ABSTRACT

Title of thesis: PHYSIOLOGICAL AND MOLECULAR STUDIES OF ETHYLENE EFFECTS ON SOYBEAN ROOT INFECTION BY SOYBEAN CYST NEMATODES

Ping Xue, Master of Science, 2007

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Soybean cyst nematode (SCN), *Heterodera glycines*, is one of the most devastating pests of soybean in the world. Several earlier reports demonstrated that ethylene is involved in nematode feeding cell formation in *Arabidopsis* and tomato. I investigated whether or not ethylene is involved in SCN feeding cell formation in soybean. My results show that SCN parasitism was increased by treatment of roots with ethylene and inhibited by suppressors of ethylene action or in an ethylene resistant soybean mutant. My results also indicate that excised soybean roots colonized by SCN produced ethylene at 1.5-3 times the rate of non-infected roots between 14 and 22 days post inoculation. To determine if ethylene was being synthesized in feeding cells, an ethylene-responsive promoter fused to a GUS reporter gene was constructed and transformed into soybean roots with *Agrobacterium rhizogenes*. Overall, the results suggest that ethylene plays an important role in SCN infection in soybean.

PHYSIOLOGICAL AND MOLECULAR STUDIES OF ETHYLENE EFFECTS ON SOYBEAN ROOT INFECTION BY SOYBEAN CYST NEMATODES

By

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2007

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Dedication

To the glorious God and my loving mother

Acknowledgements

I want to thank my advisor Dr. Theophanes Solomos and my co-advisor Dr. Mark L. Tucker for all their help and advice along the way and also thank for their patience with me. They provided much needed guidance to someone who did not have a lot of research experience before entering graduate school.

I also want to thank other people in the USDA Soybean Genomics and Improvement Lab, for all their nice help and friendship. I couldn't have finished my research without their help. I especially want to thank Qijian Song, Mindy L. Ehrenfried and Margaret H. Macdonald (Peggy) who were always available to answer my questions and to give me a lot of help with my experiments.

Of course I must thank my family for their continuous support.

Lastly, I want to thank my good friends Ivy Chaine, Wei You, Grace Ji, Xiaofang Wang, Manjing Zeng, Shengchun Wang, Hailan Wang and Zhen Li for being so understanding and supportive. They were always there to cheer me up when I was frustrated with my research.

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List of Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
AVG	aminoethoxyvinylglycine
X-Gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid
GUS	*β*-glucuronidase
β-me	*β*-mercaptoethanol
CTR	Constitutive triple response
DIG	digoxigenin
CSPD	Disodium3-{4-methoxyspiro [1,2-dioxetane-3,2′-(5′-chloro) -tricyclo[3.3.1.13,7) decan]-4-yl}phenyl phosphate
EIN	Ethylene insensitive
EIL	EIN-like
EREBP	Ethylene response element binding protein
ERF	Ethylene response factor
ERS	Ethylene response sensor
ETR	Ethylene resistant
eto2	*ethylene overproduction2*
4-MUG	4-methylumbelliferyl *β*-D-Glucuronide
4-MU	4-methylumbelliferone
1-MCP	1-methylcyclopropene
MAPK	Mitogen-Activated Protein Kinase
2,5-NBD	2, 5-norbornadiene
PCR	Polymerase chain reaction
PDF	Plant Defensin

rhd	root hair defective
RKN	Root knot nematode
SCN	Soybean cyst nematode
SIMK	Salt-Stress-Inducible MAPK

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Chapter 1: Introduction

1.1 *Soybean cyst nematode*

The soybean cyst nematode, *Heterodera glycines*, is currently the most economically damaging pest of soybean (Wrather and Koenning, 2006). Estimated losses caused by SCN and other soybean pests are depicted in Figure1-1. From 1996 to 2006, SCN losses were much more than those of any other diseases in the United States (Wrather and Koenning, 2007). Between 1999 and 2002, SCN loss slightly declined from 16.92 million bushels to 14.28 million bushels, which was much lower than the 27.90 million bushels reported for 1998 (Wrather and Koenning, 2007). The decline in yield loss from 1998 to 2002 was possibly due to greater farmer awareness of SCN through the efforts of the SCN coalition in the north central states and increased planting of resistant cultivars in infested fields (Wrather *et al.*, 2003). Crop rotation, nematicides and planting soybean varieties resistant to various SCN races provide some protection; nevertheless, substantial economic loss from SCN is still incurred annually, approximately 12.37 million bushels in 2006. From 2004 to 2006, SCN was still ranked #1 on the list of diseases that suppressed soybean yields in the United States (Wrather and Koenning, 2006).

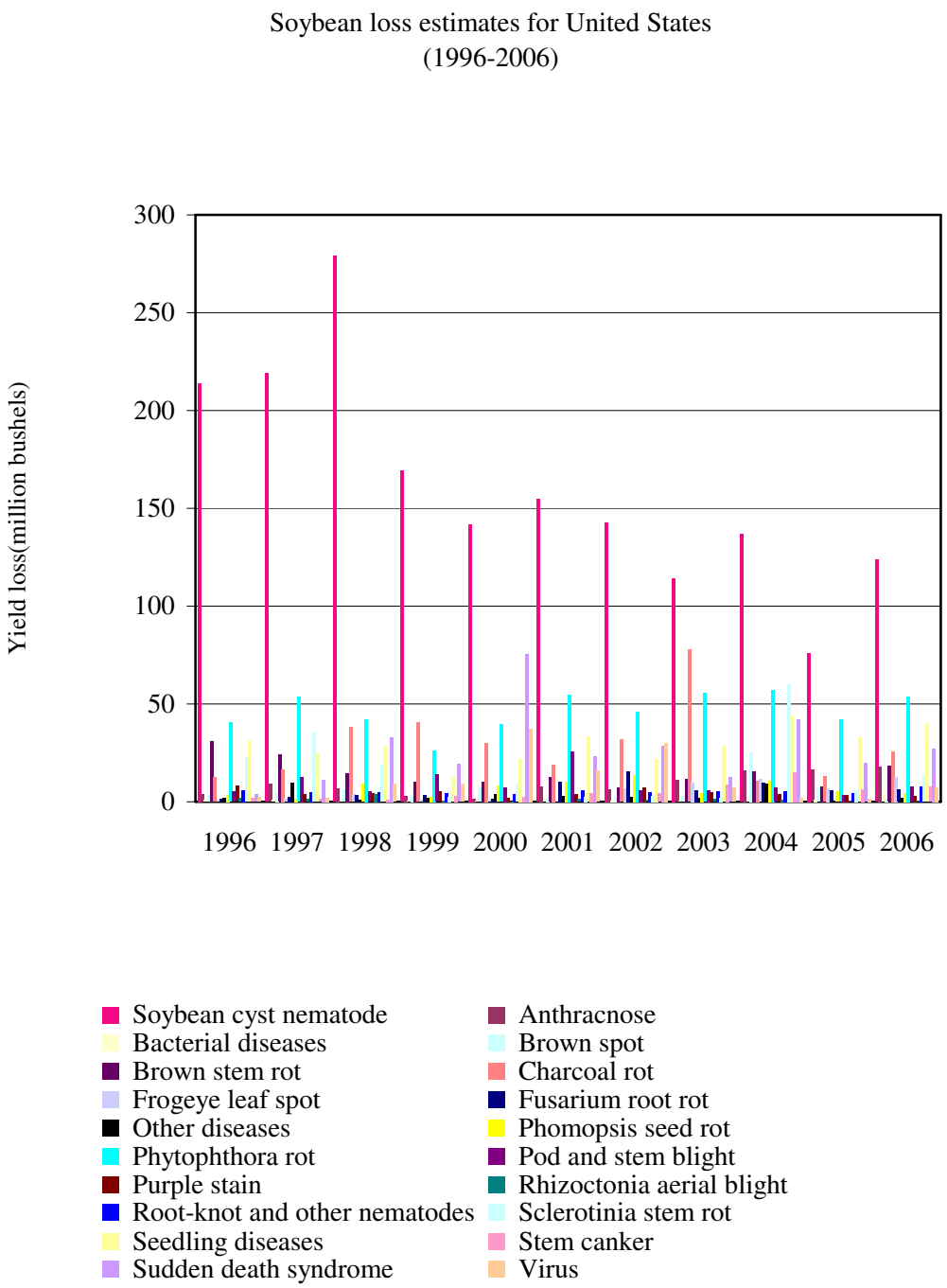


Figure 1-1. Estimated losses caused by SCN and other soybean pests from 1996 to 2006 (Wrather and Koenning, 2007).

Plant-parasitic nematodes are major pests for many important agricultural crops and soybean cyst nematode, *H. glycines,* a parasitic roundworm, only attacks the roots of soybean. SCN was first reported in Japan more than 75 years ago. In the United States, SCN was first reported in North Carolina in 1954. Since then SCN has spread to 25 states in the Midwest and southeast of the U.S., including Illinois, Minnesota, Missouri, Nebraska, Wisconsin and Iowa (Tylka, 1994). The aboveground symptoms of SCN in the field are small areas of stunted, yellowed, less vigorous plants. As they are similar to various other causes of damage e.g. drought, nutrient deficiencies and other plant diseases, the yield loss caused by SCN are often under-estimated. Underground observation of adult females and cysts on plant roots is the only accurate way to determine whether SCN damage exists. However, severe damage has already occurred by the time cysts can be seen on the underground roots. Most nematodes can only be observed under a microscope and are not visible on above-ground plant organs. Adult females and cysts are tiny lemon-shaped objects on the roots. They are first white, then turn to yellow, finally go brown as they mature. A mature female nematode is approximately 0.08 centimeter long and is the only stage in the nematode life cycle that is visible to the naked eye (Tylka, 1994) (Figure1-2).

Figure 1-2. Adult female of soybean cyst nematode with egg filled egg sac **(Tylka, 1994)** (magnified approximately 25 times).

The life cycle of SCN, (*Heterodera spp.)* includes six stages**:** an egg, four juveniles and an adult, while the second stage juvenile (J2) is the only stage to infect plant roots. Under optimum conditions, the SCN life cycle can be completed in about 30 days (Wyss, 1992). In a typical life cycle for SCN, an infective second stage juvenile (J2) penetrates into the host roots and migrates to the vascular cylinder by cell-wall degrading enzymes secreted by the nematode (Figure 1-3; Williamson and Hussey, 1996; Wang *et al.*, 1999; Davis *et al.*, 2004; Baum *et al.*, 2007). Then J2 uses its stylet, a hollow mouth spear, to pierce plant cell walls and induce cells to transform into a syncytium, which is a multinucleate feeding cell formed by the fusion of neighboring cells through partial cell wall dissolution (Jones, 1981; Endo, 1986; Wyss and Grundler 1992; Gheysen and Fenoll, 2002; Jasmer, 2003). Significant physiological and morphological changes occur during syncytium

formation: dissolution of surrounding cell walls, increased density of the cytoplasm with numerous organelles, accumulation of endoplasmic reticulum and enlarged hypertrophied nuclei (Wyss and Grundler 1992; Mahalingam and Skorupska, 1996; Hussey *et al.,* 2002). Initiation and formation of the syncytium is a complicated process requiring an unknown host signal transduction pathway triggered by secretions from the nematode esophageal glands (Williamson and Hussey, 1996; Davis *et al*., 2004). After the feeding site is initiated, J2 molts to J3 and then J4, and develops into a female or male adult. The female remains sedentary at the feeding site while the mature male becomes mobile in the root. The female extracts nourishment from the syncytia to support the production of several hundred eggs, most of which stay inside the female's body, while others are excreted as a gelatinous mass into the soil. After the female dies, the body remains intact and hardens into a tough leathery sac known as a cyst. Eggs and larvae can survive in the cyst body for several years until they are stimulated to hatch in the soil under optimum conditions (Williamson and Hussey, 1996; Gheysen and Fenoll, 2002; Jasmer *et al*., 2003).

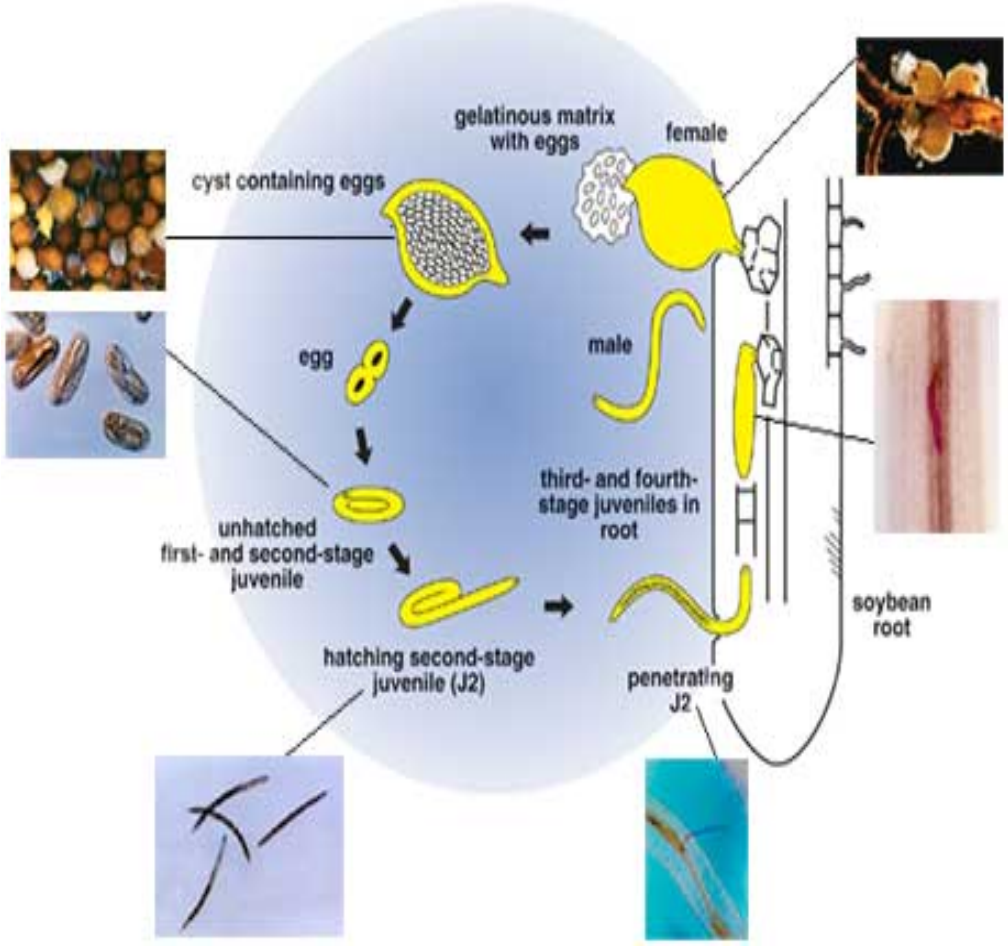


Figure 1-3. Life cycle of the soybean cyst nematode (Sketch by Dirk Charlson, Iowa State University).

