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Biological And Chemical Assessment Of Glycine Max Modified With Gm-Xth52 Gene Resistant To Attack Of Nematode Heterodera Glycines

Ismail Khan

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Biological and chemical assessment of *Glycine max* modified with *Gm*-XTH52 gene resistant to attack of nematode *Heterodera glycines*

By

Ismail Khan

A Thesis
Submitted to the Faculty of Mississippi State University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Sustainable Bioproducts in the Department of Sustainable Bioproducts

Mississippi State, Mississippi

May 2017
Biological and chemical assessment of *Glycine max* modified with *Gm*-XTH52 gene resistant to attack of nematode *Heterodera glycines*

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Soybean (*Glycine max*) yield is significantly affected by soybean cyst nematode (SCN), *Heterodera glycines*, and causes an annual loss of billions of US dollars. In this study, *Glycine max* xyloglucan endotransglycosylase/hydrolase gene (*Gm*-XTH52) was transformed into a nematode susceptible *G. max* [Williams 82/PI 518671] variety of soybean to test whether the protein expression has a role in resistance to *H. glycines*, and possible chemical changes the expression may cause in the plant composition. Expression level of the *Gm*-XTH52 gene was three times higher than in controls. Significant reduction in the number of SCN cysts suggested suppression of *H. glycines* parasitism upon transformation. While total sugar amounts did not significantly differ between the transformed and control plants, xyloglucan amounts of loosely bound sugars of genetically mosaic plants were significantly lower in comparison to controls. Control plants showed lower molecular weight sugars than the transformed plants not subjected to *H. glycines* infection.
DEDICATION

This thesis is dedicated to my parents.

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CHAPTER I
INTRODUCTION

1.1  Soybean plant and soybean cyst nematode (SCN)

Soybean (*Glycine max*) is an important agricultural crop belonging to the family Fabaceae. Soybean production is the second highest in the United States, preceded by Brazil (USDA, 2016). Soybean has a number of commercial uses and it is an important source of oil and protein. Soybean seeds serve as the richest and the cheapest vegetable protein source. Therefore, soybean crops are of significant economic importance.

Soybean cultivation was first recorded five thousand years ago in Asia (XongHong et al., 1997, Hymowitz, 1970, Noel et al., 1992). Reports also mention soybean domestication in 1100 B.C. in China. The seeds of soybean have been brought to the U.S. in 1765 and cultivated since then (NCSPA, 2014).

Soybean crops require great efforts for maintaining high yields. Part of this problem is caused by different pathogens. Plant parasitic nematodes (PPNs) are one of the most damaging crop pathogens in general, causing more than 100 billion dollars annual losses worldwide (Sasser and Freckman, 1987, Chitwood, 2003). With regard to soybean, more than twenty genera of PPNs have been reported in soybean cultivated crops (Backman and Sinclair, 1989). Among these PPNs, soybean yield is mostly decreased by the soybean cyst nematode (SCN), *Heterodera glycines*. *H. glycines* was reported as early as the late nineteen century in Asia (Ichinohe, 1952, Noel et al., 1992).
However, *H. glycines* was first identified in the U.S. as an invasive species in 1954 in North Carolina (Winstead et al., 1955). Subsequently, *H. glycines* spread very quickly to other states including Mississippi (Spears, 1957). *H. glycines* now causes annual losses of more than 1 billion dollars in the U.S. alone (Chen, 2011). Furthermore, the economic loss due to *H. glycines* is much higher than all other pathogens of soybean together (Wrather and Koenning, 2006). Thus, *H. glycines* is the most economically important pathogen of soybean.

*H. glycines* are classified into 16 races based on their ability to successfully parasitize *G. max* genotypes that exhibit varying levels of resistance (Arelli et al., 2000). This diversity of *H. glycines* makes it difficult to manage.Broadly, *H. glycines* and most PPNs that infect *G. max* penetrate soybean roots and cause stunted growth by weakening the root which is followed by disturbance and manipulation of the metabolic processes of root cells leading to depletion of nitrogen, iron and potassium, microelements essential for growth of soybean plants (Chen, 2011). Ultimately, this process can lead to early death (Chen, 2011). As endoparasites of roots, PPNs secrete different types of proteins and enzymes, such as cellulases, xylanases, polygalacturonases, pectate lyases, arabinases and invertases into the roots (Abad et al., 2008). These enzymes break down hemicellulose, cellulose, pectin and other polysaccharides in the root cell walls. Also, these enzymes, secreted from the sub-ventral glands of the nematode, act as cell wall modifying agents and help the nematode to reach a target site in roots (Abad et al., 2008). Some of the secreted proteins seem to be specific to *H. glycines*, such as chorismate mutase (Bekal et al., 2003), and β-1,4-endoglucanase (Gao et al., 2004). For example, the chorismate mutase of *H. glycines*, manipulates the plant's shikimate pathway which
controls plant cell growth and structure, but also has a role in a defense mechanism against pathogens (Bekal et al., 2003).

1.2 Life cycle of soybean cyst nematode

The life cycle of *H. glycines* lasts approximately 30 days, varying with respect to the temperature and other factors (Lauritis et al., 1983, Koenning, 2004). The 30-day life cycle of *H. glycines* consists of four juvenile and a final, adult stage through which a successive growth of nematode from egg to adult happens (Chen, 2011). Initially, the eggs are enclosed in the carcass of the dead female, a structure referred to as a cyst. Upon release from the cyst, eggs enter a pre-infective, second juvenile stage (pi-J2s). To fulfill the requirements of their growth, reproduction, and development, *H. glycines* infect soybeans (Jung and Wyss, 1999) thus becoming infective second stage juveniles (i-J2s) (Chen, 2011, Noel et al., 1992). During infection, *H. glycines* use a sharp stylet to cut cell walls forming openings through which they penetrate and further move toward the vascular cylinder. *H. glycines* then release their infective enzymes and proteins from their sub-ventral glands that degrade cell walls and make openings near or at plasmodesmata. The parasitic juveniles (p-J2s) cause infection within a few hours. The increased openings created by *H. glycines* cause the organelles, nuclei and cytoplasm to flow across the infected cells, creating a permanent feeding structure called syncytium that consists of about two hundred cells having common cytoplasm (Jones, 1981, Jones and Northcote, 1972, Jung and Wyss, 1999, Lauritis et al., 1983, Chen, 2011). Once the syncytium forms, the feeding of *H. glycines* continues until the end of J3 stage, upon which the male nematode stops feeding and develops into a vermiform J4. The vermiform J4 exits the roots and becomes ready for copulation. In contrast, the p-J2s that become females
remain sedentary after establishment of their feeding site. While passing through juvenile J3 and J4 stages, the size of the female nematode increases and its posterior end erupts out beyond the root epidermis. This condition gives the male the opportunity for copulation. After copulation, the female continues to grow, producing approximately 40 to 600 eggs within the female adult nematode. The life of the female concludes, concomitant with its color change to yellow-tan as the female dies. The dead female body that encases the eggs becomes hard and protective (Niblack, 2005). Under unfavorable conditions this cyst can stay dormant in soil for nine, and in some cases up to 11 years (Inagaki and Tsutsumi, 1971). Once favorable conditions reestablish, eggs hatch.

