1996; Holtmann et al., 2000). Histochemical GUS staining was greatest in the stele of SCN infected roots (Fig. 4a). However, as the nematode and syncytium matured GUS stain disappeared at the immediate site of the nematode attachment (Fig. 4e) but remained high near the ends of the expanding syncytium (Fig. 4g). PG11a may play a role in opening up the pectin
matrix to provide access for other enzymes to the cellulo-xyloglucan framework and therefore 
GUS expression is most evident in cells at the initial phase of incorporation into the syncytium 
and not when the cell wall material is being digested inside an established syncytium boundary. 

A low level of GUS expression was also observed in cortical cells near the syncytium, but 
cortical cells are presumably not incorporated into the syncytium (Goverse et al., 2000). It’s 
possible that PG expression in the cortical cells may be part of a loosening process that allows 
the syncytium to expand into the cortex without breaking the cortex cells.

Regulation of gene expression is a complex process that often includes multiple cis- and 
trans-acting factors. Identification of sequence motifs in gene promoters for genes with similar 
expression patterns can lead to the identification of shared regulatory mechanisms (Romer et al., 
2007). In this regard, an 8 nt motif, SCNbox1 (WGCATGTG), was identified in many of the 
promoters for genes highly up-regulated in SCN infected root pieces and also correlated to some 
extent with up-regulation in other cell wall loosening processes (Table I). Of interest here is that 
Cel12a/b and PG10a/b, which were expressed in SCN infected root pieces but not abscission or 
aerenchyma, do not include the SCNbox1 motif. Expression of Cel12a/b and PG10a/b may be 
more closely linked to cell wall synthesis than degradation and be regulated differently than 
genes linked to cell wall dissolution.

The SCNbox1 sequence includes the CATGTG motif sometimes referred to as an E box, 
which has been most extensively characterized in animal systems (Aksan and Goding, 1998; 
Vallone et al., 2004). Nevertheless, in plants, the CATGTG motif was reported to function in the 
water stressed induction of erd1 in Arabidopsis (Simpson et al., 2003). The same sequence was 
also found in other drought induced genes. In a subsequent more precise examination of an 
Arabidopsis 63 nt erd1 fragment that was required for induction by drought it was discovered 
that a NAC-domain transcription factor bound to this element and the sequence requirements for 
NAC binding were best represented by CACGCATGT, which includes all but the last nucleotide 
of the original CATGTG motif (Tran et al., 2004). NAC transcription factors are of interest as 
possible candidate regulatory components in the current study because they’ve been 
demonstrated to be involved in root tip development and in lateral root initiation as well as 
defense and biotic and abiotic stress responses (Olsen et al., 2005). Arabidopsis includes more 
than 100 NAC domain transcription factors (Ooka et al., 2003), but only a few different NAC 
DNA binding sites have been characterized (Tran et al., 2004) and none match perfectly with the
WGCATGTG motif found in the soybean genes described here. The role, if any, for the
SCNbox1 motif remains to be determined; nevertheless, the occurrence of this motif in the
promoters for genes up-regulated by SCN infection does not appear to be random and therefore
of interest for further study.

Knowing which plant developmental processes and what parts of the process are most
similar to SCN infection provides clues and helps set priorities for testing and comparing
signaling events in SCN infected roots. All the tissues examined include multiple developmental
events. Nevertheless, ethylene is a plant developmental signal common to all the processes
examined, i.e., adventitious rooting (Kemmerer and Tucker, 1994), aerenchyma formation
(Bailey-Serres and Voesenek, 2008) and SCN feeding structure development (Goverse et al.,
2000; Tucker et al., 2010). Ethylene may also play a role in root tip maturation (Ivanchenko et
al., 2008). Clearly no one signal or molecular mechanism or transcription factor regulates all the
cell separation events examined but clustering the processes by shared gene expression profiles
identifies genes that bear further scrutiny as does the identification of a shared sequence motif
found in many of the genes up-regulated in the same processes.