1.2 *Ethylene biosynthesis and signaling transduction*

The plant hormone ethylene, a simple two-carbon olefin, plays important roles in many aspects of plant growth and development, including seed germination, root nodulation, abscission of various organs, flower senescence and fruit ripening (Abeles *et al.*, 1992; Roman *et al*., 1995; Ecker, 1995; Johnson and Ecker, 1998; Giovannoni, 2004). In higher plants, ethylene is produced from methionine, which is converted to S-adenosyl-methionine (S-Ado-Met) through catalysis by S-adenosyl-methionine

synthase. S-Ado-Met is converted into 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase with pyridoxal phosphate as a co-factor (Adams and Yang, 1979; Yang and Hoffman, 1984; Sato and Theologis, 1989). Formation of ACC is the rate-limiting step in ethylene biosynthesis (Kende, 1993). Production of ethylene from ACC is catalyzed by ACC oxidase (Yang and Hoffman, 1984; Hamilton *et al.*, 1991; Bleecker and Kende, 2000). This reaction is oxygen-dependent and under anaerobic conditions, ethylene formation is completely suppressed. ACC synthase and ACC oxidase are encoded by multigene families and many of the signals that influence ethylene synthesis induce expression of single members in the ACC synthase and ACC oxidase gene families (Kende, 1993; Barry *et al.*, 2000).

Exposure of dark grown (etiolated) germinated seedling to ethylene or its precursor, ACC, causes a radial swelling of the hypocotyl, an exaggeration in the curvature of the apical hook and an inhibition of root and hypocotyl growth, which is termed the triple response. Over the past two decades the triple response has been used to screen for mutants that display a defective triple response in the presence or absence of ethylene (Bleecker *et al.*1988; Guzman and Ecker, 1990; Chao *et al.*, 1997). These mutant screens identified many ethylene insensitive and resistant mutants which include *etr1, etr2, ein4, ein2, ein3, ein5, ein6* and *ein7*. In addition, several ethylene constitutive mutants (i.e., constitutive triple response in the absence of ethylene) were also identified, such as *ctr1, eto1, eto2* (Kieber *et al.*, 1993; Roman and Ecker, 1995; Chang, 2003, Guo and Ecker, 2004).

The action of ethylene in higher plants is mediated by ethylene receptors. In *Arabidopsis thaliana*, five ethylene receptors have been identified (ETR1, ETR2, ERS1, ERS2 and EIN4) (Bleecker *et al.*, 1988; Chang, 1993; Hua *et al.*, 1995; Sakai *et al.*, 1998; Hall *et al.*, 2000). Each has been shown to bind ethylene via a copper cofactor and to function as homodimers (Schaller and Bleecker, 1995; Schaller *et al.*, 1995; Rodriguez *et al.*, 1999). The ethylene receptors are similar to bacterial two-component His protein kinase receptors, which transmit the signal through autophosphorylation of histidine in the His kinase domain, and then transfer the phosphate to a conserved aspartate residue in the receiver domain (Chang *et al.*, 1993). On the basis of structural similarities, the ethylene receptor family can be divided into two subfamilies, subfamily 1 (ETR1, ERS1) and subfamily 2 (ETR2, ERS2, EIN4). Members of the ETR2 subfamily lack some motifs of the bacteria histidine-kinase domain, and have an extended hydrophobic subdomain in the amino-terminus. It has been shown that the ethylene receptors are negative regulators of ethylene action, which means that receptors actively repress responses in the absence of ethylene but when ethylene binds to the receptor the receptors are inactivated (Hua and Meyerowitz, 1998; Chang and Stadler, 2001).

The relative order of ethylene signaling pathway components in *Arabidopsis thaliana* has been established by epistasis analysis of many ethylene response mutants (Stepanova and Ecker, 2000; Guo and Ecker, 2004; Chang and Bleecker, 2004; Chang, 2003) (Fig. 1-5). CTR1, a Raf-like kinase that interacts with the cytoplasmic portions of ETR1, functions as a negative regulator in the ethylene signaling pathway

(Kieber *et al.*, 1993; Clark *et al.*, 1998). The similarity of CTR1 to members of the Raf family of mitogen activated protein kinase kinase kinases (MAPKKKs) suggests that ethylene signaling in plants is mediated by a MAPK pathway (Kieber *et al.*, 1993; Novikova *et al.*, 2000; Quaked *et al.*, 2003; Chang, 2003). Another key factor in ethylene signaling is EIN2, a novel integral membrane protein that functions upstream of EIN3 and downstream of CTR1 as the first positive regulator in ethylene signaling pathway (Guzman and Ecker, 1990; Alonso *et al.*, 1999). Ethylene signaling downstream factors EIN3 and EILs regulate the expression of other transcription factors, such as ERF1, which encodes a protein that belongs to the ethylene response element binding protein (EREBP) family of DNA binding protein that binds to the GCC box (Chao *et al.*, 1997; Solano *et al.*, 1998). The GCC box is a *cis*-element found in the promoters of some ethylene-responsive pathogen related (PR) genes in plants (Hart *et al.*, 1993; Ohme-Takagi and Shinshi, 1995).

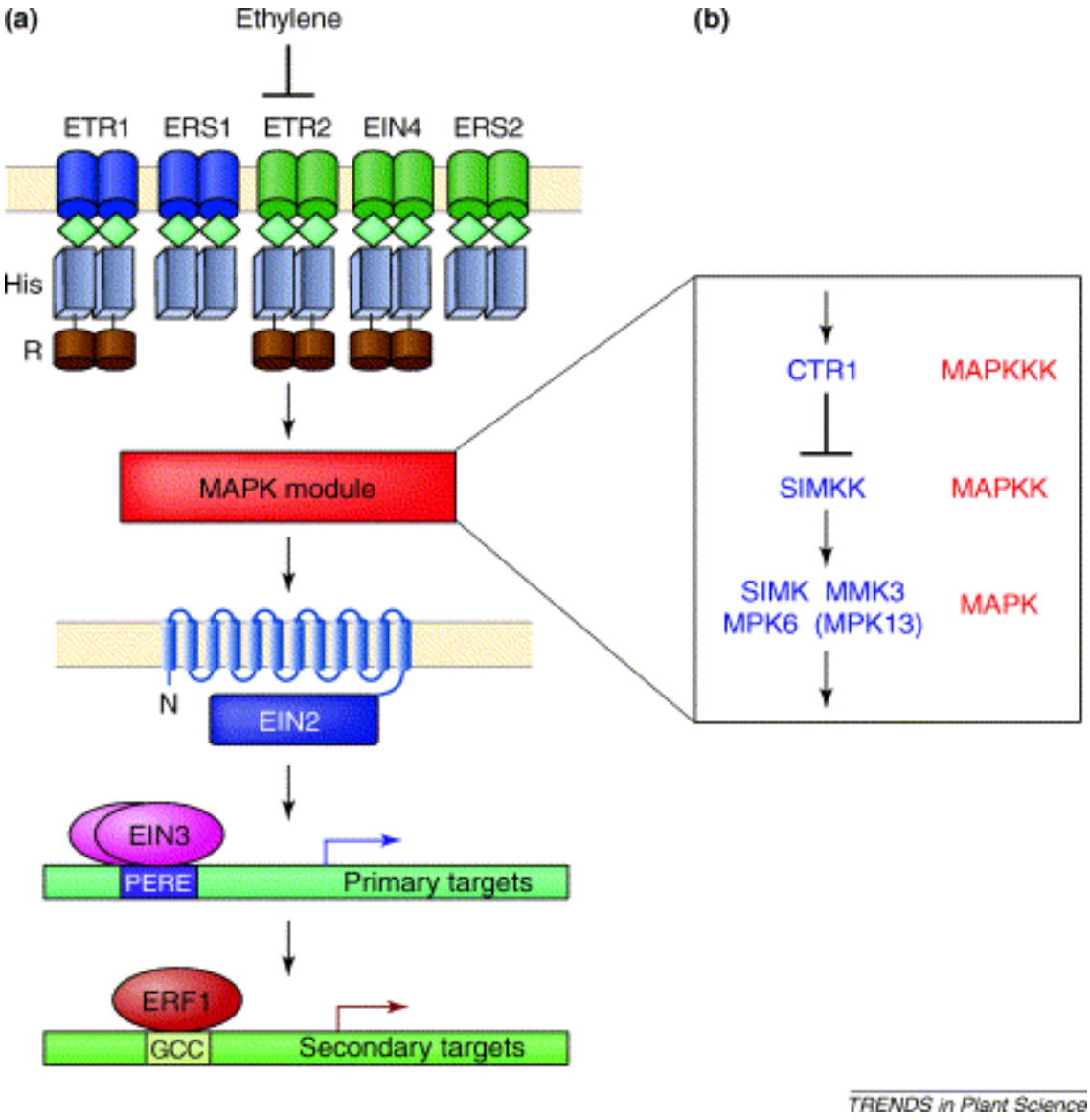


Figure 1-4. The model of the ethylene signaling pathway in A. thaliana (Chang 2003).

(a) The five ethylene receptors (ETR1, ERS1, ETR2, EIN4 and ERS2) are thought to be dimers and are members of the two-component receptor family, which is characterized by a histidine kinase domain (His) and a receiver domain (R).The receptors fall into two subfamilies; ETR1 and ERS1 in subfamily 1 have all the conserved motifs of functional histidine kinases, whereas subfamily 2 receptors have degenerate histidine kinase domains and an additional N-terminal transmembrane domain. One member of each family lacks the receiver domain. The receptors are negative regulators of ethylene responses such that ethylene binding by the N-terminal transmembrane domain represses receptor signaling. However, the biochemical mechanism of ethylene receptor signaling remains unclear. CTR1, the next known component downstream of the receptors, is a negative regulator of responses. CTR1 is a Raf-like protein kinase and the findings of Ouaked et al. (2003) suggest that CTR1 might act in the MAPK module shown in (b). CTR1 is possibly regulated through direct interaction with the ethylene receptors. It is deduced that inactivation of CTR1 results in activation of EIN2, a positive regulator of responses, whose signaling mechanism is unknown. The EIN2 N-terminal transmembrane domain has similarity to the N-ramp family of metal ion transporters, and the hydrophilic C-terminus is

novel. In the nucleus, an ethylene-dependent transcriptional cascade occurs. When activated by ethylene, members of the EIN3 transcription factor family bind as dimers to the primary ethylene response element (PERE) in the promoters of primary response genes such as ETHYLENE-RESPONSE-FACTOR1 (ERF1). ERF1 encodes an ethylene response-element-binding-protein (EREBP). ERF1 and perhaps other EREBPs bind to the GCC-box of secondary response targets, such as basic chitinase and the defensin PDF1.2, activating their transcription. (c) The proposed MAPK module in ethylene signaling is based on Ouaked et al. (2003). In the absence of ethylene, the CTR1 Raf-like kinase is activated, negatively regulating SIMKK (a MAPKK from Medicago). When CTR1 is inactivated by ethylene, SIMKK becomes activated and in turn activates two *Medicago* MAPKs (SIMK and MMK3) or the presumed Arabidopsis orthologs of SIMK and MMK3 (MPK6 and MPK13), respectively. The direct downstream targets of the MAPKs have yet to be determined (**Chang, 2003**).

1.3 *Ethylene in plant disease responses*

Although the role of ethylene in responses to plant pathogens is important, it is nevertheless very complicated. Enhanced ethylene production is one of the earliest active responses of plants to diverse pathogens, such as fungi and bacteria (Yang and Hoffman, 1984; Glazer *et al.*, 1985; Boller *et al.* 1991; Avni *et al.*, 1994; Rojo *et al.*, 1999; Nimchuk *et al.*, 2003; Glazebrook, 2005), but it is not always clear that this ethylene improves disease resistance or increases disease susceptibility. When treated with exogenous ethylene, some plants are more resistant, some are more susceptible, and some are not affected by ethylene (Marte *et al.*, 1993; van Loon and Pennings, 1993; Hoffman *et al.*, 1999; Broekaert, *et al.*, 2006). Ethylene may be a stimulus for defense responses that lead to resistance, or it might play a role in disease symptom development and in the breakdown of endogenous resistance (Boller *et al.* 1991; Abeles *et al.* 1992; Lund *et al.*1998; Bleecker and Kende, 2000; Broekaert, *et al.*, 2006).

The roles of ethylene in the activation of plant defense have been implicated on several different levels. First, ethylene biosynthesis in plants is highly regulated

through complex transcriptional and post-transcriptional controls of the enzymes involved in ethylene biosynthesis after recognition of a specific pathogen attack. Many studies have demonstrated that differential transcription of the various members of the *ACS* gene family is a very important factor regulating ethylene production in response to different pathogens (Peck and Kende, 1998; Barry *et al.*, 2000; Tsuchisaka and Theologis, 2004). In addition to ACS gene expression, root colonization by bacteria has been shown to enhance ACO activity *in vivo* (Hase *et al.*, 2003). Different transcriptional activation of *ACO* genes has been described in response to *potato virus A* in potato (Nie *et al.*, 2002), tobacco mosaic virus and fungi in tobacco (Kim *et al.*, 1998; Xu *et al.*, 2005). More recently, two *ACO* genes were shown to be induced by *Pesudomonas syrinage* infection in tomato (Cohn and Martin, 2005). Moreover, the Genevestigator database records the differential expression of several members of *ACO* gene family in response to several different biotic stresses, which were then confirmed with additional experiments (Zimmermann *et al.*, 2004). The complexity of expression patterns for the *ACS* and *ACO* gene families might have evolved to induce an appropriate ethylene response for a particular pathogen.