1.3 Resistance mechanism of soybean to SCN

Different strategies have been employed to manage *H. glycines* infections. Included in these strategies are biological control methods such as the use of bacteria, fungi and even fungal proteins. Other methods include crop rotations with crops possessing natural resistance to *H. glycines*. Furthermore, resistant *G. max* cultivars are another viable option. Some of the naturally resistant *G. max* varieties have been used in breeding programs (Ross and Brim, 1957, Ross, 1958, Epps and Hartwig, 1972). From original variety trials, a total of 118 *H. glycines*-resistant genotypes have been identified (Concibido et al., 2004). From those, about seven of the resistant genotypes have been selected for further development of resistant cultivars in the U.S. (Shannon et al., 2004). Among these varieties, *G. max*[Peking/PI 548402] and *G. max*[PI 88788] genotypes have been used to identify a number of candidate resistance genes expressed during the resistant reaction (Shannon et al., 2004).
Genetic studies have identified five important loci that provide resistance to *H. glycines* (*rhg*) (Caldwell et al., 1960, Matson and Williams, 1965, Rao-Arelli, 1994). These studies have identified the recessive *rhg1*, *rhg2*, *rhg3* and dominant *Rhg4* and *Rhg5*. These genes cause degeneration of the feeding cells leading to the death of the nematode. The loci have been shown to contain resistance genes that can be used to develop resistance when transferred into susceptible soybean varieties (Endo, 1965, Riggs et al., 1973, Acedo et al., 1984, Matsye et al., 2011, Matsye et al., 2012).

In addition, several other resistant soybean germplasms are studied for development of resistant cultivars in other parts of the world (Ma et al., 2006, Li et al., 2011). The understanding of the resistance mechanism has enabled development of effective resistance opportunities (Steeves et al., 2006, McLean et al., 2007, Matsye et al., 2012, Klink et al., 2009a).

Some of the resistance genes that have been identified showed activity only when expressed collectively (Cook et al., 2012). In contrast, the expression of alpha soluble NSF attachment protein (α-SNAP) (*Gm*-α-SNAP) functions effectively when expressed on its own (Matsye et al. 2012; Sharma et al. 2016). The α-SNAP gene has been originally identified as *Sec17* in *Saccharomyces cerevisiae*, functioning in membrane fusion events during vesicle transport (Novick et al., 1980). Thereby, the *Gm*-α-SNAP could function to deliver materials involved in the development of cell wall apposition structures developed as a part of a defense response (Wilson et al., 1992, Matsye et al., 2011, Matsye et al., 2012). For example, a protein whose expression is activated along *Gm*-α-SNAP is a syntaxin known as *Gm*-SYP38 (Pant et al., 2014). Proteins that are homologous to *Gm*-SYP38 reside on cis face of the Golgi apparatus and bind α-SNAP-
like proteins. This process facilitates the fusion of transition and secretory vesicles to target membranes and, ultimately, the secretion of materials into the apoplast where the cell wall is constructed (Matsye et al., 2011, Matsye et al., 2012, Pant et al., 2014, Pant et al., 2015). Experiments have been performed to identify genes that are expressed specifically within the syncytium undergoing the process of resistance (Klink et al., 2007, Klink et al., 2009b, Klink et al., 2010, Klink et al., 2011, Matsye et al., 2011). The analysis resulted in the identification of 1,787 genes expressed exclusively in syncytia of *G. max* resistant phenotypes. Xyloglucan endotransglycosylase/hydrolase (XTH) genes have been identified among these 1,787 candidate resistance genes (Klink et al., 2007, Klink et al., 2009b, Klink et al., 2010, Klink et al., 2011, Matsye et al., 2011). XTHs are secreted proteins known to enzymatically modify hemicellulose and believed to be good candidates for understanding the process of resistance in *G. max* to *H. glycines* parasitism (Fry et al., 1992).

More recently, Pant et al. (2014) reported the role of *Gm*-XTH43 gene in *G. max* resistance to *H. glycines*. Prior experiments showed that *Gm*-XTH43 expression is suppressed during susceptible reaction to parasitism, but it is induced during the defense response (Matsye et al., 2011). The overexpression studies of the same gene showed co-expression of the closely related genes or their isoforms that translate enzymes involved in synthesis and metabolism of xyloglucan (Matsye et al., 2011, Matsye et al., 2012, Pant et al., 2014, Sharma et al., 2016).

1.4 Xyloglucan endotransglycosylase/hydrolase and xyloglucan

Xyloglucan endotransglycosylase/hydrolases (EC 2.4.1.207) are found in all land plants (Fry et al., 2008, Hayashi, 1989, Van Sandt et al., 2006, Sandt et al., 2007, Eklöf
and Brumer, 2010). XTHs function in a biochemical pathway that metabolizes and synthesize xyloglucan, the major hemicellulosic component of dicots and non-graminaceous monocots (Rose et al., 2002, Hayashi and Maclachlan, 1984, Eklöf and Brumer, 2010, Hayashi et al., 1984, Fry, 1989). Xyloglucan (XyG) chains cross-link cellulose microfibrils (Fry et al., 1992, Hayashi, 1989, McCann et al., 1990, Rose et al., 2002). Xyloglucans’ main chains are composed of β-(1→4) linked glucose residues. The backbone is supplemented with α-(1→6) linked xylose side chains to which galactose and/or fucose monomers are attached at varying patterns. XyGs in different species and tissues have different structural patterns. They are structural sugars found in most plant cell tissues, and are a critical component for plant growth and development. In majority of dicots, XyG is a major hemicellulose making up to 20% of cell walls constituents, whereas in monocots it presents 5% of growing cell walls (Fry, 1989, Hayashi, 1989, Carpita, 1996, Gibeaut et al., 2005, Schultink et al., 2014). It is a major component of flowering plants and can make up to 25% of the cell wall matrix (Hsieh and Harris, 2009). XyG was initially considered a seed storage polysaccharide in nasturtium, tamarind and other plants (Kooiman, 1957) and it was later found an important component of primary cell walls of many species (Hayashi, 1989). XyGs are the most abundant in the primary cell wall of dicots, while secondary cell walls have minimal to no XyGs (Pauly and Keegstra, 2016). XyGs in the cross-section of the primary cell walls and the middle lamellae are uniformly distributed (Moore et al., 1986). In the primary cell walls, they provide mechanical strength (Bootten et al., 2004, Harris and Stone, 2008). XyGs support the rigidity of the plant cell walls by binding tightly to cellulose chains through hydrogen bonds (Terry et al., 1981), thereby creating an XyG and
cellulose network (Cosgrove, 2001, Cosgrove, 2005). XyGs in the primary cell walls are also the major source of active oligosaccharides that regulate cell wall expansion (Aldington and Fry, 1993, Cote and Hahn, 1994). During the expansion, XyGs allow cellulose microfibrils to slide relative to each other (Keegstra et al., 1973, Van Sandt et al., 2007). The function of XyGs in secondary cell wall of seeds is to serve as a carbohydrate reservoir. XyGs are successively transferred to seeds during their active stage of germination (Buckeridge et al., 2000).