MATERIALS AND METHODS

SCN Colonized Root Pieces, Root Tips and Abscission Zones

Plants were grown and tissues prepared as previously described (Tucker et al., 2007).
Briefly, soybean (Glycine max, cv Williams 82) were germinated and grown in the greenhouse in
Perlite for 2 weeks. For each time interval (8, 12 and 16 dpi) six plants were transferred to a
shallow plastic box and the roots inoculated with 5,000 J2 SCN (Heterodera glycines, population
NL1-RHg, HG-type7, race 3) or mock inoculated. Root pieces from SCN and mock inoculated
roots (1 to 5 mm in length), and root tips from non-infected roots were dissected from the roots
under a stereomicroscope and then frozen in liquid nitrogen. Stem-petiole explants for
abscission zone and petiole collection were prepared from 2 week-old plants that were exposed
to 25 μL L⁻¹ ethylene at 25°C for 0, 1 or 2 days. Abscission zones (approximately 2 mm) were
harvested from the upper abscission zone immediately below the leaf blade. The lower
abscission zone was not collected to avoid the possibility of inadvertently collecting part or all of
the lateral bud. The petiole material was taken between the two abscission zones at either end.
All tissues were frozen in liquid nitrogen and pulverized with a mortar and pestle. Total RNA
was extracted from pulverized tissues using a Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, CA).

Flooded Plantlets

Seeds were surface sterilized in 95% ethanol for 3 min followed with 10% bleach for 10 min and then rinsed with distilled water. Seeds were planted in Perlite and grown 3 weeks in a growth chamber at 23°C with 15 hr light. Perlite was removed from the roots and the roots submerged in water up to the base of the stem. At 1, 3 and 7 days post submergence the entire root systems of each plant was harvested at the root stem juncture and frozen in liquid nitrogen. For microscopy, pieces of lateral and taproots were fixed in an ethanol acetic acid mixture at a ratio of 3:1.

Adventitious Rooting

Intact auxin and ethylene response pathways are necessary to get a profuse emergence of adventitious roots (Kemmerer and Tucker, 1994). To maximize adventitious rooting, 16-day-old plants were cut approximately 1 cm above the soil and the hypocotyls of the explants with the leaves still attached were submerged approximately 4 cm into a beaker containing 10^{-5} M IBA in water. The beakers were then placed in a 9 L glass chamber (desiccator) and ethylene injected to achieve a concentration of 1 μL L^{-1} ethylene. The desiccator was placed in a growth chamber at 23°C with a 15 hr light and 9 hr dark cycle. Every 24 hr the jars were opened for a few minutes, closed and the ethylene replenished. Approximately 4 cm of hypocotyl tissue was collected from 5 explants at the beginning of the experiment (0 day) and then again after 1, 4 and 8 days of treatment and frozen in liquid nitrogen. For histochemical GUS assay and sectioning for microscopy the hypocotyls were treated and collected in a similar fashion and stained for GUS activity and then fixed in ethanol and acetic acid (3:1) or fixed immediately without GUS staining.

QPCR

Procedures for Semi-quantitative real-time PCR and the PCR primers used to examine gene expression for cell wall modifying proteins were described previously (Tucker et al., 2007). A single bulk cDNA synthesis reaction (5 μg of DNased RNA) was performed and the cDNA
diluted to a larger volume to accommodate a large number of PCR reactions and thereby reduce

1 differences that might occur between cDNA synthesis reactions. Real-time PCR reactions were
2 completed using a Brilliant II SYBR Green QPCR Master Mix in an Mx3000P instrument
3 (Stratagene, La Jolla, CA). The real-time PCR signal for the constitutively expressed soybean
4 elongation factor (\textit{GmEFlb}, accession AK243885), which encodes a protein that’s part of the
5 ribosomal protein translation complex, was used to normalize all the RNA samples. Expression
6 for an actin (accession AK285258) and ubiquitin (accession AK285252) gene were also included
7 for comparison. The mean thresholds (Ct) and Ct standard deviations for EF1b, actin and
8 ubiquitin for the 65 cDNAs used in the experiments reported here were respectively 24.63 +/-
9 1.39, 24.97 +/- 1.27 and 21.31 +/- 1.00. In the heat map display only, the expression value was
10 set to 0.0005 if the relative expression of a gene was equal to or less than 0.0005. A relative
11 concentration of 0.0005 represents a PCR product detected at approximately 38 cycles. Setting a
12 lower limit of 0.0005 reduces potential artifacts associated with numerous PCR cycles and
13 eliminates ratios with a denominator of zero.