Secondly, it is known that ethylene production subsequently induces transcription of a series of pathogenesis-related (PR) protein genes, such as β-1, 3-glucanases, vacuolar basic-chitinases and plant defensins (*PDFs*), through the activation of ERF-type transcription factors, and most of these PR genes are associated with fungi and bacteria pathogens (Felix and Meins; 1987; Broglie *et al.*,

1989, Ohme and Shinshi, 1990; Penninckx *et al.*, 1996; van Loon and Van Stein, 1999; van Loon *et al.*, 2006). ERFs have been identified in several plant species as proteins that bind to the GCC box element, a conserved ethylene responsive promoter element found in many ethylene induced PR genes. The GCC box is a *cis*-acting ethylene response element, consisting of an 11-bp conserved sequence (TAAGAGCCGCC) that is necessary and sufficient for ethylene regulation of ethylene responsive PR genes in several different plant species (Broglie *et al.*, 1989; Ohme-Takagi and Shinshi, 1990; Meller *et al.*, 1993; Hart *et al.*, 1993; Ohme-Takagi and Shinshi, 1995; Shinshi *et al.*, 1995; Penninckx *et al.*, 1996; Gu *et al.*, 2000; Brown *et al.*, 2003). Although most ERFs function as transcriptional activators, ERF transcriptional repressors from several plant species have also been reported (Yang *et al.*, 2005; Kazan, 2006). ERFs can function as activators or repressors of particular defense pathways, which results in resistance or susceptibility to different pathogens. For instance, *AtERF2* or *AtERF4* over-expression results in opposite disease resistant phenotypes after infection by the fungus *Fusarium. oxysporum* (McGrath *et al.*, 2005). Furthermore, transcriptional activation of *AtERF1* enhances resistance to several pathogens including *F. oxysporum* and *Botrytis cinerea* but increases susceptibility to the bacteria *Pseudomonas syringae* (Solano *et al.*, 1998; Berrocal-Lobo *et al.*, 2002; Berrocal-Lobo and Molina, 2004). These examples illustrate the complexity of the regulation of activators and repressors of ERFs during pathogen challenges and their potential to fine-tune the expression of different defense genes to different pathogen attacks.

Finally, plant defenses are regulated by complex signaling pathways that involve not only ethylene but also jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA) (Berrocal-Lobo *et al.*, 2002; Brown et al, 2003; Lorenzo *et al.*, 2003). Deciphering the crosstalk between ethylene, JA, SA and ABA-dependent pathways in plant cells is a major challenge to elucidate the means by which these plant hormones cooperate with each other to respond to different pathogens and stresses. Moreover, the role of ethylene in plant diseases is complicated by the fact that ethylene is involved in many other aspects of plant physiological processes including root structure and root cell differentiation, flowering, ripening, chlorosis, senescence and cell death (Abeles, 1992). These physiological responses to ethylene play both positive and negative roles in different pathogen attacks.

1.4 *Roles of plant growth regulators on nematode infection*

The relationship between root knot nematode (RKN, *Meloidogyne spp.*) galls development and plant growth regulators has been studied for many years, and the levels of auxins, cytokinins and gibberellins have been determined in plant tissues infected with RKN (Jones, 1981; Roy, 1981; Orion and Wergin, 1982; Glazer *et al.*, 1986; Lohar *et al.*, 2004). Cultured tomato roots were inoculated with RKN, *Meloidogyne incognita* on STW (Skoog, Tsui and White) (Murashige and Skoog, 1962) agar medium with or without kinetin (2 μM) and processed for transmission electron microscopy at different time points after inoculation. It was found that the development and ultra-structure of the plastids in galls from infected roots were different from those in non-inoculated roots when exogenous cytokinin was added to

the growth medium. It was concluded that plastid differentiation in the inoculated tissue may be influenced by an accumulation of kinetin in the gall, which was induced by the nematode and served as the nutrient sink for its feeding (Orion and Wergin, 1982). Lohar *et al.* (2004) used the gene promoter for a cytokinin-responsive Arabidopsis response regulator (ARR) fused to a β-glucuronidase (GUS) reporter gene and cytokininoxidase (*CKX*) from *Arabidopsis thaliana* to investigate the role of cytokinins in *Lotus japonicus* after RKN infection. Cytokinin oxidases are known to selectively degrade unsaturated *N6*-isoprenoid side chains and convert active cytokinins such as zeatin and i6Ade to adenine (Mok, 2001). Lohar *et al.* (2004) found that transgenic *CKX* roots had fewer nematode-induced feeding cells per plant than control hairy roots. This result indicated that cytokinins facilitate root knot nematode infection in *Lotus japonicus*. They also found that root penetration and migration of RKN second-stage larvae (L_2) failed to increase ARR5 expression, but a high level of ARR5 expression was induced when L_2 reached the vascular tissue and also during the early stages of nematode infection. Furthermore, ARR5 expression was absent in mature feeding cells although dividing cells around the feeding cells continued to express this reporter gene (Lohar *et al.*, 2004). The above evidence suggests that cytokinins are involved in the feeding cell formation in RKN infected roots.

Myuge and Viglierchio (1975) showed that IAA promoted root mass and galls (feeding cells) in tomato roots that were parasitized by *Meloidogyne incognita*. Other studies showed that RKN galls contained higher auxin levels than non-infected root

tissue (Yu and Viglierchio, 1964; Kochba and Samish, 1972). It was also found that application of indole acetic acid (IAA) (>0.6 μM) to RKN infected cultures resulted in an increase in gall fresh weight, and the level of IAA in infected roots was higher than that in non-infected tissues, reaching the highest level at 10 days after inoculation (Glazer *et al.*, 1986). It was shown that cyst nematode parasitism was inhibited in the auxin resistant *Arabidopsis thaliana* mutant *axr2*, which is a mutant that lacks auxin-inducible ethylene production (Goverse *et al.*, 2000). The experimental evidence indicates that, like galls formation in RKN infection, auxin is also important in formation of the syncytium by cyst nematode infection. Moreover, the use of an auxin-responsive reporter construct indicated the observation of a local accumulation of auxin in developing syncytia and abnormal feeding cells when auxin transport was chemically inhibited (Goverse *et al.*, 2000). In addition to the auxin effects on syncytium formation, it was found that inoculation of the ethylene-overproducing Arabidopsis mutants *eto1, eto2* and *eto3* with *Heterodera schachtii* resulted in hyper-infection, enhanced female development, and more extensive syncytia compared with the control plants. It was also observed that protoplasts fusion by cell wall dissolution was promoted in ethylene overproducing mutants. It was discussed the importance of cross-talk between auxin and ethylene in syncytium formation and proposed that ethylene might result in a local activation of cell wall degrading enzymes in plants during nematode infection (Goverse *et al.*, 2000).

1.5 *Interaction between ethylene and nematode infection*

Two decades ago, it was demonstrated that an increase in ethylene production was closely associated with root knot nematode, *Meloidogyne spp.* infection and feeding cell formation in tomato (Glazer *et al.*, 1983; Glazer *et al.*, 1984; Glazer *et al.*, 1985; Glazer *et al.*, 1986). Excised tomato roots infected with root knot nematode, *Meloidogyne javanica* produced ethylene at 3-6 times the rate of non-infected roots. This increase started at 5 days and then peaked between 9 and 16 days after inoculation (Glazer *et al.*, 1985). It was demonstrated that the rate of gall growth was accelerated by stimulators of ethylene production and suppressed when the production or action of the hormone was inhibited; more specifically, the feeding cell growth and ethylene production in infected roots were increased by ethylene precursor ACC and inhibited by ethylene inhibitors, aminoethoxyvinylglycine (AVG) or silver thiosulfate (STS) (Glazer *et al.*, 1984; Glazer *et al.*, 1985). These findings suggest that ethylene plays a major role in the pathogenic symptoms displayed by RKN-infected plants. More recently, it was demonstrated that ethylene signal transduction positively influences plant susceptibility to cyst nematode. Wubben *et al.* (2001) showed that *Arabidopsis thaliana* ethylene insensitive mutants *etr1-1*, *ein2-1* and *ein3-2* were less susceptible to sugar beet cyst nematode, *H. schachtii,* than the wild type Col-0. Moreover, the ethylene overproducing mutants *eto1-1*, *eto2*, and *eto3* were hyper-susceptible to sugar beet cyst nematode (Wubben *et al.*, 2001). Wubben *et al.* (2001) also showed that an *Arabidopsis thaliana* mutant *rhd1-4* (root hair defective) was hyper-susceptible to the sugar beet cyst *H. schachtii,* and treatment of *rhd1-4* with ACC or AVG revealed that *rhd1-4* morphology was the result of an increased ethylene response. Further experiments found that the *rhd1-4*

hyper-susceptibility to cyst nematode infection, and increased root elongation were dependent upon the ethylene signaling genes EIN2 and EIN3 (Wubben *et al.*, 2004). All above evidence proves that ethylene plays an important role in nematode root infection.

Although the experimental evidence strongly suggests a key role for ethylene in nematode infection, the current data do not define the role of ethylene in SCN infection but only that the presence or absence of ethylene can alter the number of nematodes that colonize roots. An actual role for ethylene in nematode infection in plants is still unresolved. As previously mentioned, Goverse *et al.* (2000) suggested that ethylene might play a role in regulating gene expression associated with cell wall dissolution during nematode infection. It was demonstrated that extensive cell-wall changes are necessary for giant cell and syncytium development and cell wall hydrolases might play a fundamental role in feeding cell-wall architecture. It was demonstrated that several endo-β-1, 4-glucanases are up-regulated within the feeding cell during its formation in tobacco roots (Goellner *et al.*, 2000). In addition, endo-β-1, 4-glucanases were shown to be up-regulated within the roots upon infection by both root-knot and cyst nematode infection in *Arabidopsis thaliana* (Vercauteren *et al.*, 2002). The *Arabidopsis* cel1 endo-1, 4-β-glucanase showed activity only in giant cells but not in syncytia. This result demonstrated that the specific regulation of cell-wall-degrading enzymes is probably required for cell-wall modifications to build feeding cells (Mitchum *et al.*, 2004). Recently, the Affymetrix GeneChip was used to examine SCN-induced gene expression in soybean roots and many cell-wall

modifying genes were significantly changed during nematode infection, including endo-1, 4-β-glucanase, expainsins and pectatelyases (Puthoff *et al.*, 2007; Tucker *et al.*, 2007). Similar results were obtained by Ithal *et al.* (2007). In *Arabidopsis thaliana*, expansin genes *AtEXP*3, *AtEXP*6, *AtEXP*8, *AtEXP10* and *AtEXP16* were found to be up-regulated specifically in syncytia, but not transcribed in surrounding root tissue 5-7days after sugar beet cyst nematode infection (Wieczorek *et al.*, 2006). An expansin gene in tomato, *LeEXPA5* was shown to be induced in gall cells with RKN infection, and it was also shown that the ability of a nematode to complete its life cycle was reduced in antisense *LeEXPA5* transgenic roots (Gal *et al.*, 2006). It was suggested that *LeEXPA5* is necessary for a successful parasitic nematode-plant interaction. Though some progress has been made on ethylene in plants infected with nematode, the role of ethylene in the susceptibility of soybean roots to soybean cyst nematode, especially in forming the syncytium, remains obscure. Understanding the role of ethylene in nematode infection will provide us more useful information to create new approaches to increase the resistance to SCN in soybean.

1.6 *Hypothesis and experimental approaches*

Based on previous studies of ethylene with nematode infection in plants, I hypothesize that ethylene is involved in feeding cell formation in soybean roots after SCN infection. In addition, several sub-hypotheses are proposed to be tested as follows: (1) SCN development is increased by enhanced ethylene concentrations and inhibited by ethylene inhibitors or in ethylene insensitive mutants. Chapter 2 describes results to demonstrate that SCN development was decreased in ethylene

resistant soybean *etr1-1* mutant and also in the roots when the ethylene response was inhibited with 1-MCP or 2, 5-NBD treatment but increased in ethylene treated root. (2) Ethylene is induced in soybean roots after SCN infection. In chapter 3, results are presented that suggest that ethylene production in SCN infected excised soybean roots was 1-2 times greater than in non-infected roots. (3) Ethylene is synthesized in the syncytium or around the syncytium. In chapter 4, transgenic soybean hairy roots were prepared that included an ethylene responsive promoter GCC box promoter**::**GUS construct which acts as a reporter for the synthesis of ethylene in the feeding cells after SCN infection.

Chapter 2: Effects of Ethylene on the Development Of SCN in Soybean Roots

2.1 *Introduction*

As discussed in Chapter 1, it has been demonstrated that ethylene positively influences plant susceptibility to nematode infection through studies with ethylene precursors, ethylene inhibitors, ethylene insensitive and overproducing mutants in tomato and *A. thaliana* (Glazer *et al.*, 1985; Wubben *et al.*, 2001). My hypothesis is that SCN parasitism in soybean roots should be increased by ethylene treatments and reduced when ethylene action is chemically or genetically inhibited. To test this hypothesis, I investigated the effects of ethylene on soybean cyst nematode infection in an ethylene resistant soybean mutant (*etr1-1*) and in its wild-type Hobbit 87, and also in soybean (*G. max*, cv. Williams) cultured roots exposed to ethylene or the ethylene action inhibitors 1-MCP and 2, 5-NBD.