1.1 Structure of xyloglucan

Different subunits of XyG can be found in different species and tissues with much variable pattern. The complex structure of XyG has been elucidated and described (Fry et al., 1993). The diversely modified xyloglucan is described by the system of abbreviated names for xyloglucan oligosaccharides where X stands for α-D-Xyl-(1→6)-β-D-Glc part of the chain. L has been used for β-D-Gal-(1→2) attached to X, while S for α-L-Ara-(1→2) also attached to X residue. Furthermore, F was used for α-L-Fuc-(1→2) attached to L.

The plants with seeds contain mostly XXXG pattern of XyG while other plant species have different patterns (Vanzin et al., 2002, Hoffman et al., 2005). Peña et al. (2008) proposed that fucosylated XyG is first produced in a conjoint ancestor of vascular plants and hornworts (non-vascular plants). Such pattern can be described as XXFG or XLFG. Fucosylated XyG can be found in flowering plants in XXFG form, along with XXXG, but they do not have any XLFG (Hsieh and Harris, 2009). XyGs found in primary cell walls of eudicots are mostly XXXG with some X units substituted with different residues like fucose and galactose (Vincken et al., 1997, Pauly and Keegstra,
2016). These XyGs are known as fucogalactoxyloglucans (Pauly et al., 2013, Vanzin et al., 2002). Notably, XyGs of monocots are often less branched than those of eudicots. They also have lower amounts of xylose in comparison to glucose and significantly lower amounts of galactose and almost no fucose residues (Fry, 1989; Vincken et al., 1997). Grasses, for example, have XXG without any fucose associated with it (Hsieh and Harris, 2009). Other than monocots and eudicots, XyGs are also found in some classes of green algae, such as Charophycean (Sørensen et al., 2010, Sørensen et al., 2011).

1.2 Xyloglucan biosynthesis

XyGs are synthesized from nucleotide sugars in Golgi bodies, transported into the vesicles and finally delivered to the plant cell wall. This transport process is facilitated by the action of many different enzymes during different stages of the cell development (Fry, 2004, Lerouxel et al., 2006). The exact pathway of XyGs synthesis and the transport mechanism of XyG to extracellular matrix and its assembly in the cell wall are not fully revealed, but some discoveries have been made. The presence of XyG is visualized in the Golgi apparatus by immune-labeling assay (Zhang and Staehelin, 1992). However, it is still unclear in which part of Golgi apparatus the XyG-synthesizing enzymes function. Different types of enzymes used in XyG biosynthesis are (1→4)-β-glucan synthases, glycosyltransferases, xyloglucan endotransglycosylases, xylosyltransferases, and fucosyltransferases (Keegstra and Raikhel, 2001, Fry, 2004, Lerouxel et al., 2006, Hsieh and Harris, 2009). For example, synthesis of XLFG subunits, is encoded in minimum by: one (1→2)-α-fucosyltransferase, one (1→4)-β-glucansynthase, two (1→2)-β-galactosyltransferases and three (1→6)-α-xylosyltransferases (Zabotina, 2012).
1.3 **Enzymatic reactions involved in xyloglucan modification**

Besides the enzymes that contribute to the biosynthesis of XyG in the Golgi apparatus, there are many other enzymes that act on XyGs, whose activities can be classified into two major categories: xyloglucan endotransglucosylase (XET) and xyloglucan endohydrolase (XEH) activity. Xyloglucan endo-transglucosylases (XETs) non-hydrolytically cleave and ligate XyG chains, and xyloglucan endohydrolases (XEHs) irreversibly shorten the chains (Smith and Fry, 1991, Fry et al., 1992, Eklöf and Brumer, 2010). These enzymes and their activities were discussed in literature under different names (Smith and Fry, 1991, Fry et al., 1992, Nishitani and Tominaga, 1992, Okazawa et al., 1993), until (Rose et al., 2002) unified the nomenclature to xyloglucan endo-transglucosylase/hydrolases (XTHs).

The functions of XTHs are different in different cell types. In the cell walls, their function is generally described as to perform cell wall loosening (Thompson and Fry, 2001, Cosgrove, 2005) or cell wall tightening activity (Nishikubo et al., 2011). XTHs, as well as some expansin proteins, act on cellulose-XyG crosslinks, breaking them and hence causing cell enlargement (Rose et al., 2002). The function of XTHs also varies with different isoforms. Sometimes even the same isoform of an enzyme can perform different functions and act either on cell wall loosening or cell wall strengthening (Herbers et al., 2001, Nishikubo et al., 2011, Liu et al., 2007, Cho et al., 2006, Osato et al., 2006, Cosgrove, 2005, Shin et al., 2006, Thompson and Fry, 2001).

1.4 **Xyloglucan modification by gene regulation**

The identification and characterization of XTH genes have opened a window for understanding the biosynthetic and regulatory mechanisms of XyGs. In *Arabidopsis*
*Arabidopsis thaliana*, for example, 33 XTH genes are reported (Yokoyama and Nishitani, 2001, Osato et al., 2006, Rose et al., 2002). The knowledge of genes grants strategies for gene manipulation, i.e. enzyme expression and gives an insight into diverse functions of XyGs. It has been shown that the overexpression of XTH genes in *Populus* sp. resulted in the decreased amounts of loosely bound XyGs in primary-walled xylem cells (Nishikubo et al., 2011). Moreover, the tightly bound XyG amounts decreased in secondary-walled xylem cells. Nishikubo et al. (2011) further reported that XyG molecular weight decreased when XTH genes were overexpressed. In tobacco, similarly, the length of the XyG increased by down-regulation of the XTH gene (Herbers et al., 2001), while it increased with overexpression of XTH gene in *A. thaliana* (Liu et al., 2007). XTHs are also reported to cause cell wall loosening especially in elongating tissues with increased expression of XTH gene in rice and tomato plants (Ma et al., 2001, Jia et al., 2003).