14 \textbf{Generation of Transgenic Plants:}

15 A soybean phage genomic library was screened for clones with similarity to a PG11 cDNA.
16 A 17 kb genomic insert was sequenced and when the soybean genomic sequence became
17 available the two sequences were compared. Only a few differences were found between the two
18 sequences. PCR was used to fuse a 1951 bp \textit{GmPG11a} gene promoter to a GUS reporter gene
19 immediately downstream from the ATG in the \(\beta\)-glucuronidase (GUS) open reading frame. The
20 promoter included an 88 bp 5´ untranslated region in the \textit{PG11a} mRNA and approximately 1860
21 bp upstream from a start of transcription predicted based on the 5´ end of the cDNA sequence
22 and the presence of a TATA-box motif 27 bp upstream from the end of the cDNA sequence.
23 The GUS open reading frame included an intron from the castor bean catalase gene 18 bp down
24 from the start of translation (Tanaka et al., 1990). The PG11a-GUS-NOS3 construct was cloned
25 into the \textit{Bam} H1/\textit{Eco} R1 site of pTF101.1 in the opposite orientation to the hygromycin
26 selectable marker gene. The Iowa State University Transformation Facility transferred this
27 construct into \textit{A. tumefaciens} (EHA101) and then transformed and regenerated transgenic
28 soybean (\textit{Glycine max}, Williams 82). Seven independent events displayed hygromycin
resistance and all seven tested positive for the GUS gene in a PCR genomic DNA assay. Five events displayed strong GUS staining in an abscission assay.

**GUS Assays**

Several methods of partial fixation and infiltration of 500 mg mL$^{-1}$ of GUS substrate (X-Gluc, 5-Bromo-4-chloro-3-indolyl b-d-glucuronic acid) in histochemical staining buffer (10 mM Disodium EDTA, 0.5 mM K$_4$Fe(CN)$_6$, 0.5 mM K$_3$Fe(CN)$_6$, 0.1% Triton X-100, 100 mM NaH$_2$PO$_4$, pH 8.0) were tested. Vacuum infiltration of the X-Gluc in histochemical staining buffer without any prior treatment produced superficial staining patterns at the cut ends or regions where the epidermal cells were damaged. Tissue fixation for 1 hour in 90% acetone at -20°C and then washing twice with a 5 minute vacuum infiltration in washing buffer (0.1 M phosphate, pH 7.0, 10 mM EDTA, 2 mM K$_3$Fe(CN)$_6$) followed with a third vacuum infiltration with 500 mg mL$^{-1}$ X-Gluc in histochemical staining buffer worked well for roots and was used for staining of root tissue (Weijers et al., 2001). In our hands, the procedure that worked best for the above ground plant parts was to collect the tissue in ice cold histochemical staining buffer without the X-Gluc and then heat the tissue samples in buffer to 55°C for 30 minutes. After heating the sample, the tissue and buffer were cooled on ice, X-Gluc added to 500 mg mL$^{-1}$ and the solution vacuum infiltrated for 2 min at approximately 29 inches of mercury (0.95 atm) and the vacuum released and repeated again. To improve infiltration of hypocotyls, which are relatively large, the hypocotyls were first sliced down the middle before the heat treatment.

**Microscopy**

Small pieces (1 cm) were cut from the taproots approximately 1 cm below the soil line and from the middle of 4 cm sections of hypocotyls. The taproot pieces were fixed in ethanol acetic acid (3:1) for >2 hr and then several changes of 100% ethanol. The hypocotyl pieces were fixed in 2.5% glutaraldehyde (v/v) in 0.2M Na Cacodylate buffer for 2 hr and then transferred to 100% ethanol. All tissues were then placed into 50/50 (v/v) L.R White/ethanol overnight followed by 100% L.R. Whites for 24 h. The L.R. Whites were replaced with fresh resin and cured in an oven at 60°C for 16 hr. Tissues were sectioned to a thickness of 1 to 15 μm on a Reichert/AO Ultracut microtome with a Diatome diamond knife and adhered onto glass slides. Some sections were stained with 0.1% (w/v) toluidine blue to enhance the outline of cells.
Supplemental Data

The following materials are available in the online version of this article.

Supplemental File S1. Fasta file of genomic sequences for cell wall modifying proteins used in this study. The coding sequence for an alcohol dehydrogenase (named *GmADH4*) identified in the *Glycine max* genomic sequence is also included.

ACKNOWLEDGMENTS

We thank Autar Mattoo and Edward (Pete) Masler for suggestions and review of the manuscript.

LITERATURE CITED


Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. The Plant Journal 3: 1-30


Table I. Summary of expression and position of WGCATGTG motif in the sequence 5’ to the start of translation of all the genes for cell wall modifying proteins included in Fig. 1.

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<th>Rooting hypocotyls</th>
<th>Abscission</th>
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a, b or c after the gene name indicate a paralogous gene.