2.2 *Materials and methods*

2.2.1 SCN infection

Soybean cyst nematode, *H. glycines* race 3 was isolated from soybean fields in Maryland and has been maintained for many years on sterile cultures of soybean *G. max*. cv. Kent roots in the Nematology Lab at USDA, Beltsville Agricultural Research Center. The sterile SCN used in the following experiments have been maintained on similarly cultured soybean, *G. max.*, cv. Williams roots where fresh

root radicals were inoculated every month with mature females from earlier culture plates. Soybean seeds of the ethylene resistant mutant (*etr1-1)* and wild-type Hobbit87 were kindly provided by Dr. Andrew Bent, University of Wisconsin (Madison, WS). Soybean *G. max,* cv. Williams seeds were provided by Dr. Perry Cregan at USDA Soybean Genomics and Improvement Lab in Beltsville.

Soybean seeds of the ethylene resistant mutant (*etr1-1)* and its wild-type Hobbit 87 were surface sterilized with 95% ethanol for 3 minutes, followed by 15% bleach for 10 minutes and then rinsed several times with an excess of sterile water. Seeds were germinated at 26°C in the dark on 1.5% (w/v) *Type* A agar plates. After 3-4 days, approximately 2 cm of root radicals was excised from geminated seeds and placed on 1.2% Noble agar containing Gamborg's B5 salts and vitamins pH 6.2 (Sigma, St. Louis, MO, USA) supplemented with 2% sucrose. Roots (two per plate) were incubated at 26°C for 3 days and then five mature axenic SCN females with egg masses were placed around each root tip and crushed to release eggs. Plates were maintained at 26°C on a cycle of 30 days in the dark, and the number of first generation swollen females was then counted in each plate carrying Hobbit 87 or *etr1-1* mutant line. Susceptibility of soybean roots to SCN was evaluated by comparing the different number of mature SCN females on Hobbit 87 and *etr1-1* mutant roots. Each treatment was replicated five times and the experiment was conducted twice.

2.2 2 Ethylene, 1-MCP and 2, 5-NBD treatments

1-MCP was purchased from SmartFresh Technology and 2, 5-NBD was purchased from Sigma-Aldrich.

For each treatment, four plates of soybean *G. max*., cv. Williams roots were inoculated with SCN (5 mature females per root and two roots per plate) and then placed in a 2.5 liter desiccator. Ethylene, 1-MCP and 2, 5-NBD were injected through a needle into an airtight desiccator to obtain a final concentration of 1 µl/L ethylene, 2 µl/L 1-MCP and 5000 µl/L 2, 5-NBD, respectively. Four plates soybean roots with SCN were placed in a desiccator (no treatment) was used as a control. The four desiccators were incubated at 26°C for 30 days in the dark. Every four days, all of the desiccators were opened in the hood for 5-10 minutes to exchange O_2 and CO_2, and the same amount of 2, 5-NBD (5000 µl/L), 1-MCP (2 µl/L) or ethylene (1 µl/L) was injected into the desiccator after closure. At 30 days, the number of first generation females was counted on the roots of all four plates for each treatment. Susceptibility of soybean roots to SCN was evaluated by comparing the different number of fully mature female SCN among the different treatments. The complete experiment was replicated twice.

2.3 *Results*

2.3.1 SCN development in the roots of soybean *etr1-1* mutant and its wild type Hobbit 87

The soybean *etr1-1* mutant was identified in a screen of seedlings that displayed reduced sensitivity to ethylene after mutagenesis of the seeds with

nitrosoguanidine (Hoffman *et al.*, 1999). Further tests demonstrated that the soybean *etr1-1* mutant was not completely insensitive to ethylene but had a significantly diminished response to ethylene (Hoffman *et al.*, 1999). The ability of a compatible soybean cyst nematode, *H. glycines*, race 3 to infect and develop on roots from soybean *etr1-1* mutant and its wild-type Hobbit 87 plants was also examined. In two independent experiments, significantly fewer females developed on *etr1-1* mutant than those on wild-type Hobbit 87 control roots one month after inoculation ($P<0.05$, mean ± standard error of mean=118±36 and 67±10 for *etr1-1* mutant in the two respective experiments, and 182±27 and 126±13 for its wild type Hobbit 87). The root morphology of infested roots was similar for Hobbit 87 and the *etr1-1* mutant (Bent *et al.*, 2006).

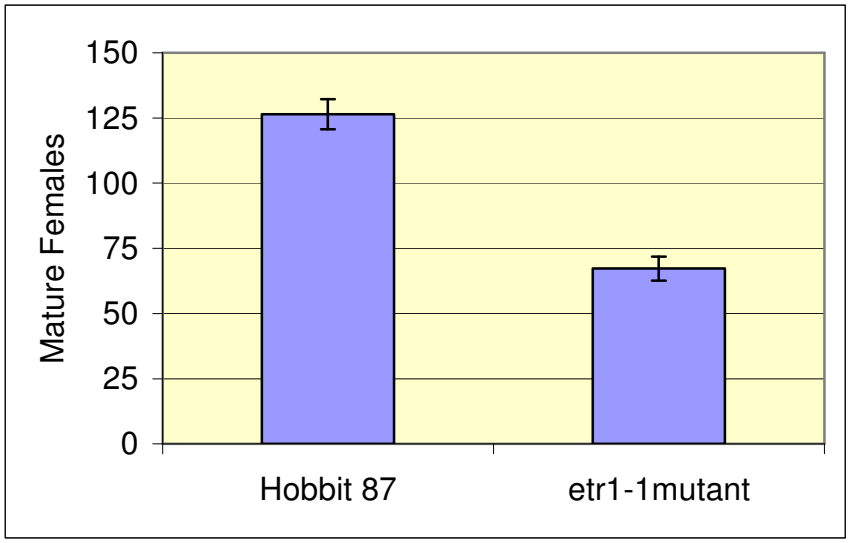


Figure 2-1. SCN development on the root of etr1-1 mutant and Hobbit 87.
The data presented are from one representative experiment out of two with comparable results ($P<0.05$).

2.3.2 Effects of ethylene and its inhibitors on SCN development in soybean excised roots

Both 2, 5-NBD and 1-MCP can bind to ethylene receptor to inhibit ethylene response. 2, 5-NBD binds to the receptor and prevents ethylene responses but requires continuous exposure, and high level of ethylene will overcome the effect by competition. However, 1-MCP can bind to the receptor and a single exposure can prevent ethylene responses for up to 12 days, during which time ethylene does not overcome the response (Sisler and Serek, 1999). The ability of a susceptible soybean cyst nematode, *H. glycines*, race 3 to infect and develop on cultured roots from soybean (*G.max,* cv. Williams.) treated with ethylene and its inhibitors was examined. Treatment of SCN infected Williams roots with 1 µl/L exogenous ethylene significantly increased the number of mature SCN females by 23% compared to non-treated control SCN infected roots. Relative to control roots, the number of SCN significantly decreased 87% on the roots treated with 1-MCP (2 µl/L) or 2, 5-NBD (5000 µl/L). In two independent experiments, significantly fewer females developed on 1-MCP or 2, 5-NBD treated roots than those on control roots or ethylene treated roots 1 month after inoculation ($P<0.05$, mean ± standard error of mean=0.67±0.67 and 8.25±1.89 for 1-MCP treated roots and 3.33±1.76 and 7.25±1.49 for 2, 5-NBD treated roots, 30.67±3.71 and 73.50±2.40 for ethylene treated root, and 21.67±6.23 and 60.00±5.34 for control roots in the two respective experiments). The root morphology of infested roots was different among the ethylene, ethylene inhibitor treated roots and control non-treated roots. Ethylene treated roots were shorter and thicker, whereas the inhibitors treated roots were thinner and longer compared to the

control roots. 1-MCP treated roots were much thinner and longer compared to 2, 5-NBD treated roots (pictures were not shown here).

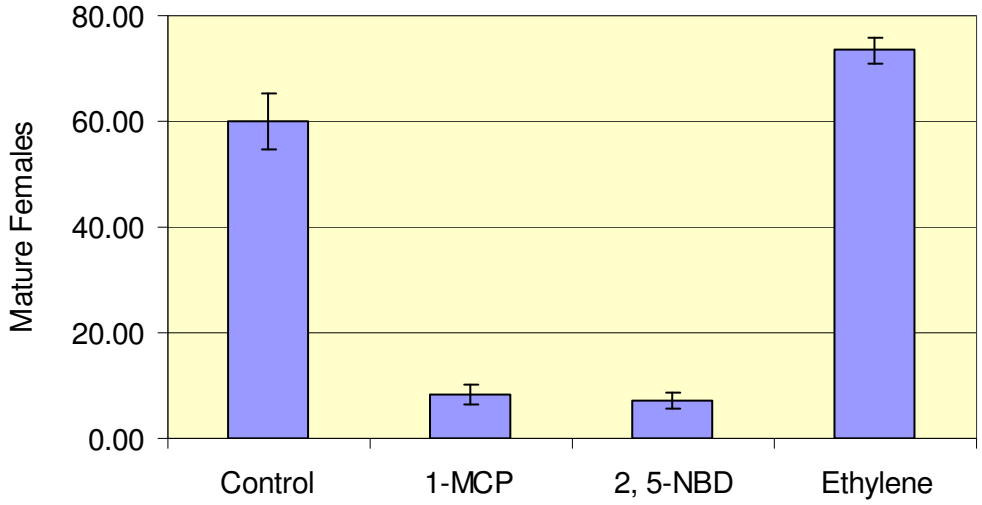


Figure 2-2. Effects of ethylene and its inhibitors 1-MCP and 2, 5-NBD on SCN development in soybean root cultures.
The data presented are from one representative experiment out of two with comparable results ($P<0.05$).

2.4 *Discussion*

The goal of this study was to investigate the impacts of ethylene, ethylene inhibitors and ethylene resistant mutant on SCN development in soybean roots. Ethylene insensitive (resistant) mutants provide a unique opportunity for such studies because external ethylene response inhibitors do not need to be applied for the studies to proceed. The SCN number might be decreased much more than 40% if *etr1-1* mutant was completely ethylene insensitive rather than only resistant to ethylene. A completely insensitive mutant might be more similar to 1-MCP or 2, 5-NBD treatment. Although the root morphology was slightly different between the 2, 5-

NBD and 1-MCP treatments, no significant difference was observed in the number of SCN that matured on the treated roots, both inhibitors significantly reduced SCN numbers by 87%. Both 2, 5-NBD and 1-MCP were tested to reduce the possibility that effect of the inhibitors was secondary and not due to an inhibition of ethylene action. Compared to the control, ethylene treatment increased SCN numbers by 23%, this smaller increase might be because infected roots produce enough ethylene to support SCN development but not at optimal concentrations. Overall, my observations are consistent with earlier results (Glazer *et al.*, 1985; Wubben *et al.*, 2001). Although these experiments demonstrate a requirement for ethylene, still many questions on the role of ethylene need to be resolved including a determination of how much ethylene is produced in SCN infected soybean roots compared to non-infected roots.

Chapter 3: Ethylene Production in SCN (*H. glycines)* Infected Soybean Roots

3.1 *Introduction*

It was observed that excised tomato roots infected with RKN *M. javanica* produced 3-6 times as much ethylene as non-infected roots and the rate of gall growth was accelerated by stimulators of ethylene production and suppressed when the production or action of the hormone was inhibited (Glazer *et al.*, 1985). To explore the relationship between ethylene and formation of SCN feeding cells in soybean roots, I examined the rate of ethylene production in SCN infected and non-infected soybean excised roots, emphasizing the relationship between the production of ethylene and SCN development in soybean roots.

3.2 *Materials and methods*

3.2.1 Ethylene production in SCN infected roots

Excised soybean (*G.max* cv. Williams) roots were grown in 25-ml Erlenmeyer flasks containing Gamborg's B5 media with Noble Agar (12 g/L). Two excised roots were placed in each flask and both of them were inoculated with 10 mature SCN females with egg masses as described earlier in Chapter 2, then covered the top of the flask with parafilm. Non-infected roots were used as controls. The rate of ethylene production by non-infected and infected excised soybean roots was

determined every 7 days after SCN inoculation. 24 hr before ethylene determinations, the parafilm was replaced by a sterilized rubber cap to prevent loss of ethylene. Ethylene production by roots growing in the Erlenmeyer flasks was measured by gas chromatography. After 30 days post inoculation (dpi), the roots in each flask were pulled out of the agar and weighed. The number of SCN in each Erlenmeyer flask was counted under the microscope. Each treatment included 7 replicate flasks.

3.3 *Results*

3.3.1. Ethylene production in SCN infected soybean excised roots

The rate of ethylene production in SCN infected soybean roots and non-infected roots is plotted in Figure 3-1. Ethylene production for both infected and non-infected roots at 7 days was too low to be accurately measured by the procedures used. At 14 days, non-infected roots produced about 0.47 nl/g h ethylene and SCN infection caused a 1.6-fold increase to 0.74 nl/g h. At 22 days, non-infected roots produced ethylene at the rate of 0.55 nl/g h and SCN infected roots produced the highest measurement of ethylene production of 1.6 nl/g h, which is about a 2-fold increase in ethylene compared to non-infected roots. Ethylene production was too low to be measured in both infected and non-infected roots at 30 days. The average number of mature SCN females on the roots in each 25 ml Erlenmeyer flask was about 30, which was much lower than that on the roots in Petri plates.