### 1.5 Study objectives

The study presented here is based on the observation that a number of genes is expressed specifically in the *G. max* root cells undergoing a defense reaction to *H. glycines* parasitism (Klink et al., 2007, Klink et al., 2009b, Klink et al., 2010, Klink et al., 2011, Matsye et al., 2012). Among these genes, there is an XTH gene family comprising of 53 different members (Pant et al., 2014). While a defense role for one of these paralogs (*Gm*-XTH43) had been demonstrated, the role of other members of the XTH gene family remained to be characterized. In the study presented here, *Gm*-XTH52 is functionally examined. The examination involves the molecular cloning of *Gm*-XTH52, determination of whether it performs a defense function, and analysis of whether it has a role in inducing chemical changes of xyloglucan in the root. In particular, the analysis of a
potential role in inducing chemical changes of xyloglucan in the *G. max* roots provides the first molecular and structural data regarding actual cell wall modification as an actual part of the defense process that *G. max* has toward *H. glycines*. To accomplish these goals, *Gm*-XTH52 has been cloned, transferred into the susceptible *G. max* [Williams 82/PI518671] variety through *Agrobacterium rhizogenes* K599-mediated root genetic transformation (Matsye et al., 2012). The outcome of these experiments has been the identification of a defense role for *Gm*-XTH52. With the identification of this defense role for *Gm*-XTH52, a possible role in cell wall restructuring that could be revealed by a cell wall polysaccharide analysis had been performed. The work presented here links the defense response driven by *Gm*-XTH52 and changes in cell wall composition to earlier work by Nishikubo et al. (2011).
CHAPTER II
MATERIALS AND METHODS

2.1 Introduction

Using published methodologies developed in the G. max-H. glycines pathosystem, the current work is based on the isolated Gm-XTH52 gene cloned into the pRAP15 destination vector (Matsye et al., 2012). The genetically engineered pRAP15 plasmid containing Gm-XTH52 was transformed into Agrobacterium rhizogenes strain K599 (K599). These experiments allowed for the genetic transformation of G. max, producing genetically mosaic plants that have transgenic roots that develop from unengineered shoots (Matsye et al., 2012). The procedures undertaken for these steps are described (Matsye et al., 2012). The methods of the work performed in the current study are given in the subsequent sections.

2.1.1 RNA extraction and complimentary DNA (cDNA) preparation

Roots of G. max [Peking/PI 548402] were harvested and used for total RNA isolation. RNA was isolated using RNeasy Plant Mini Kit® following the protocol of the manufacturer (Qiagen®). The cDNA synthesis reaction was performed through reverse transcription polymerase chain reaction (RT-PCR) using SuperScript III First-Strand Synthesis System® kit according to the manufacturer’s protocol (Invitrogen®) (Matsye et al., 2012). Genomic DNA contamination was assessed through the use of PCR by using β-conglycinin primer pair which amplifies DNA across an intron (Klink et al.,
2009b). By performing this reaction, yielding different sized products based on the presence or absence of that intron (DNA or mRNA) can be determined (Klink et al., 2009b). All DNA primers used in the analysis for cloning genes, quality control and quantitative PCR are provided (Table 2.1).

Table 2.1  PCR primers used for all overexpression experiments

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession</th>
<th>Primer type</th>
<th>Primer 5'→3'</th>
<th>G/C</th>
<th>Length</th>
<th>TM</th>
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<td>Gm-XTH52</td>
<td>Glyma20g27380</td>
<td>PCR-F-OE</td>
<td>CACCATGAAAAACTTCCACACAA</td>
<td>39.1</td>
<td>23</td>
<td>60.6</td>
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<td></td>
<td></td>
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<td></td>
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<td></td>
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<td>qPCR-R</td>
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<td></td>
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<td></td>
<td>PCR</td>
<td>CTACCAGGCTTACGCGGATTTTC</td>
<td>52</td>
<td>25</td>
<td>67.4</td>
</tr>
</tbody>
</table>

### 2.1.2  Entry cloning and transformation into *E. coli*

The forward and reverse polymerase chain reaction (PCR) primers for the *G. max* [Peking/PI548402] candidate resistant gene *Gm*-XTH52 that encodes XTH protein were used in gene cloning experiments (Matsye et al., 2012). After the PCR reaction had been performed, the reaction contents that included the *Gm*-XTH52 amplicon were separated on 1% Tris-acetate (TAE)-EDTA agarose gel (1% agarose gel). The *Gm*-XTH52 amplicon (891bp) were excised out from the gel, isolated and purified using QIAquick Gel Extraction® kit (Qiagen®). The gel-purified *Gm*-XTH52 cDNA was cloned into pENTR/D-TOPO® vector (Invitrogen®) containing a kanamycin resistant gene for
chemical selection. After the completion of the ligation reaction, the reaction contents were transformed into the One Shot® chemically competent TOP10® *E. coli* cells (TOP10®) cells using the pENTR/D-TOPO Cloning Kit® (Invitrogen®). The TOP10® cells were plated on Luria-Bertani (LB)-kanamycin (5 μg/ml) (LB-K) agar media and grown at 37°C overnight to facilitate colony selection (Matsye et al., 2012) (Appendix A.1.1).

2.1.3 Sub-culturing and gene confirmation

The grown colonies were selected by collecting them on a sterile pipette tip. The bacterial colonies were transferred and subcultured in 5 ml LB-K broth (Appendix A.1.2). This culture was shaken for 16-24 hours at 37°C. Plasmid DNA (pDNA) containing the 891bp *Gm*-XTH52 amplicon was isolated from the subcultured colonies and purified using Wizard® Genomic DNA Purification Kit® (Promega®). PCR was performed on pDNA using AccuPrime® Pfx DNA Polymerase® (Life Technologies®) according to the manufacturer’s instructions. Gel electrophoresis on a 1% agarose gel was performed to confirm the expected size of the *Gm*-XTH52 amplicon (891bp). The pDNA was then sequenced and the *Gm*-XTH52 gene confirmed by comparing the obtained sequenced with the sequence housed at Phytozome.net (www.phytozome.net) (Goodstein et al., 2012).

2.1.4 Recombination and transformation of vectors

Transfer of *Gm*-XTH52 amplicon from the pENTR/D-TOPO entry vector to the pRAP15 destination vector was performed using the Gateway LR Clonase® enzyme mix kit (Invitrogen®). The resultant ligation reaction was then transformed into TOP10®
cells. The TOP10® cells were plated on LB tetracycline (LB-T) (5μg/ml) agar medium (Appendix A.1.1) and incubated at 37° C for 16-24 hours. A permanent -80°C freezer stock was made.

2.2 Current studies

2.2.1 Sub-culturing and gene confirmation

The transformed TOP10® colonies were thawed, plated on LB-T agar medium and sub-cultured in 5 ml LB-T broth on a shaker for 16-24 hours at 37°C. pDNA was extracted from the sub-cultured colonies and purified using Wizard® Genomic DNA Purification Kit®. The presence of Gm-XTH52 in the pRAP15 destination plasmid was confirmed by PCR using gene specific primers (Table 2.1). The PCR reaction was then run on 1% agarose gel and the Gm-XTH52 confirmed on the basis of molecular weight band size.