PCR amplification predicted using MacVector’s Test PCR Primer Pairs. If a primer mismatch occurred at the 3’ end or there were multiple mismatches in a primer, amplification was predicted to not be likely.

Number of nucleotides upstream from the start of translation (ATG).

N/A - not applicable because the gene fragment was predicted to not be amplified with the primers used.

Large intron 2385 and 2211 bp in 5’ UTR of PG03a and PG03b, respectively.

Predicted start of translation (ATG) based on largest ORF 5’ to a predicted intron.
FIGURE LEGENDS

Figure 1. Heat map displaying the change in gene expression (log base 2 ratios) for multiple cell wall modifying proteins in different plant tissues and treatments. Abbreviations: WR, whole root system; AZ, leaf abscission zone. A dark box indicates strong up-regulation of gene expression whereas a white box indicates strong down-regulation. No change in expression is indicated by a neutral grey box and in-between expression levels as shades of grey. The QPCR results were all normalized to the elongation factor 1 beta (EF1b, accession AK243885). Also included for comparison are gene expression for an actin (accession AK285258) and ubiquitin (accession AK285252). SCN root pieces are log2 ratios for SCN infected tissue relative to non-infected root pieces aged and collected at the same time as the SCN inoculated tissues. The log2 ratios for all the other tissues and treatments are the expression levels relative to the expression of the control displayed in the left most column (neutral grey boxes) in each cluster of columns. The control root pieces used for comparison to root tips were root pieces taken further up into the root system not colonized with SCN and the branch roots trimmed off. Alcohol dehydrogenase (ADH) gene expression is included as an indicator of anaerobiosis in the flooded roots and hypocotyls. Gene name abbreviations: alcohol dehydrogenase (ADH), cellulase (Cel), expansin (EXP), pectate lyase (PL), polygalacturonase (PG), xyloglucan endotransglucosylase/hydrolase (XET). An a or b after the gene name indicates a paralogous gene. Where the paralogous gene is not included (Supplemental File S1), one or more mismatches occurred in one or both PCR primers that would likely prevent PCR amplification of the paralogous gene. Gene names underlined and in bold include at least one WGCATGTG motif in their 5’ upstream promoter sequence.

Figure 2. Thin sections prepared from the tap root of an explant after 7 days in well aerated conditions (Air) or 7 days of flooded conditions (Flooded). Magnified insets shown to the right are marked with a dashed line. Abbreviations: Co, cortex; Ph, phloem; Pi, pith; Sc, sclerenchyma; Xy, xylem.

Figure 3. Explants were submerged in aqueous solution of 10^{-5} M IBA and exposed to 1.0 \mu L L^{-1} ethylene in the gas phase to induce a proliferation of adventitious roots on the hypocotyls.
Hypocotyls were collected at 1, 4 and 8 days (a, e, j) and 1 μm sections prepared (b, f, k) and magnified insets (c, g). Hypocotyls from similarly treated explants were collected at 0, 4 and 8 days from transgenic plants and the hypocotyls sliced longitudinally through the middle and stained for GUS activity (d, h, m). A 15 μm section was prepared from a darkly stained 8 day hypocotyl (l) and thicker hand sections (~100 μm) were prepared from 4 and 8 day hypocotyls (i, n). Abbreviations: Adv R, adventitious root; L-Aer, lysigenous aerenchyma; Co, cortex; Ph, phloem; Pi, pith; Sc, sclerenchyma; Xy, xylem.

Figure 4. Histochemical staining for GUS activity in root pieces, abscission zone and leaf piece. The 1 μm sections b, c, and f were stained with toluidine blue to enhance cell outlines. The root-piece section labeled GUS (g) is 5 μm thick. Longitudinal sections f and g are several microns apart from the same SCN-infected root piece. Abbreviations: Nem, nematode; Syn, syncytium; Co, cortex; AZ, abscission zone; Pet, petiole; Xy, xylem.

Figure 5. Graphical display of the linear gene expression profiles for one of each class of cell wall protein, polygalacturonase (PG), pectate lyase (PL), cellulase (Cel) and expansin (EXP), that were strongly up-regulated in SCN infected roots and also include at least one copy of the WGCATGTG motif. Note that the scale changes for some graphs in order to better illustrate differences in gene expression. The means and standard error bars for root pieces, abscission zones (AZ), petioles (Pet) and leaves are for two replicate experiments. The hypocotyl experiments were replicated twice for 0 and 8 day time points and only once for 1 and 4 day samples and the dissected 8 day hypocotyls. The root tip fraction from 12 to 50 mm behind the apex was also only collected once.