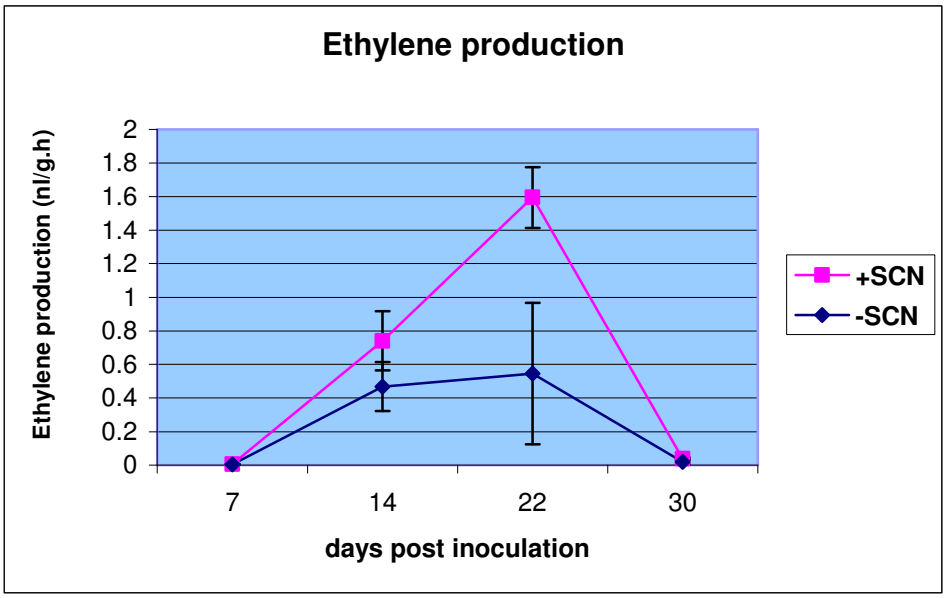


Figure 3-1. Ethylene production at different time points after inoculation in soybean excised roots non-infected and infected by SCN.

3.4 *Discussion*

The data from this experiment indicate that ethylene production is very low in the first 7 days and at 30 days. The lack of measurable ethylene in the first 7 days might be because it takes time for SCN eggs to develop to J2s on B5 medium before they can infect the roots and the root mass of both the inoculated and non-inoculated roots is too small to produce enough ethylene to be measured in the experiment. At 30 days the roots were wilt, which might account for the low undetectable levels of ethylene. Nevertheless, the rate of ethylene production was significantly increased in the SCN inoculated roots compared to the control roots at 14 and 22 dpi. It is possible that the ethylene production might have been much higher if the SCN infection of roots cultured in the Erlenmeyer flasks was as high as that on Petri plates.

Earlier attempts to measure ethylene production in the Petri dish failed because it can not be easily sealed and it was difficult to attach a septum to the Petri plate surface for penetration of a gas syringe to extract a sample from the gas environment.

An increase in ethylene production from 14 to 22 dpi is consistent with syncytium development in the roots. The results indicate that ethylene increases during feeding cell formation after nematode infection, which supports previous findings with gall formation in tomato after infection with RKN. The next step in my research is to determine where ethylene is synthesized in SCN infected roots and if ethylene is synthesized specifically in SCN feeding cells.

Chapter 4: Preparation of an Ethylene Reporter Gene Construct and Transformation of Soybean Roots

4.1 *Introduction*

As discussed in chapter 1, the GCC box, an 11-bp sequence (TAAGAGCCGCC), is a *cis*-element found in the promoters of several ethylene-response PR genes. It has been shown that the GCC box is necessary and sufficient for ethylene regulation of ethylene responsive PR genes in plants (Felix and Meins; 1987; Broglie *et al.*, 1989; Ohme and Shinshi, 1990; Penninckx *et al.*, 1996). It has also been demonstrated that the GCC box confers ethylene responsive transcription when incorporated into a heterologous promoter (Ohme and Shinshi, 1995). In addition, an *EREBP* homolog was isolated from a soybean cDNA library and gel mobility-shift assays revealed that the translation product of this cDNA bound specifically to GCC box (Mazarei, *et al.*, 2002). To study local changes of ethylene action during SCN infection in soybean roots, a GCC box promoter::GUS construct was made and expressed in soybean roots. My hypothesis is that the activity of the GUS reporter gene should be detectable during SCN infection or after exogenous ethylene treatment, and GUS staining should be localized in expanding syncytia and in cells around the syncytia. This hypothesis was formulated based on the literature and results described in Chapters 1, 2 and 3, which suggest that ethylene might mediate feeding cell formation in soybean roots after SCN infection.

Multiple copies of a GCC box element were fused upstream from a -50_35S minimal promoter to promote expression of a GUS reporter gene (GCC-50_35S-GUS). This construct was transferred into the soybean genome by hairy root transformation. The -50_35S promoter used in this construct includes the minimum sequence required for recognition by RNA polymerase, i.e., TATA box (Tucker *et al.*, 2002). Genomic DNA was isolated from the transgenic hairy roots, and PCR analysis and non-radioactive Southern blots were performed to make sure that the GCC fragment had been integrated into soybean genome.

After hairy root transformation, the ethylene-dependent expression of GUS from the GCC-50_35S-GUS gene was tested by histochemical and fluorometric GUS assays (Jefferson *et al.*, 1987). However, it was discovered by GUS fluorometric assay that cultured *Agrobacterium* that included the GUS construct produced high level of GUS activity even when no plant root material was present. To eliminate the GUS activity from the *Agrobacterium*, a new GCC-GCC-5035-GUS construct with a catalase intron (a 190 bp fragment inserted into GUS gene sequence) was made and the ethylene responsiveness of this construct was tested by a GUS transient expression assay using particle-gun bombardment of the GCC-GCC-50_35-GUSi construct.

4.2 *Materials and methods*

4.2.1 Construction and transformation of GCC-50_35S-GUS in soybean roots

Small scale plasmid DNA extraction was performed by following the protocol of QIAprep Miniprep Kit from QIAGEN (Valencia, CA).

4.2.1.1 Preparation of the GCC fragment

The GCC fragment was prepared by single-strand synthesis (Invitrogen, Carlsbad, CA) of each strand of the double GCC-box found in the 5' upstream promoter of the tobacco *Gln2* gene encoding *β*-1, 3-glucanase (nucleotides -1164 to -1118) (Ohme and Shinshi, 1990). *Hind*III and *Not*I restriction endonuclease sites were added at the 5' end and 3' ends, respectively in order to insert the GCC box into the minimal -50_35S-pBI221 vector. The sequence of both strands (54 bp each) was shown below (Each of two 11 bp GCC boxes was underlined):

5' agctt**CATAAGAGCCGCCACTAAAATAAGACCGATCAAATAAGAGCCGCCAT**gc **3'**

3' a**GTATTCTCGGCGGTGATTTTATTCTGGCTAGTTTATTCTCGGCGGTA**cgccgg **5'**

4.2.1.2 Construction of GCC-50_35S-GUS and 50_35S-GUS constructs (no intron).

The minimal promoter construct -50_35S-Luc was prepared previously (Tucker *et al.*, 2002). Plasmid DNA for the -50_35S-Luc construct was extracted and digested with *Hind*III/*Bam*HI, and a 60 base pair (bp) DNA fragment (-50_35S) was purified from 1.2% low melting agarose gel. The pBI221 vector plasmid was digested with *Hind*III/*Bam*HI, and pBI221 vector (without CaMV35S promoter) was purified from the gel. The -50_35S was inserted into pBI221 vector to create a 50_35S-GUS (pBI221) construct. The complementary GCC box single stranded

DNAs were heated to 95°C, cooled down slowly to 50°C to create a double stranded DNA. The -50_35S-GUS (pBI221) was digested with *Not*I first, and then with *Hind*III, and the GCC double-strand DNA was inserted into pBI221-50_35S vector with T4 DNA ligase (Sambrook *et al.,* 1989). The recombinant DNA was analyzed using restriction enzymes and sequenced using PCR-based dideoxynucleotide terminator protocol and an ABI automated sequencer in Dr. Van Berkum's lab at USDA Soybean Genomics and Improvement Lab in Beltsville.

GCC-50_35S-GUS (pBI221) plasmid DNA was digested with *Hind* III/*Eco*RI., and a 2.3Kb fragment was gel purified. *Agrobacterium* binary vector pBI121 was digested with *Hind*III/*Eco*RI., and an 11.8 kb fragment was purified from the gel. The GCC-50_35S-GUS insert was then cloned into pBI121 to create GCC-50_35S-GUS (pBI121). The recombinant DNA was digested with *Hind*III/*Eco*RI to make sure that the GCC box was correctly inserted into pBI121 binary vector. A diagram of the GCC-50_35S-GUS constructs was shown in Figure 4-1.

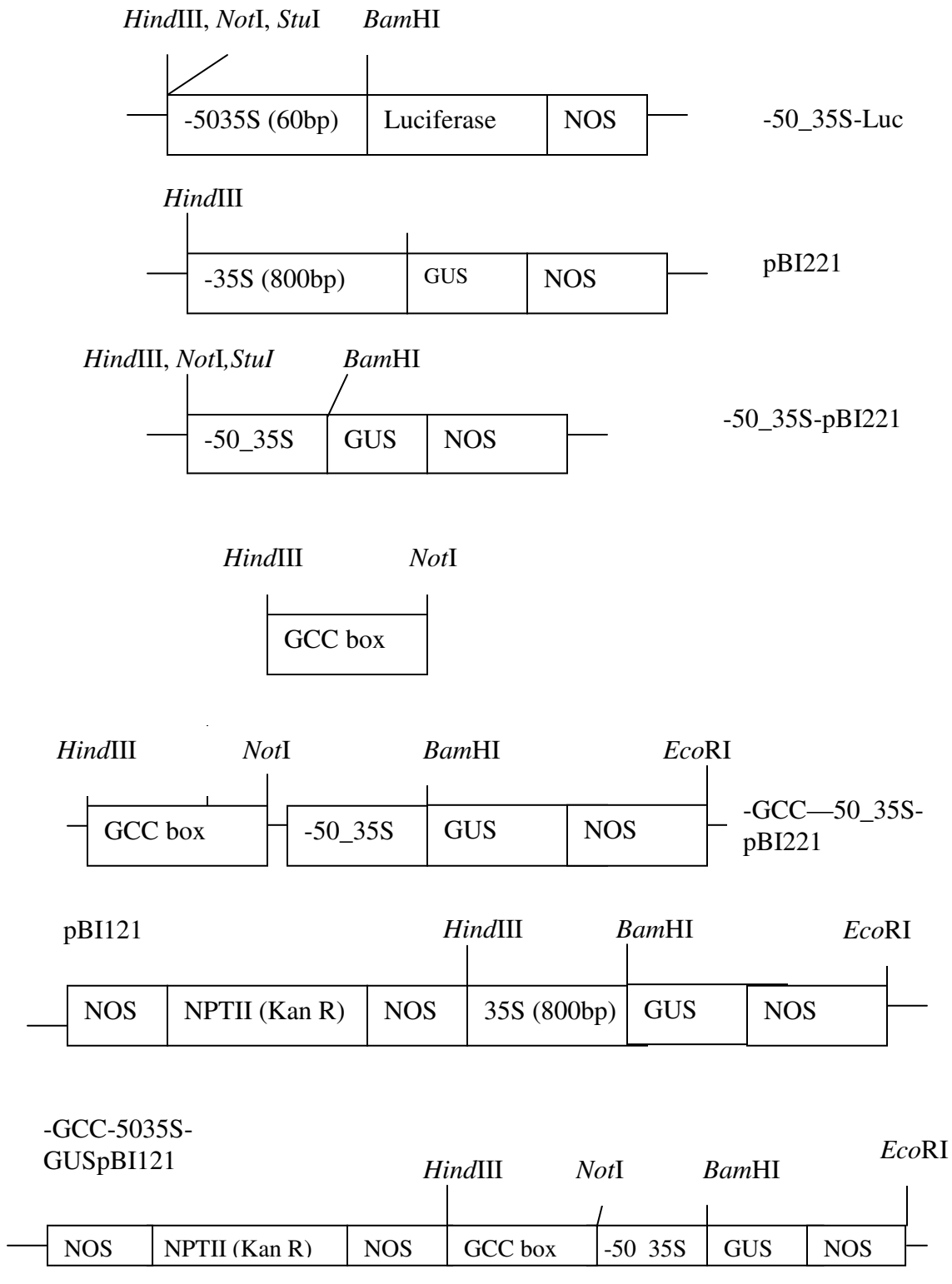
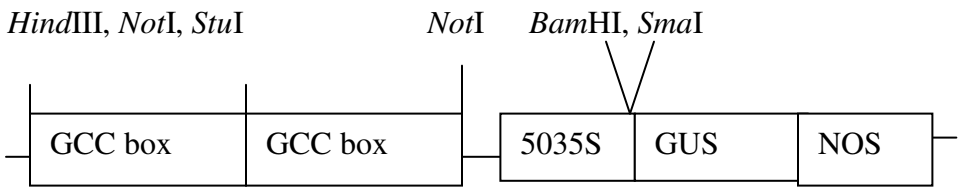
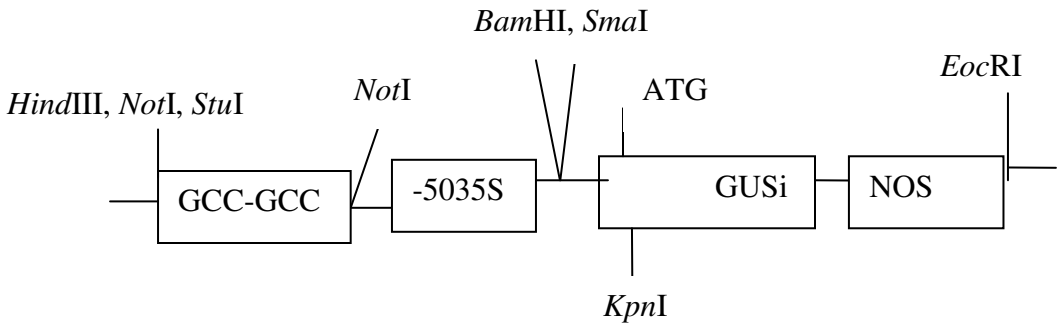


Figure 4-1. Construction of GCC-50_35S-GUS in pBI121

-GCC--5035S-pBI221



(2GCC-50_35S-pBI221)



(-50_35S-pBI221)