2.2.2 Agrobacterium rhizogenes (K599) transformation and gene confirmation

The pRAP15 vector containing Gm-XTH52 was transferred into K599 cells by freeze-thaw method (Höfgen and Willmitzer, 1988). Control K599 cells were transformed with pRAP15 vector without the gene of interest, but having the endogenous ccdB gene in its place. The ccdB gene present in the pRAP15 vector is used to maintain archival, unengineered laboratory stocks of pRAP15 in ccdB Survival® E. coli cells (Matsye et al., 2012). Because the pRAP15 control has the ccdB gene located in the position where, otherwise, the Gm-XTH52 is inserted during the LR clonase reaction, those control vectors also control for non-specific effects caused by gene overexpression (Klink et al., 2009b, Matsye et al., 2012, Pant et al., 2015, Pant et al., 2014, Sharma et al.,
2016, Matthews et al., 2013, Klink et al. 2016). Therefore, by definition, the pRAP15-ccdB serves as a control. The transformed K599 cells were grown on LB-T agar plates at 28°C for 2 to 3 days. The grown colonies were selected and sub-cultured in 5 ml LB-T broth at 28°C for 16-24 hours. The presence of the pRAP15+\textit{Gm-XTH52} gene, enhanced green fluorescent gene (eGFP) reporter and root inducing (Ri) gene were confirmed by PCR using gene specific primers for each gene, as noted in the protocol listed in the Appendix A.2 (Table A.2) using primers provided in Table 2.1. The K599 cells, transformed with an un-engineered pRAP15-ccdB destination vector or vector containing \textit{Gm-XTH52} were mixed with 30% glycerol and stored at -80°C for further use.

\subsection{2.2.3 Plant transformation and its replanting}

The frozen K599 cells were restored from the -80°C stock and cultured in LB-T broth at 28°C for two days. The cultures were then centrifuged at 4,000 rpm for 30 minutes and re-suspended in Murashige and Skoog (MS) media (Biggs et al., 1986). Over 150 one-week-old susceptible \textit{G. max} [Williams82/PI 518671] plants were cut at the hypocotyl with an unused sterile razor blade while submerged in MS media (Matsye et al. (2012). The plants were placed into beakers containing K599 cultures in MS media. Afterwards, the plants were subjected to vacuum for 30 minutes and left in the recombinant K599 suspensions for 15 minutes. The plants were replanted in coarse vermiculite in 3-4 cm deep holes and kept in a growth room under light for 1 week during which time the plants were watered. In addition, over 150 one-week old control plants were transformed with engineered K599 lacking \textit{Gm-XTH52-OE} and planted along with the experimental \textit{Gm-XTH52-OE} transformed plants. The plants were moved to a greenhouse and transferred after one week from vermiculite to 4-inch clay pots having autoclaved soil. Plants were
grown at ambient temperatures and natural sunlight for 20-25 days. Miracle Grow®
fertilizer was added to the soil twice a week.

2.2.4 Transgenic root identification and trimming

After approximately 25 days in the greenhouse, the genetically transformed *Gm*-XTH52-OE and control plants were uprooted, cleaned with water and examined under a
Dark Reader® Spot Lamp (Clare Chemical Research, Dolores, CO, USA). Roots having
expressed eGFP reporter gene displayed green fluorescence under the lamp. The roots
without fluorescence were trimmed off while leaving the fluorescent roots attached to the
unengineered shoot. These genetically mosaic plants were then placed back into the pots
with autoclaved soil containing 1:1 mixture of sand and clay. The plants were kept in the
greenhouse to grow and recover for a week, followed by infection with *H. glycines*.

2.2.5 Isolation of SCN and infection of soybean

*G. max*[Williams 82/PI 518671] are naturally susceptible to the *H. glycines*[NL1-Rhg/HG-type
7/race 3] race used in the present study (Klink et al., 2005). *H. glycines* female carcasses that
contain the eggs (cysts) were isolated from the root-soil debris of stock plants through
sucrose floatation method (Jenkins, 1964, Matthews et al., 2003, Matsye et al., 2012).
Successively, the *H. glycines* cysts were crushed and sieved through 200 mesh sieve in
order to obtain the eggs. The inoculum for each plant was prepared by suspending 2,500
eggs in 3 ml of water. For infection, the *H. glycines* egg suspension was administered
near the plant stem into a hole 1 cm wide and 4-5 cm deep. The holes were subsequently
covered with soil and the plants were allowed to grow in the greenhouse for the duration
of their life cycle (~30 days).
2.2.6 Cyst extraction

At the end of the infection period, the plants were uprooted and massaged in individual buckets through three washes of water to separate the cysts from the roots (Klink et al., 2009c). The washings were filtered through 20 mesh sieves to separate out the debris, and additionally washed through 100 mesh sieves to collect the cysts. The cysts were then enumerated.

2.2.7 Female index calculation

The effect of a treatment on *H. glycines* parasitism is presented as the female index (FI) (Golden, 1970). For the purposes of the experiments presented here, the FI is calculated per Equation 2.1 as a percentage of the average number (Nx) of cysts produced per modified (transgenic) plant divided by the average number (Ns) of cysts produced on control plants (Golden, 1970, Schmitt and Shannon, 1992, Matsye et al., 2012, Pant et al., 2014). Three sets of biological replicates of Gm-XTH52 plants and controls were used. Statistical analysis was performed using Mann–Whitney–Wilcoxon (MWW) Rank-Sum test (Mann and Whitney, 1947) to test whether a randomly selected value from transgenic plants will be smaller or greater than a randomly selected value from a control sample.

\[ FI = \frac{N_x}{N_s} \times 100 \% \]  \hspace{1cm} (2.1)

2.2.8 Quantitative real-time PCR (qPCR)

The experimentally induced expression of the *Gm*-XTH52 gene in transgenic plants was determined through quantitative PCR (qPCR) of cDNA synthesized from total RNA isolated at 0 days post infection (dpi), and compared to that of control plants. Gene expression was quantified using *Gm*-XTH52 primers as compared to the ribosomal S21
control gene (Klink et al., 2005, Alkharouf et al., 2006). In these experiments, total RNA was isolated and cDNA was synthesized from these samples as described in Section 2.1.1. The primers for Gm-XTH52 and S21 have been provided in Table 2.1. The qPCR reaction included TaqMan® 6-carboxyfluorescein (6-FAM) and Black Hole Quencher (BHQ1) (MWG Operon®) probes. The qPCR reaction conditions included a 10 µl TaqMan Gene Expression Master Mix (Applied Biosystems®; Foster City, CA), 0.4 µl of 100 µM forward and reverse primers for Gm-XTH52 or S21 gene, 0.2 µl of probe mix and 3 µl of template cDNA to final volume of 20 µl adjusted by RNase-Free® (Ambion®) distilled water. The qPCR experiments were run at 50˚C for 2 minutes, followed by 95˚C for 10 minutes, 95˚C for 15 seconds, and 60˚C for 1 minute for a total of 40 cycles. These reactions were performed on an ABI 7300® (Applied Biosystems®). The statistical analysis of gene expression data was performed using 2-ΔΔCT method as described by Livak and Schmittgen (2001) in Excel.