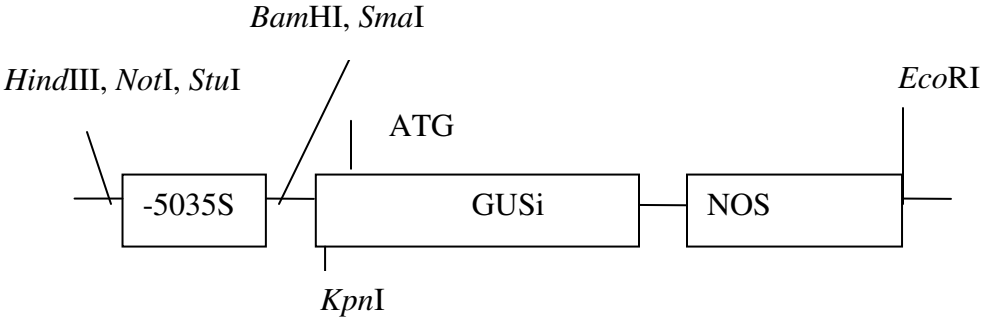


Figure 4-2. Construction of GCC-GCC-50_35S-GUSi (intron)

4.2.1.3 Hairy root transformation

Introduction of the binary vectors GCC-50_35S-pBI121 and 50_35S-pBI121 into *Agrobacterium rhizogenes* K599 was performed by electroporation (Cho *et al.*, 1998). Hairy root transformation was conducted according to Cho *et al.* (2000) as follows: Cotyledons from 4-5 day-old seedlings were inoculated with an overnight culture of the *A. rhizogenes* strain being tested. Cotyledons were incubated in the dark for 3 days before transfer to MXB medium [MS (Murashige and Skoog, 1962) basal nutrient salts, B5 (Gamborg *et al.*, 1968) vitamins and 3% sucrose (pH 5.7)] solidified with 3 g/L Gelrite (Greif Bros. Corp., East Coast Division, Spotswood, N.J., USA) in Petri dishes (100 mm diameter, 25 mm deep). Carbenicillin disodium (500 µg/ml) was added to inhibit the growth of *A. rhizogenes*, and kanamycin (200 µg/ml) was added to the MXB medium to select for transgenic roots. About 10-14 days after root emergence, 1 to 2 cm long root tips were transferred and were freed from bacteria by two or three passages on the same medium at 25°C in the dark. The established root cultures were then transferred every 4 to 5 weeks on medium without carbenicillin.

4.2.1.4 Soybean Hairy root genomic DNA extraction

Total genomic DNA was isolated from hairy roots by the CTAB method according to Rogers and Bendich (1985) as follows: 7.5ml extraction buffer (50 mM Tris pH 8.0, 1% CTAB, 50 mM EDTA, 0.7 M NaCl) with 0.1% β-me (added just before using). The extraction buffer was added to 300 mg plant tissue, mixed thoroughly, and incubated at 60°C for 1 hr. Six milliliters of Chloroform/Isoamyl alcohol (24:1) was added and mixed well. The aqueous phase was transferred to a

new tube after being centrifuged at 3500 rpm for 10-15 mins. Isopropanol equal to 2/3 volume of the above aqueous phase was added to the tube and mixed well. The DNA pellet was rinsed with ethanol wash buffer (80% ethanol, 15 mM NH_4OAC, and 20% ddH_2O) and carefully transferred to a clean 1.5 Eppendorf tube. The DNA pellet was dried and re-suspended in TE Low (10 mM Tris, 1 mM EDTA). RNaseA was added and the DNA pellet was incubated at 37ºC for 1 hr, then resuspended well and quantified spectrophotometrically (Nanodrop) and visualized in an agarose gel.

4.2.1.5 PCR reaction to detect transgenic hairy root lines

20 µl of PCR reaction mixture contained: 2 µl 10xPCR reaction buffer (-$MgCl_2$), 0.6 µl 50 mM $MgCl_2$, 0.5 µl 1 mM dNTPs, 0.5 µl (10 U/µl) Taq, 100 ηg hairy root genomic DNA, Kanamycin synthesized 5' and 3' primers, 1 µM each and sterile ddH_2O. The cycling conditions were as follows: denature at 94°C 1 minute for 1 cycle, then 94°C for 30 second, anneal at 58°C 30 second, 72°C 1 minute, after 28 cycles of amplification. The reaction was incubated at 72 °C for 7 minutes to assure complete extension.

4.2.1.6 Histochemical and fluorometric GUS assays for GCC-50_35S-GUS gene

Soybean hairy roots carrying the GCC-50_35S-GUS reporter gene construct were incubated for 48 hrs in a desiccator treated with final concentration of 10 µl/L ethylene before the GUS assays were performed. *Agrobacterium* K599 (empty vector), pBI121 (CaMV35S) binary vector and -50_35S-GUS gene constructs hairy root lines controls were in the same desiccator. As a control, hairy roots carrying the

GCC-50_35S-GUS reporter gene construct were placed in a separate desiccator which no ethylene was added. GUS assays were conducted according to the procedure described in Jefferson *et al.* (1987). Histochemical reactions with the indigogenic substrate X-Gluc were performed with the final X-Gluc concentration of 500 µg/ml of GUS histochemical assay buffer (100 mM NaH_2PO_4, pH7.0, 10 mM Na_2-EDTA, 0.5 mM $K_4Fe(CN)_6$, 0.5 mM $K_3Fe(CN)_6$, 0.1% Triton-X100) in a 1.5 ml tube at 37°C for several hours (one hairy root tip per tube). After staining, plant tissues were rinsed with 3:1 Ethanol: Acetic acid for a few minutes, and then stained hairy roots were observed by microscopy.

Fluorometric assays for GUS activity were performed as described by Jefferson et al. (1987), except that samples were heated to 55ºC for 30 minutes to inhibit endogenous GUS-like activities. Hairy roots were homogenized in 500 µl lysis buffer (100 mM $NaPO_4$, pH 7.0, 10 mM dithiothreitol (DTT), 1 mM Na_2EDTA, 0.1% Sodium Lauryl Sarcosine, 0.1% Triton X-100, 0.1% β-me in an Eppendorf tube. The homogenized tissue was heated at 55ºC for 30 minutes, and then centrifuged for 5 min at 4°C at 14,000 rpm. A 90 µl extract was taken to a new tube, 1/10 volume 10 mM 4-MUG was added to the tube, and the tube was incubated at 37ºC in the dark for 3 to 15 hrs. Immediately after adding extract, t=0 time point, 10 µl extract was taken out to a new tube and 1 ml 0.2M Na_2CO_3 added to stop the reaction. After 3 and 15 hrs, samples were taken and stored in the dark at room temperature. Fluorescence was measured using a Bio-Rad fixed wavelength fluorometer, excitation 365 nm and emission 455 nm. Immediately before reading the samples, 4-MU standards were

prepared and kept in the dark: 1 ml 10 ηM, 50 ηM and 100 ηM 4-MU in 2 ml 0.2 M Na_2CO_3. The machine was blanked with 0.2 M Na_2CO_3, and set to auto concentration with the 4-MU standards. 1.0 ml of each time point sample was diluted with 2.0 ml of 0.2 M Na_2CO_3, mixed well, and then fluorescence was measured. Protein assays were performed using a BSA standard curve and Bio-Rad assay kit, which was based on the Bradford protein assay (Bradford, 1976). The protein content of samples was read with a Beckman spectrophotometer by the absorbance of light at 595 nm.

4.2.1.7 Southern blot hybridization

Approximately 5 µg soybean total genomic DNA (0.5 µg/µl) was digested with *Hind*III at 37ºC for 3 hrs. As controls, 75 pg pBI121 and 25 pg pSAC1 plasmid DNA were also digested with *Hind*III. The pBI121 plasmid DNA was the control for pBI121 hairy roots, and the soybean abscission cellulase gene pSAC1 was used to make sure the hybridization worked well and equal amount of genomic DNAs was loaded into each lane. The fragments were separated by electrophoresis in 0.8% agarose gel without ethidium bromide to avoid high backgrounds observed after autoradiography. The fragments were transferred from the agarose gel to a nylon membrane (Hybond-N; Amersham) and cross-linked to the membrane by using a UV Stratalinker (1200 Joules).

Hybridization was carried out according to the instruction manual for DIG High Prime DNA labeling and Detection Starter Kit II (Roche Applied Science,

Indianapolis, IN). DIG-labeled DNA probes were generated with DIG-High Prime according to the random primed labeling technique. DIG-High Prime is a specially developed reaction mixture containing digoxigenin dUTP and other reagents. The membrane was subjected to immunological detection with anti-digoxigenin-AP conjugate and then visualized with the chemiluminescence ready-to-use substrate CSPD. Enzymatic dephosphorylation of CSPD by alkaline phosphatase leads to a light emission at a maximum wavelength of 477 nm which is recorded with an appropriate imager or on X-ray film. Film exposure times are in the range of 5 to 30 min only.

Approximately 1 µg pBI221 (or SAC1) plasmid DNA was labeled with DIG-High Prime. The labeling efficiency of probes was determined by comparing them with the control DNA in the Kit before hybridization. The membrane was pre-hybridized in DIG Easy Hyb for 30 minutes at 45ºC in a hybridization tube, and then approximately 2 µg denatured DIG-labeled DNA probe was added to pre-heated DIG Easy Hyb and the membrane in Hyb buffer was incubated at 45ºC overnight. The membrane was washed sequentially with 2×SSPE/0.1%SDS, 1×SSPE/0.1%SDS SSPE and 0.1×SSPE/0.1%SDS at 55ºC. The wash was followed by a brief rinse in washing buffer (maleic acid buffer with 0.3% Tween 20). The membrane was subjected to immunological detection with anti-digoxigenin-AP conjugate and visualized with CSPD ready-to-use. Finally, the membrane was exposed to X-ray film for 15-25 minutes at room temperature.

4.2.2 Construction and transient expression assay of GCC-GCC-50_35S-GUSi

4.2.2.1 Construction of GCC-GCC-50_35S-GUSi (with intron) and -50_35S-GUSi control (with intron)

A new GCC-GCC-50_35S-GUSi (with intron) construct was made to eliminate GUS activity coming from contaminating *Agrobacterium* in soybean hairy roots and two additional GCC boxes was used to increase the ethylene response compared to the double GCC boxes in the original clone. The pBI221-GCC-50_35S-GUS plasmid DNA 10 μg was digested with *Hind*III first, then subjected to phenol extraction to denature the endonuclease, and then the overhanging ends filled in with Accuprime DNA polymerase (Invitrogen, Carlsbad, CA). The polymerase was denatured with phenol and the DNA digested with *Eco*RI. The smaller 2.5 kb GCC-5035S-GUS fragment was recovered from a low melt agarose gel. Approximately 600 ηg of the original pBI221-GCC-5035S-GUS plasmid DNA was digested with *Stu*I and *Eco*RI, and the larger fragment (2.4 kb) recovered from a low melting agarose gel. The GCC-5035S-GUS (2.5kb) was inserted into pBI221-GCC (2.4 kb) vector to make GCC-GCC-50_35S-GUS (no intron). The GCC-GCC-50_35S-GUS (no intron) plasmid was digested with *Bam*HI and *Eco*RI to remove GUS (no intron), and a *Bam*HI and *Eco*RI digested GUSi fragment from T7/T3 α19 vector that included an intron inserted into this plasmid DNA. The pBI221-GCC-GCC-5035S-GUSi recombinant DNA was sequenced using PCR-based dideoxynucleotide terminator protocol and an ABI automated sequencer in Dr. Van Berkum's lab at USDA Beltsville Research Center. The GCC-GCC-5035S-GUSi gene construct was then excised from the pBI221 vector and ligated into the pBI121 binary vector. A diagram

showing the steps in the construction of the GCC-GCC-50_35S-GUSi construct was shown in Figure 4-2.

4.2.2.2 Coating DNA on gold particles

Large scale plasmid DNA extraction was carried out by following the protocol of QIAGEN Plasmid Midi Kit from QIAGEN (Valencia, CA). The gold particles (1.6 µm in diameter, Bio-Rad) were coated with the plasmid DNA (4 µg DNA/mg particle) by co-precipitation in ethanol before shooting. The procedure was used as described in the manual for the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad).

4.2.2.3 DNA shooting with Biolistic PDS-1000/He Particle Delivery System

DNA shooting was performed as described by Tucker *et al.* (2002): Soybean leaves harvested from 2 week-old seedlings from the greenhouse were surface sterilized in 1% bleach for 1 minute, rinsed with sterile water and placed on 1% water agar Petri plates with 200 µg/ml ampicillin before DNA shooting.

A Biolistic PDS-1000/He particle gun (Bio-Rad) was used to bombard explants. Bombardment parameters included a helium pressure of 1,350 psi, 1/4 inch distance between the rupture disk and macrocarrier, and 5 cm distance between the stopping screen and the Petri plate.

4.2.2.4 GUS Fluorometric assay for 2GCC-50_35S-GUS reporter gene

After DNA shooting, Petri plates containing explants were placed inside a 2.5 L desiccator, and ethylene and 1-MCP was injected through the needle into the sealed desiccators to obtain a final concentration of 10 µl/L ethylene and 2 µl/L 1-MCP, respectively. Desiccators were held at 26ºC in the dark. After 48 hrs, explants (approximately 0.1 g) were used for GUS fluorometric assay as previously described in **4.2.1.6** (Jefferson *et al.*, 1987).

4.3 *Results*

4.3.1 Construction of GCC-5035S-GUS (pBI121)

Figure 4-3 showed the restriction enzyme (RE), *Hind*III and *Eco*RI digestion for GCC-5035S-GUS (pBI121) and CaMV35S-GUS (pBI121) vector, and no RE plasmid DNA was used as a control. Lane 1 showed the CaMV35S-GUS (pBI121) vector digested with *Hind*III and *Eco*RI. The CaMV35S-GUS-NOS was approximately 3.0 kb, and the remaining fragment was approximately 11 kb (the total plasmid DNA was approximately 14kb). Lane 2 was the RE digestion for GCC-5035S-GUS (pBI121), which included a GCC-5035S-GUS-NOS fragment of about 2.5 kb, and the remaining vector was about 11.5 kb. From the gel picture, it may be seen that the GCC-5035S- fragment was successfully inserted into the pBI121 expression vector.