2.3 Chemical analysis

2.3.1 Material preparation

The roots of G. max [Williams 82/PI 518671] transformed with Gm-XTH52 and control plants were analyzed for chemical differences. Roots of ~65-85 modified and control plants were crushed in liquid nitrogen in an autoclaved and cooled mortar and pestle. Wet roots were weighed before and after crushing. The crushed samples were kept at -80˚C and freeze-dried (Labconco, Freezone® 4.5 Plus) until constant weight. The dry weight of the samples were recorded and the samples were then stored at 4˚C.
2.3.2 Removal of extractives

Extractives were removed from the roots according to NREL/TP-510-42619 standard (Sluiter et al., 2008). The freeze-dried samples of Gm-XTH52 and control roots were wrapped in Kimwipes and stapled into small packets. The samples were first Soxhlet extracted in water for 15 hours and air-dried overnight. Air-dried samples were returned into Soxhlets and extracted in 95% ethanol of 24 hours.

Upon evaporation of ethanol under room temperature for two days, the samples were additionally dried in an oven at 50°C to constant mass (Heratherm™ OMS60, Thermo Fisher Scientific™). Dry mass of the samples was recorded before further processing.

2.3.3 Removal of starch, protein and pectin

According to the literature, 1 g of dry tissue of soybean roots contains 2.5 mg of proteins (Rodrigues et al., 2012) and 25 mg of starch (Finn and Brun, 1982).

In order to remove starch, weighed roots of the transgenic and control extracted samples were treated with porcine pancreas α-amylase (Sigma-Aldrich®). 25 units of amylase (capable of releasing 25 mg of starch) were added per 1 g of sample. Amylase was diluted in sufficient volume of 100mM Tris-HCl buffer (pH 7.0) to cover the sample. The destarching reaction was performed at 37°C for 3 hours. The solution was centrifuged at 10,000 rpm for 5 minutes and the supernatant was discarded.

To each gram of the root sample, 0.025 mg of Pierce™ Trypsin Protease (Thermo Scientific™), capable of removing 2.5 mg of protein, was added in sufficient amount of 50mM acetic acid (pH 8) to cover the destarched material and the reaction was carried out at 37°C for 4 hours. The solution was centrifuged at 4000 rpm for 8 minutes, and the
pellet washed with deionized water (10ml). Upon removal of the supernatant, the pellet was subjected to depectination by boiling at 100°C in 50mM EDTA (pH 6.8) for 15 minutes three times. The extracted pectin was removed by centrifugation and depectinated samples were further fractionated in 4% and 24% KOH solutions for extraction of polysaccharides.

2.3.4 **Polysaccharide fractionation**

Polysaccharide fractionation was performed according to the procedure described by Nishikubo et al. (2011). Loosely bound hemicelluloses were obtained by extracting the polysaccharides with three portions of 4% (w/v) KOH at room temperature on a rocker platform (first for 2 hours, then overnight, and finally for 2 minutes). The supernatants of each step were combined and labeled as 4% KOH extracts.

Remaining tissues of *Gm*-XTH52 and control roots were extracted in three portions of a solution containing 24% (w/v) KOH and 0.02% (w/v) NaBH4 in a similar manner (first for 2 hours, then overnight, and finally for 2 minutes). Three of these supernatant extracts (tightly bound polysaccharides) were combined and labeled as 24% KOH extracts.

Both 4% KOH and 24% KOH extracts were neutralized with concentrated acetic acid on ice, and dialyzed in a dialysis tubing (Spectra/Por6® 1000 MWCO; Spectrum®) against water until the conductivity was lower than 1 mS. The dialyzed material was freeze-dried and weighed.
2.3.5 **Xyloglucan quantification**

Lugol’s solution (Fisher Scientific™) was diluted 10 times to obtain approximately 20mM I₂ and 60mM KI solution. Freeze dried samples of 4% and 24% KOH-extracted polysaccharides were diluted in water (app. 8µg/µl) and heated at 80°C for 10 minutes. 30µl of each sample was mixed with 15.1µL of diluted Lugol’s solution and 151µl of a sodium sulfate solution (1.4 M), as per Kooiman (1957). Upon cooling, the absorbance at 660 nm was measured (Epoch™, Biotek®). Xyloglucan contents of the samples were calculated and expressed in mg/g freeze-dried tissue according to the standard curve of tamarind (*Tamarindus indica*) xyloglucan (Megazyme™). Tamarind xyloglucan standards were prepared at concentrations of 100, 200, 400, 600 and 1,000 ppm. Difference in xyloglucan amounts of 4% and 24% KOH extracts between transgenic and control samples was assessed by analysis of variance (ANOVA) Tukey test using SAS (SAS® Institute) software.

2.3.6 **Molecular weight analysis by gel permeation chromatography (GPC)**

The dry polysaccharide samples were diluted to (app. 8µg/µl) in distilled water to analyze the molecular weight of polysaccharides. Five dextran GPC analytical standards (Sigma-Aldrich®) in the range of 5 kDa to 1,400 kDa were used for molecular weight calibration. The samples and the standards were filtered through 0.2 µm filters and then analyzed by GPC using Refractomax 520 RI (Thermo Fisher Scientific™) detector and HPLC Finnigan Surveyor system (Thermo Fisher Scientific™) controlled by Chromeleon™ v. 7 software (Thermo Scientific™). The separation of MWs was performed by Superose™ 6 10/300 column (GE Healthcare™), using water mobile phase at flow rate of 0.15-0.2 ml/min (back pressures 240 psi) over a period of 100 minutes.
GPC spectra were area normalized and compared by principal components analysis (PCA) using Unscrambler® (CAMO Software AS).
CHAPTER III
RESULTS

3.1 *Agrobacterium rhizogenes* transformation and gene confirmation

*Agrobacterium rhizogenes* strain K599 transformed with pRAP15-ccdB destination vector (positive control) or pRAP15 vector containing *Gm*-XTH52 were cultured on LB-T plates along with untransformed K599 as a negative control. As can be seen from Fig. 3.1, the growth of the positive control (A) and *Gm*-XTH52 transformed (B) colonies was not considerably different, as both of the cells contained vector with a tetracycline resistant gene. However, no remarkable growth was recorded for negative, untransformed controls (C), which confirmed successful transformation experiments.

Figure 3.1 K599 colonies grown on LB-T plate

Note: *Agrobacterium* colonies grown on LB-T plate after 2 days of incubation at 28°C. A –Positive control colonies transformed with pRAP15-ccdB destination vector. B – Colonies of *Agrobacterium* transformed with pRAP15 vectors containing *Gm*-XTH52 gene. C – Colonies of untransformed *Agrobacterium*. 
PCR products of pDNA extracted from the transformed K599 colonies were run on 1% agarose gel in order to confirm presence of Gm-XTH, eGFP and Ri gene. As shown in Figure 3.2, all expected genes were successfully amplified and visible on the gel.

![Image](image.png)

**Figure 3.2** Separation of PCR products of *Agrobacterium* pDNA transformed with pRAP15 vector containing *Gm*-XTH52 gene

PCR products of pDNA extracted from *Agrobacterium* transformed colonies run on 1% agarose gel. Lane 1: 1kb ladder, Lane 2: *Gm*-XTH52 gene band (891bp), Lane 3: Ri gene band (690bp), Lane 4: eGFP gene band (770bp).