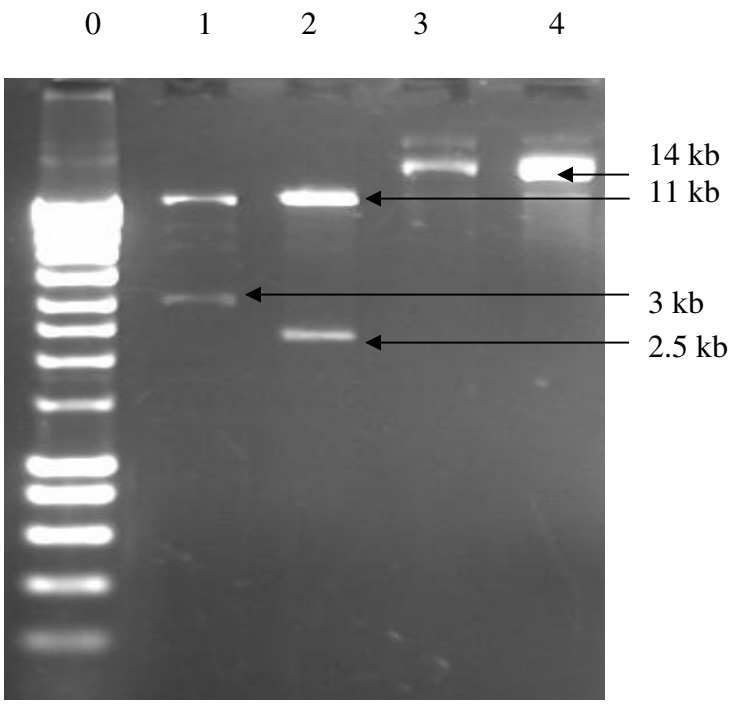


Figure 4-3. Hind*III*/Eco*RI *digestion for -GCC-50_35S-GUS construct.
Lane0: HyperladderI 1: pBI121 (*Hind*III+*Eco*RI) 2: pBI121 (-GCC-50_35S) *Hind*III+*Eco*RI 3: pBI121plasmid DNA control. 4: pBI121 (-GCC-50_35S) plasmid DNA control

4.3.2 PCR analysis of transgenic cultures

Figure 4-4 showed the PCR result for hairy root lines of GCC-5033S (pBI121) using primers for the kanamycin gene. Lane 1 was a PCR reaction with no DNA added (ddH_2O, negative control); Lane 2 was K599 *Agro.* DNA; Lane 3 was pBI121 vector, which was used as a positive control; Lanes 4-7 were genomic DNA from (-GCC-5035S-pBI121), and Lanes 8-14 were genomic DNA from -5035S-pBI121 roots. Based on the PCR results, it would appear that all the hairy root lines were transgenic. However, it was not clear that all the positive results (400 bp DNA bands) from lanes 4-7 and lanes 8-14 were real transgenic roots or contamination from *Agrobacterium* K599.

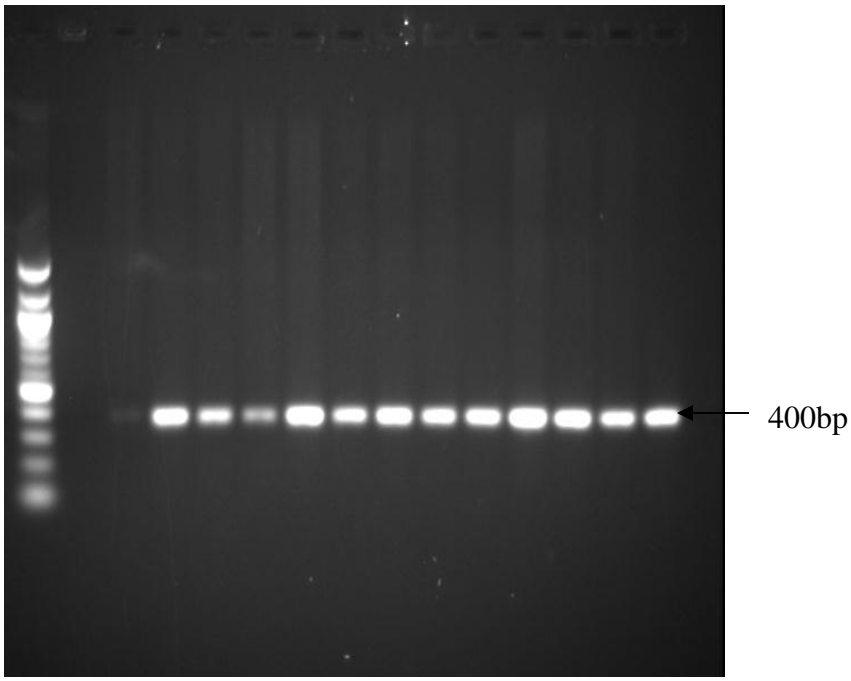


Figure 4-4. PCR analysis (with Kanamycin primers) of transgenic cultures
Lane0:100bp DNA ladder.1: -H2O. 2: K599. 3: k599 (pBI121). 4-7: K599 (pBI121-GCC-5035S). 8-14: K599 (pBI121-5035S control)

4.3.3 Histochemical and fluorometric GUS assays for GCC-50_35S-GUS gene

GUS fluorometric and histochemical assays were performed to identify which transgenic lines expressed GUS and where in the root GUS was accumulated and therefore where ethylene was being synthesized. GUS staining with blue color was only observed in the positive control pBI121 (CaMV35S binary vector). Moreover, GUS stain was only detected in the root tips. No blue color was observed in hairy root lines transformed with either the GCC-50_35S-GUS gene or the -50_35S-GUS gene with or without exogenous ethylene treatment (results not shown). Similar results were obtained for the GUS fluorometric assays, and there was no difference for GUS gene expression between ethylene treated and non-treated hairy root lines;

nevertheless, high GUS activity was measured in *Agrobcaterium* that contained these constructs (Table 1).

Agrobacterium	GUS activity (pmol 4-MU/m.mg of protein)
K599	644.83
K599(pBI121)	44695.73
K599(pBI121-5035S)	89705.31
K599(pBI121-5035S-GCC)	65053.46

Table 4-1 GUS fluorometric assay for Agrobacterium contamination on hairy roots

4.3.4 The detection for quantification of labeled probes

GUS assays failed to detect GUS activity in hairy roots, genomic Southern blot analysis was performed to determine which hairy root lines truly had the T-DNA integrated into the soybean genome. Plasmid DNA including a GUS gene or a soybean abscission cellulase gene (SAC1) was used to probe a genomic Southern blot, respectively. The SAC1 probe was used to confirm that the procedure worked well and that equal amount of DNA was loaded in each lane. After DIG labeling, the yield of the labeling probes pBI221 and SAC1 was determined through dilution series, showing that pBI221 and SAC1 probes reached the expected labeling efficiency (Figure 4-4), i.e., 1 pg and 0.1 pg dilution spots of pBI221 and SAC1 probes and of the control are visible, then the labeled probe has reached the expected labeling efficiency and can be used in the recommended concentration in the hybridization.

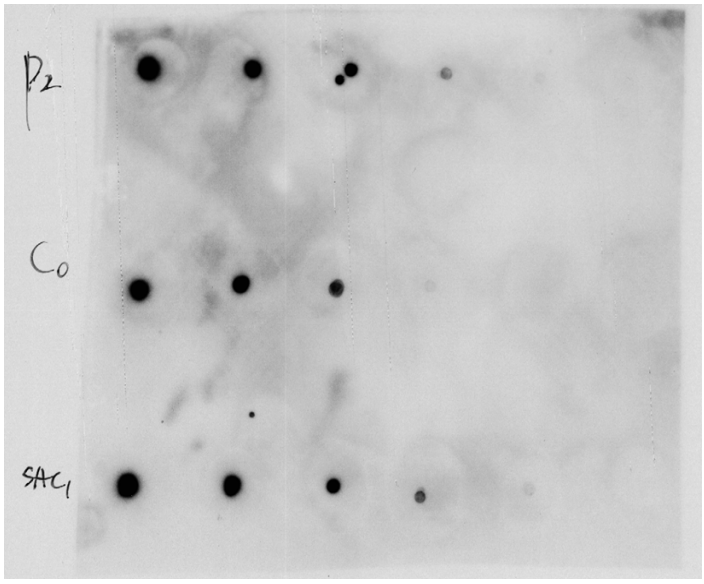


Figure 4-5. Dot blot quantification of labeled probes.
Serial dilutions between 1000 pg/µl and 0.01 pg/µl of DIG-High prime labeled probe were spotted on a membrane. P2: GCC-5035S-GUS-pBI221 plasmid DNA, C0: Control DNA from DIG-High Prime DNA Labeling and Detection Starter Kit II, SAC1: Soybean cellulase1 plasmid DNA.

4.3.5 Southern blot analysis with GCC-5035S-(pBI221) plasmid DNA probe

Southern blot analysis was performed with genomic DNA samples (Lanes 1-7) digested with *Hind*III using the 5.0 kb GCC-5035S-(pBI221) plasmid DNA as a probe. The hybridization signal (bands) corresponding to the GCC-5035S-GUS fragment was detected in samples isolated from hairy roots transformed by *A. rhizogenes* K599 containing GCC-5035S-GUS (pBI121) binary vector but not in roots transformed with the *Agrobacterium* (K599) that did not contain the binary vector (Figure 4-6). Since *Hind*III cuts at a unique site in the binary vector, the

presence of more than one variable size fragment(s) in the genomic DNA indicated the insertion of one or more copies of the T-DNA into the plant genome.

The 14 kb fragment in lane 8 of Figure 4-6 represented 75 pg of *E. coli* pBI121 plasmid DNA after *Hind*III digestion. This 14 kb fragment was the size fragment expected for *Agrbacterium* contamination. The minimum size of a *Hind*III T-DNA fragment integrated into the genome would be approximated by the 2.5 kb *Bam*HI/*Eco*RI digestion of pBI121 plasmid in lane 9. The presence of variable size fragments was greater than 2.7 kb in lanes 2-7 but not 14 kb indicated an integrated copy of the T-DNA insertion.

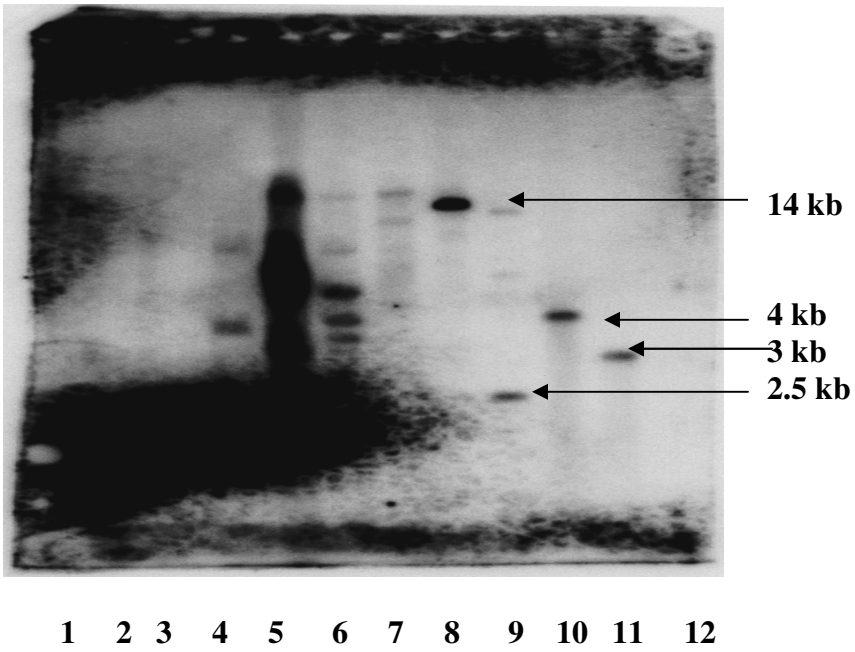


Figure 4-6. Autoradiographs of Southern blot analysis of GCC-5035S-GUS transformed hairy root lines (GCC-5035S-(pBI221) plamid DNA probe).
All soybean hairy roots genomic DNAs were digested with *Hind*III. Lane 1: Empty lane. 2: K599 control hairy root. 3: K599 negative control. 4: GCC 20 hairy root. 5: GCC3 hairy root. 6: -5035S control hairy root line (F7g). 7: pBI121 hairy root, positive control. (All above lanes 1-7 hairy root genomic DNA were digested with *Hind*III). 8: pBI121 plasmid

DNA(*Hind*III). 9: pBI121 plasmid DNA (*Eco*RI+*Bam*HI) 10: SAC plasmid DNA (*Hind*III). 11: SAC1 plasmid DNA (*Bam*HI). 12: Empty lane

4.3.6 Southern blot analysis with pSAC1 plasmid DNA probe

Southern blot analysis was performed with the same membrane as above after it was boiled in 0.1XSSPE/0.1%SDS for 5 minutes and then cooled down to room temperature to remove the probe from the previous hybridization. The membrane was then hybridized with the SAC1 probe. Digestion of soybean genomic DNA with *Hind*III should yield a 14 kb band after hybridization with the labeled pSAC1 probe (Kemmerer and Tucker, unpublished data). All of the hairy roots should have a 14kb band (Fig.4-7).