### 3.2 Plant transformation and transgenic root identification

*Agrobacterium*-transformed soybean plantlets grown in vermiculite are shown in Figure 3.3. The overexpression of the genetically engineered genes was confirmed by visualization of the roots under blue light. Both roots of positive controls and transgenic plants fluoresced (Figure 3.4), and showed similar root growth and maturity, which was in the agreement with previous studies (Matsye et al., 2012, Pant et al., 2014, Klink et al., 2009c).
Figure 3.3  Soybean (*G. max* [Williams 82/PI 518671]) transgenic plantlets cultured in vermiculate

Figure 3.4  Genetically engineered roots of *G. max* [Williams 82/PI 518671] under blue light

Note: A – *Gm*-XTH52 (OE), B – Control (pRAP15 vector) plant roots showing green fluorescence under the lamp.
3.3 Generation of *G. max* plants having experimentally induced levels of *Gm*-XTH52

Induced levels of relative transcript abundance of *Gm*-XTH52 gene in engineered *H. glycines* susceptible genotype (*G. max* [Williams 82/PI 51867]) measured by qPCR showed almost three times higher expression level in comparison to positive controls (Figure 3.5).

![Fold change bar graph](image_url)

**Figure 3.5** Relative expression of *Gm*-XTH52 in engineered transgenic *G. max* roots

Note: Relative levels of transcript abundance in transgenic *Gm*-XTH52-OE roots, as compared to controls, revealing experimentally induced mRNA levels. Error bar represents standard deviation.
3.4 Induced XTH52 expression leads to a defense response

Genetically mosaic Gm-XTH52-OE and pRAP15-ccdB control plants were infected with H. glycines and allowed to have their respective infections proceed for the duration of one complete life cycle (approximately 30 days). After 30 days, the cysts were extracted from the soil and enumerated. From these data, a female index (FI) was calculated for three independently run experiments. The results of the experiment demonstrated that the G. max plants genetically engineered to have induced levels of Gm-XTH52 expression, also have suppressed levels of H. glycines parasitism as shown in Figure 3.6.

![Figure 3.6 Level effect of overexpression of Gm-XTH52 on H. glycines parasitism in G. max as indicated by its female index (FI)](image)

Note: Asterisk (*) denotes statistically different values according to Mann–Whitney–Wilcoxon (MWW) Rank-Sum Test at p < 0.05. The solid bars represent cyst per whole root, and lined bars represent cyst per gram of root tissue.
3.5 Amounts of loosely and tightly bound sugars

Gravimetrically determined percent of loosely bound sugars (4% KOH extractions) in transformed plants accounted for 1.87% of dry Soxhlet-extracted root material, but the variation between the replicates was large (1.87%). This large deviation is possibly caused by one of the samples being analyzed at a different time, and the overall small amount of that sample (Appendix A.3). In case of the control plants, loosely bound sugars accounted for 2.31±0.57% of dry Soxhlet-extracted root material. Tightly bound sugars (24% KOH extractions) in general contributed in higher amounts to the dry Soxhlet-extracted root material with 5.87±2.84% of transformed, and 3.88±1.80% of control plants. The total sugar amount between the transformed and control plants did not significantly differ, as shown in Figure 3.7.

![Figure 3.7](image)

**Figure 3.7**  Total sugar % of Soxhlet-extracted transformed and control soybean plants
3.6 Xyloglucan amounts

The difference between control and modified samples was tested for xyloglucan amount in loosely bound, tightly bound, and total sugars by Kooiman (1957) method. All the results were interpreted at 5% significance level.

XyG amount in loosely bound sugars was shown to be significantly different between control and transformed plants, with average values of 1.14±0.2% and 1.72±0.2%, respectively (Figure 3.8). XyG accounted for 13.68±3.11% and 10.86±4.41% of tightly bound sugars of control and transformed plants, respectively. These XyG amounts were significantly higher than XyG amounts of loosely bound sugars. However, XyG amounts of tightly bound sugars did not significantly differ between the two plant types (Figure 3.9).

![Figure 3.8 Xyloglucan % amounts of loosely bound sugars of transformed and control soybean plants](image-url)
According to this study, the overall amounts of xyloglucan in the soybean root system presented only 12-15% of the total sugar amount with no significant differences between the control and modified plant root systems. The data on the amounts of xyloglucan in the root systems of soybean plants are not reported, to our knowledge, so it is impossible to compare the current data with the work of others. In addition, no root information could be found for a total chemical analysis of other legume root systems. In order to verify the data, it would be useful in the future studies to potentially perform acid hydrolysis of the KOH-extracted material, and perform monosugar analysis as an indicator of xylan and glucan amounts in the plants.
3.7 GPC analysis

3.7.1 Standards

The GPC chromatograms of dextran standards are given in Figure 3.10. It can be seen that the separation of the standards was clear, with the major 5kDa peak eluting at app. 73 min, and the 1400 kDa peaks eluting between 18-40 minutes. The peak visible in all spectra, eluting at app 86 min is a methanol peak that served as an internal standard. From the consistent retention time of this peak, it is fair to conclude that the instrument performance was very stable throughout the analysis.

Figure 3.10 Area normalized GPC chromatograms of dextran standards (5-1400kDa)
3.7.2  **Tightly bound sugars**

Chromatograms of two GPC analyses of each replicate sample of tightly bound sugars (24% KOH extracts) are shown in Figure 3.11. The chromatograms overall showed stable runs as MeOH peak remained constant elution time. However, some differences between the replicate runs were seen, indicating that the differences from sample analyses could be due to settling of the sample, despite the fact that the samples were filtered. It is not anticipated that the samples changed over a week period, during which all of the samples were analyzed.

In general, as visible from the Figure 3.11, and confirmed by PCA analysis (Appendix A.4.1), the difference between the control (red chromatograms) and transformed plants (blue chromatograms) lies mainly in the section of the peaks eluted after 33 minutes, i.e. in sugars with size comparable to dextran standards with MW<150kDa. Control samples appear to have sugars with lower MW in this region in comparison to the modified plants. This observation is somewhat contrary to the expectation that modified plants would have shorter XyG chains, and therefore smaller spaces for nematode enzyme penetrations. However, it is important to note that the sugar profiles in the GPC spectra do not reflect MW of XyG, but MW of all sugars.
Figure 3.11  Area-normalized chromatograms of tightly bound sugars

Note: Blue – spectra of transformed plant root extracts; Red – spectra of control plant root extracts.
3.7.3 **Loosely bound sugars**

Loosely bound sugars (4% KOH extracts) showed very similar spectral difference between the control and modified plants to the differences between the tightly bound sugars (Figure 3.13). The major difference was also seen in region of dextran MW<150kDa, with control samples showing lower MW peaks in this region. PCA analysis differentiated control from modified plants by PC2 as shown in Appendix A.4.2.
Figure 3.13  Area-normalized chromatograms of loosely bound sugars

Note: Blue – spectra of transformed plant root extracts; Red – spectra of control plant root extracts.
Figure 3.14  Magnified lower-MW spectral region of area-normalized chromatograms of loosely bound sugars

Note: Blue – spectra of transformed plant root extracts; Red – spectra of control plant root extracts.
CHAPTER IV
DISCUSSION AND CONCLUSIONS

Transformation of *G. max* [Williams82/PI 518671] has confirmed that *Gm*-XTH52 functions to provide resistance of *G. max* root system to infection of *H. glycines*. Similarly, Pant et al. (2014) showed that the genetic induction of another *G. max* XTH paralog, *Gm*-XTH43, also suppresses *H. glycines* parasitism. The results presented here, in conjunction with those of Pant et al. (2014), point toward a general role of XTH proteins in impeding the ability of *H. glycines* to establish a functional feeding site.