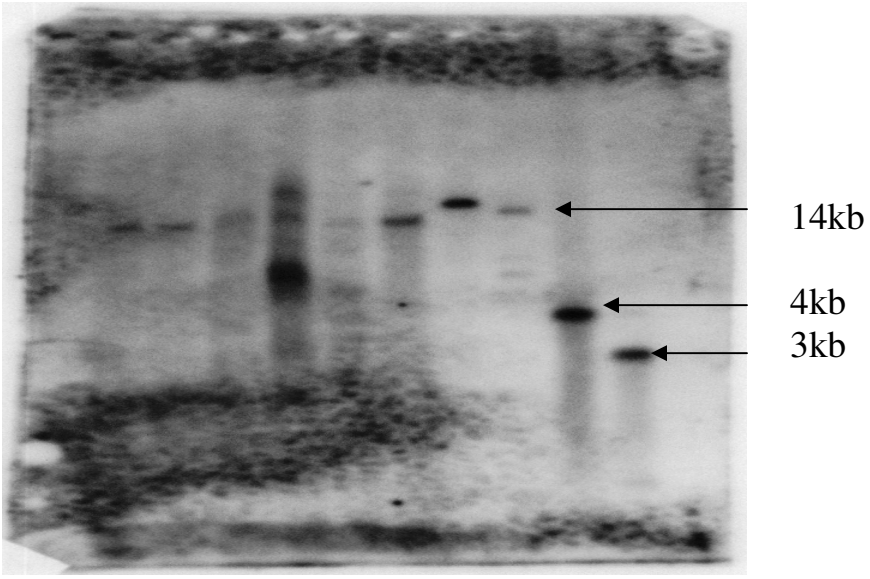


Figure 4-7. Autoradiographs of Southern blot analysis of GCC-5035S-GUS transformed hairy root lines (SAC1 plasmid DNA probe).
Lanes description and loading were the same as in Figure 4-6.

4.3.7 RE digestions for 2GCC-50_35S-GUSi (pBI121) construct.

Figure 4-8 showed the restriction enzyme (RE) digestion for GCC-5035S-GUSi (pBI121), -50_35S-GUSi (pBI121) and D35S-GUSi (pBI12). Lanes 1-3 showed the bands that all constructs were digested with *Hind*III and *Eco*RI. Lanes 4-6 showed the bands that all constructs were digested with *Kpn*I, and lanes 7-9 showed the bands after *Kpn*I and *Eco*RI digestion. According to Figure 4-2 and the gel picture, it can be seen that the GCC-5035S fragment was successfully inserted into the pBI121 expression vector. Because there is only one *Kpn*I site in all the constructs, *Kpn*I and *Eco*RI digestion cut off GUSi-NOS fragment (2.3 kb), and *Hind*III and *Eco*RI digestion cut off 2GCC-50_35S-GUSi-NOS total.

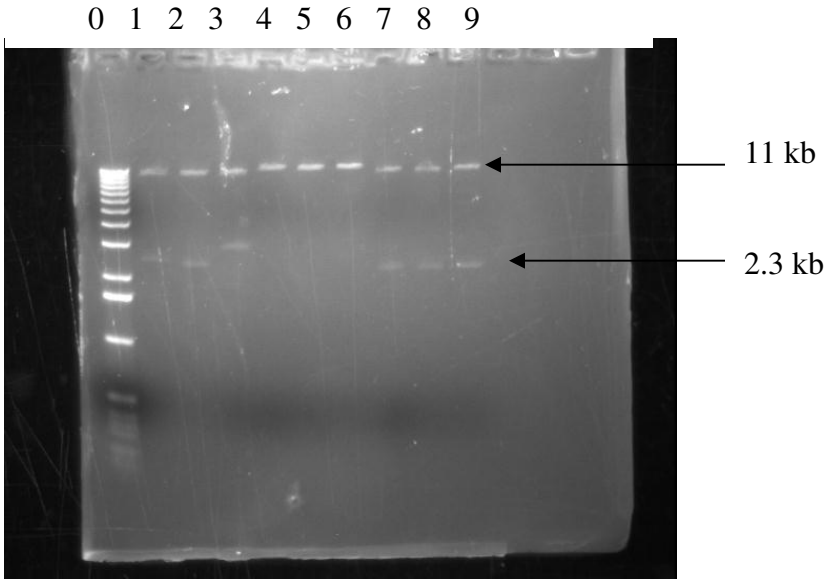


Figure 4-8 RE digestions for 2GCC-50_35S-GUSi (pBI121) construct.
0: Hyperladder 1: pBI121-2GCC-50_35S-GUSi (*Hind*III/EcoRI); 2: pBI121-50_35S-GUSi (*Hind*III/*EcoR*I) 3: pBI121-D35S-GUSi (*HindIII*/*EcoR*I); 4:, pBI121-2GCC-50_35S-GUSi (*Kpn*I); 5: pBI121-50_35S-GUSi (*Kpn*I); 6: pBI121-D35S-GUSi (*Kpn*I); 7: pBI121-2GCC-50_35S-GUSi (*Kpn*I/*Eco*RI) 8: pBI121-50_35S-GUSi (*Kpn*I/*Eco*RI); 9: pBI121-D35S-GUSi (*Kpn*I/*Eco*RI)

4.3.8 GUS transient assay for 2GCC-50_35S-GUSi (intron)

Sample ID	GUS activity(pmol 4-MU/m.mg of protein)
G1	25
G2	79
G3	77
G4	34
G5	93
G6	101
G7	75
G8	81
G9	75
G10	46
G11	75
G12	40
5035S1	99
5035S2	60
5035S3	87
5035S4	34
5035S5	53
5035S6	41
5035S7	31
5035S8	67
5035S9	61
5035S10	58
5035S11	72
5035S12	101
D1	61
D2	73
D4	413
D5	396
D6	979
D7	2514
D8	1847

Table 4-2. GUS fluorometric assay for 2GCC-50_35S-GUSi construct in soybean leaves.
G 1-4, 5035S 1-4, D 1-2, 4 were no treatment control; G5-8, 5035S5-8 and D5-6 were treated with 2 µl/L 1-MCP; G 9-12, 5035S 9-12 and D 7-8 were treated with 10 µl/L ethylene.
G: GCC-GCC-50_35S-GUSi (intron); 50_35S: -50_35S-GUSi (intron) negative control; D: D35S-GUSi (intron) positive control.

No GUS activity was detected in ethylene treated soybean leaves with 2GCC-5035S-GUSi (intron). The result was shown as follows (Table 2): GUS activity was detected in D35S-GUSi (intron) in soybean leaves but no significant levels of GUS activity were detected in any explant of 2GCC-5035S-GUSi construct, and no difference was shown between ethylene and 1-MCP treated soybean leaves bombarded with the GCC-GCC-5035S-GUSi (intron) construct.

4.4 *Discussion*

GCC-50_35S-GUS hairy roots were contaminated with *Agrobacterium* K599, and GUS activity could not be used to identify the transgenic hairy roots due to the high GUS activity from *Agrobacterium*. The new construct GCC-GCC-50_35S-GUSi that contained an intron in the GUS sequence can solve this problem. It was demonstrated that transgenic plants containing the chimeric GUS gene including the intron is spliced efficiently in plants, giving rise to GUS enzymatic activity, but no GUS activity was detected in *Agrobacterium* containing this construct due to the lack of a eukaryotic splicing apparatus in prokaryotes. Thus, the intron-containing GUS gene can be used as an optimized marker gene in transient and stable transformation experiments (Vancanneyt *et al.*, 1990). Four copies of the GCC box were used in the new promoter-GUSi construct to hopefully increase the ethylene-induced expression of GUS. However, no GUS activity was detected in the particle bombardment of soybean leaves with the GCC-GCC-5035S-GUS construct before or after ethylene treatment.

This negative result may be attributed to several causes: First, the GCC sequence used in this construct was from tobacco, not from soybean, so it might not produce an ethylene response to soybean leaves. Secondly, 48 hours of ethylene treatment might not be the optimum time to elicit an ethylene response for a high GUS transient expression in soybean leaves. Thirdly, GUS activity might be decreased by the intron insertion in GUS gene if an improper self splicing of GUS gene occurs during GUS gene transcription.

To test these possibilities the GCC construct GUS transient assay can be carried out in several other plant leaves, such as tobacco, tomato, and soybean to determine whether the GUS gene expression is higher in these other species. In addition, ethylene treated plant tissue could be harvested for GUS transient assay at time points other than 48 hrs, such as 24hrs, 36 hrs, or even 72hrs to find the optimal time after bombardment to measure GUS gene activity.

Chapter 5: Summary and Conclusion

5.1 *Background and aim of this project*

The relationship between ethylene and nematode infection has been studied in the past and it has been determined that ethylene plays a crucial role in nematode colonization of plant roots. However, the role of ethylene in SCN feeding cells in soybean roots has not been well studied. I decided it would be useful to investigate whether or not ethylene does in fact play a role in SCN colonization of soybean roots and the establishment of the nematode feeding cell.

To determine if ethylene is involved in SCN feeding cell formation in soybean roots, I used a variety of approaches that have been used in the past to study the roles of other plant hormones in nematode feeding cell formation in plants. These approaches are: (1) to quantify the effects of exogenous ethylene on SCN colonization of soybean roots and the effect of suppressing ethylene responsiveness with chemical inhibitors of ethylene action or an ethylene-resistant mutant, *etr1-1*; (2) to compare the ethylene production in SCN infected and non-infected roots; and (3) to determine where in the roots ethylene is synthesized at active levels that might influence the formation of feeding cells, syncytia, by using an ethylene-responsive promoter fused to a GUS reporter gene.

5.2 *Conclusions*

The main conclusions from this project are:

1. SCN parasitism in soybean roots is increased by ethylene treatment and reduced when ethylene action is chemically or genetically inhibited.
2. In monoxenic soybean root cultures, the rate of ethylene production increases in SCN infected roots compared to non-infected roots.
3. An ethylene-responsive promoter fused to a GUS reporter gene (GCC-50_35S-GUS) was constructed and transformed into soybean genome. In addition, a construct with two additional GCC-boxes was constructed that included a GUS gene interrupted by an intron (2GCC-5035S-GUSi) that will presumably enhance the ethylene responsiveness of the construct and remove ectopic expression of GUS from contaminating *A. rhizogenes* on the hairy root cultures.

Here I briefly summarize the evidence supporting these conclusions, attempting to make a critical assessment of them and their underlying experiments.

1. SCN parasitism in soybean roots is increased by ethylene treatments and reduced when ethylene action is chemically or genetically inhibited.

This conclusion is supported by results comparing SCN colonization of the roots in a soybean ethylene resistant mutant *etr1-1* to the wild-type mutant progenitor Hobbit 87, and also in the experiments where the roots were exposed to exogenous ethylene or the ethylene action inhibitors 1-MCP or 2, 5-NBD. The complementary results with the chemical inhibition of ethylene action using 1-

MCP and 2, 5-NBD and the genetic inhibition using the ethylene resistant mutant *etr1-1*strongly support this conclusion.

2. In monoxenic soybean root cultures, the rate of ethylene production increases in SCN infected roots compared to non-infected roots.

Although there were several complications associated with these experiments (see Chapter 3), e.g., the roots and SCN did not grow as well in 25ml Erlenmeyer flasks as they did in Petri plates, the trend towards an increase in ethylene synthesis in SCN infected cultured roots adds an additional facet to the positive role ethylene plays in SCN infection. In other words, it's possible that the SCN has evolved to take advantage of wound-induced ethylene synthesis caused by SCN penetration of the root or that the SCN actively stimulates ethylene synthesis by secretions from the nematode itself.

3. An ethylene-responsive promoter fused to a GUS reporter gene (GCC-5035S-GUS) was constructed and transformed into soybean genome. In addition, a construct with two additional GCC-boxes was constructed that also included a GUS gene interrupted by an intron (2GCC-5035S-GUSi) that will presumably enhance the ethylene responsiveness of the construct and remove ectopic expression of GUS from contaminating *A. rhizogenes* on the hairy root cultures.

I have completed only a part of this project. The presence of active ethylene concentration in the root has not yet been identified. Several ethylene responsive constructs were prepared along with control constructs to test where ethylene is being synthesized in the roots of transgenic root cultures. A few transgenic lines were identified by genomic Southern blots, but the histochemical staining for GUS activity in transgenic roots has not yet been completed successfully (see Chapter 4). Nevertheless, it is hoped that the constructs will be useful for future experiments.

Appendix

GCC sequence:

```
5′ 1 ACCTGCCCAC AGGCCGTCGA GTTTTTTGAT TTCACGGGTT GGGGTTTCTA
  51 CAGGACGTAA CATAAGGGAC TGACCACCCG GGGATCCTCT CCAAATGAAA
 101 TGAACTTCCT TATATAGAGG AAGGGTCTTG CGAAGGCCTC TGAGCTGCAG
 151 CGGCCGCATG GCGGCTCTTA TTTGATCGGT CTTATTTTAG TGGCGGCTCT
 201 TATGAAGCTT GGCGTAATCA TGGTCATAGC TGTTTCCTGT GTGAAATTGT
 251 TATCCGCTCA CAATTCCACA CAACATACGA GCCGGAAGCA TAAAGTGTAA
 301 AGCCTGGGGT GCCTAATGAG TGAGCTAACT CACATTAATT GCGTTGCGCT
 351 CACTGCCCGC TTTCCAGTCG GGAAACCTGT CGTGCCAGCT GCATTAATGA
 401 ATCGGCCAAC GCGCGGGGAG AGGCGGTTTG CGTATTGGGC GCTCTTCCGC
 451 TTCCTCGCTC ACTGACTCGC TGCGCTCGGT CGTTCGGCTG CGGCGAGCGG
 501 TATCAGCTCA CTCAAAGGCG GTAATACGGT TATCCACAGA ATCAGGGGAT
 551 AACGCAGGAA AGAACATGTG AGCAAAAGGC CAGCAAAAGG CCAGGAACCG
 601 TAAAAAGGCC GCGTTGCTGG CGTTTTTCCA TAGGCTCCGC CCCCCTGACG
 651 AGCATCACAA AAATCGACGC TCAAGTCAGA GGTGGCGAAA CCCGACAGGA
 701 CTATAAAGAT ACCAGGCGTT TCCCCCTGGA AGCTCCCTCG TGCGCTCTCC
 751 TGT 3′
```

The GUS primer used for above sequence is shown as below:

	5'	3'
GUS3-5_Nterm (20-mer):	TTGTAACGCGCTTTCCCACC	

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