Earlier experiments performed by Klink et al. (2010) identified the extent to which *Gm*-XTH43 is expressed during the defense reaction of *G. max* toward *H. glycines*. In those experiments, pericycle and root cells of control and two resistant *G. max* genotypes (*G. max*[Peking/PI 548402] and *G. max*[PI 88788]) were isolated and studied during the process of infection of *H. glycines*. The results revealed high transcript levels of *Gm*-XTH43 in the cells undergoing resistance to *H. glycines* parasitism (Klink et al., 2010, Matsye et al., 2011). The highly induced levels of *Gm*-XTH43 were proved to provide defense against *H. glycines* by Pant et al. (2014). In this work, although the *Gm*-XTH52 expression levels were not measured during the parasitism stage of the *H. glycines*, the genetically modified *G. max*[Williams82/PI 518671] did show 77% suppression of *H. glycines* parasitism, as measured by female index.
Nishikubo et al. (2011) reported decreased amounts of loosely bound XyGs in primary-walled xylem cells and tightly bound XyG in secondary-walled xylem cells of genetically engineered *Populus* sp. tissues overexpressing the XTH gene. The results of this study were not in agreement with their findings. XyG amounts in loosely bound sugars of root mass were higher in transformed than in control plants. No significant difference in XyG amounts of tightly bound sugars was found between the transformed and control plants.

According to Nishikubo et al. (2011), XyG chains shortened with the overexpression of XTH genes in *Populus* spp. Although in the present study we also attempted to determine amount of XyG in fractions of GPC eluents, the levels of XyG in 2-minute GPC collections were well below the detection limit of the instrument, despite highly concentrated GPC samples. It remains unknown how concentrated were the GPC samples of Nishikubo et al. (2011), but even 20-minute fractions of our samples could not yield measurable levels of XyG on UV spectrometer using Kooiman (1957) method. Therefore, in the present study, we only reported the MW profiles of the loosely and tightly bound sugars, which in general showed higher MW in transformed than in the control samples. It is plausible that high MW sugars observed in the transformed plants are not XyGs, as the cell walls may experience multiple polysaccharide reorganizations. Despite the GPC results, cytological observations in *G. max* undergoing a defense response to *H. glycines* did appear to lack localized expansion of the cell walls, a condition that was observed in the susceptible reaction.

For future work, it is recommended to study a significantly larger number of plants for chemical analysis. In the current study, number of plants harvested for
chemical analysis (~65-85) was about 5 times smaller than the number of plants sowed for this purpose. It is expected that planting the seedlings in individual pots instead of 50-cell flats would also significantly increase the mass of roots. It is important to keep in mind that HPLC analysis of monosugars according to Sluiter et al. (2011) requires ca. 300 mg of sugars, should this analysis be performed in the future. Transformation and processing of all the plants at the same time is expected to minimize data variability.

Although the focus of this study was transformation of soybean with XTH genes for protection from nematodes, the induced xyloglucan modification of plants used for bioproducts manufacturing can also be useful. If in fact xylan length can be manipulated through controlled expression of XTHs, then there is a possibility that xylan modification can be used for prevention of enzyme penetration of fungi, the major decaying organisms of lignocellulosic materials. As xylans and cellulose crosslink, xylan structure can affect the friction between the cell wall components, and thus its modification can be used for improvement of mechanical properties.
REFERENCES


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APPENDIX A

EXPERIMENTAL AND CHEMICAL ANALYSIS DETAILED INFORMATION
A.1 Media recipes

A.1.1 LB agar medium

LB stands for Luria-Bertani. It is a medium rich of nutrients and used for the growth of bacteria. For LB agar media, 35 g of LB broth with agar (Sigma-Aldrich) is dissolved in 1 L of distilled water and autoclaved for 20 minutes at 121°C.

A.1.2 LB broth medium

20 gram of LB broth (Sigma-Aldrich) is dissolved in 1L of distilled water and autoclaved for 20 minutes at 121°C.

A.1.3 Antibiotic amended LB media

For LB-T broth or agar, 1 μl of tetracycline stock is added per 1 ml of media upon cooling.

For LB-K broth or agar, 1μl of kanamycin stock is added per 1 ml of media upon cooling.

A.1.4 Tetracycline stock preparation

One hundred mg of Tetracycline hydrochloride powder (Sigma-Aldrich) is dissolved in 10 ml of 100% ethanol and diluted with distilled water to final volume of 20 ml.

A.1.5 Kanamycin stock preparation

Kanamycin sulfate (Sigma-Aldrich) is dissolved in distilled water to concentration of 50mg/ml, filtered and stored at -20°C.
A.2 PCR protocols

Table A.1 Accuprime Pfx DNA polymerase based PCR

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<th>Amount</th>
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<td>Accumprime Reaction Mix</td>
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<td>cDNA</td>
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<td>Polymerase</td>
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<tr>
<td>Forward Primer</td>
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<tr>
<td>Reverse Primer</td>
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Adapted from Life Technologies™

Table A.2 Taq DNA polymerase based (Taq)

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<td>dNTP</td>
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<td>Taq Polymerase</td>
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<td>Sample DNA</td>
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<tr>
<td>Reverse Primer</td>
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Adapted from Invitrogen®
### A.3 Detailed chemical analysis data

Table A.3  Detailed chemical analysis data

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<td>0.57</td>
</tr>
</tbody>
</table>

1. Number of plants processed
2. Wet mass of samples (g)
3. Dry mass of samples (g)
4. % dry mass from wet mass
5. Dry mass (mg) upon Soxhlet extraction
6. Dry mass (mg) of 4% KOH extracted sample
7. Dry mass (mg) of 24% KOH extracted sample
8. Loosely bound sugar (% extracted sample)
9. Tightly bound sugar (% extracted sample)
10. Total sugar (% extracted sample)
11. XG in loosely bound sugar
12. XG in tightly bound sugar
13. XG in total sugar amount
A.4 Principal component analysis of chromatograms

A.4.1 GPC spectral analysis of tightly bound sugars

Figure A.1  PC1/PC2 scores and PC1 loadings of transformed (blue) and control (red) spectra

A.4.2 GPC spectral analysis of loosely bound sugars

Figure A.2  PC1/PC2 scores and PC2 loadings of transformed (blue) and control (red) spectra