THE EFFECT OF LIPO-CHITOOLIGOSACCHARIDE FROM *BRADYRHIZOBIUM JAPONICUM*, ON SOYBEAN SALICYLIC ACID, PATHOGENESIS-RELATED PROTEIN ACTIVITY AND GENE EXPRESSION

by

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ABSTRACT

In this study, lipo-chitooligosaccharide (NodBj-V ($C_{18:1}$, MeFuc); LCO) 10^{-7}M, extracted from *Bradyrhizobium japonicum*, was sprayed on the leaves of soybean cv. OAC Bayfield soybean and Evans x L66-2470 (carrying the rj_1 mutation, and unable to nodulate). Leaf SA level and activities of the PR proteins chitinase, β-1,3-glucanase and guaiacol peroxidase (GPOX) were quantified. *Phenylalanine ammonia-lyase 1* (*PAL1*) and *isoflavone synthase 2* (*IFS2*) relative gene expression levels in the sprayed leaves were quantified using quantitative real-time PCR. Messenger RNA abundance was quantified using microarrays. The treatment caused a transient increase in local salicylate levels 24 h after exposure, and a systemic increase in GPOX activity 48 h after exposure, in both soybean types. Of the selected 38 genes affected by the LCO treatment, 25 were stress-related. There were no significant differences in A) chitinase and β-1,3-glucanase activity, or B) in *PAL1* and *IFS2* gene expression.

RESUMÉ

Dans la présente étude, le lipo-chitooligosaccharide (NodBj-V ($C_{18:1}$, MeFuc); LCO), de *Bradyrhizobium japonicum* a été vaporisé sur le feuillage des cultivars de soja OAC Bayfield et Evans x L66-2470. Les niveaux de SA et l'activité des chitinases, des glucanases et des peroxydases guaïacol dans les feuilles ont été quantifiés. L'expression des gènes *PAL1 et ISF2* a aussi été mesurée par RT-PCR quantitatif. En parallèle, nous avons aussi mesuré l'abondance des ARN messagers par puces à ADN. Suite au traitement LCO, une augmentation temporaire et localisée des niveaux de salicylates a été observée à 24 heures, ainsi qu'une augmentation systémique de l'activité GPOX à 48 heures dans les deux cultivars de soja. Des 38 gènes modulés, 25 sont des gènes reliés au stress. Cependant, aucune différence significative dans l'activité des chitinases et glucanases, ainsi que dans l'expression génique de *PAL1* et d'*IFS2* n'a été détectée suite à l'exposition au LCO.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

AA: arachidonic acid

AIP: 2-aminoindan-2-phosphonic acid

ASR: Asian soybean rust

BA: benzoic acid

bp: base pairs

bv: biovar

CA: *trans*-cinnamic acid

cDNA: complementary deoxyribonucleic acid

CHR: chalcone reductase

CHS: chalcone synthase

cv: cultivar

dH_2O: distilled water

DNA: deoxyribonucleic acid

DNS: dinitrosalicylic acid

EST: expressed sequence tag

FW: fresh weight

dR: end fluorescence

GLM: general linear model

GPOX: guaiacol peroxidase

GUS: β-glucuronidase

HO: heme oxygenase

HPLC: high performance liquid chromatography

HSF: heat stress/shock transcription factor

ICS: isochorismate synthase

IFS: isoflavone synthase

IFS1: *isoflavone synthase 1* gene

IFS2: *isoflavone synthase 2* gene

IFR: isoflavone reductase

LCO: lipo-chitooligosaccharide (NodBj-V ($C_{18:1}$, MeFuc)) from *Bradyrhizobium japonicum* 532C

MeFuc: methyl fucose

mRNA: messenger ribonucleic acid

N_2: molecular nitrogen

NO: nitric oxide

NPR1: 'nonexpressor of pathogenesis-related genes' protein 1

OAC: Ontario Agricultural College

OD: optical density

PAL: phenylalanine ammonia-lyase

PAL1: *phenylalanine ammonia-lyase 1* gene

PCR: polymerase chain reaction

Phe: L-phenylalanine

PR: pathogenesis-related

QRT-PCR: real-time quantitative reverse transcription polymerase chain reaction

RCBD: randomized complete block design

RNA: ribonucleic acid

rpm: revolutions per minute

RT-PCR: reverse transcription polymerase chain reaction

SA: salicylic acid

SAR: systemic acquired resistance

SATase: serine acetyltransferase

SCN: soybean cyst nematodes

SNK: Student-Newman-Keuls

TMV: tobacco mosaic virus

TNV: tobacco necrosis virus

YEM: yeast extract mannitol

1. Introduction

One of the most interesting and best-characterized plant-microbe interactions is that of legumes and rhizobia (Gage, 2004). The symbiotic relationship begins with inter-organismal communication, when the plant exudes, from its roots, phenolic signaling-molecules, generally isoflavonoids (Schultze and Kondorosi, 1998). These compounds have a number of effects on rhizobia, including the induction of chemotaxis and gene expression. In response to the plant-to-rhizobia signals, the rhizobia secrete return signaling-molecules: lipo-chitooligosaccharides (LCOs or 'Nod factors'), which are perceived by the plant, triggering a number of effects. These include the induction of plant *nodulin* genes responsible for the formation of the special root-organs that house the rhizobia and are therefore the site of nitrogen fixation, and the enhancement of: 1) plant growth and seed germination, 2) mitogenesis, 3) seedling growth, and 4) levels of particular flavonoids in the root exudate (Schmidt *et al.* 1994; Egertsdotter and von Arnold, 1998; Souleimanov *et al.* 2002; Loh and Stacey, 2003; Prithiviraj *et al.* 2003).

While the nitrogen-fixing relationship is undoubtedly symbiotic and beneficial to both partners, there are some similarities between this relationship and the interaction between a plant and a pathogen, such as: 1) reaction to compounds indicating presense of the pathogen (often chitin fragments in the case of fungal pathogens), 2) the attachment of the bacteria to the host, 3) penetration of the roots, 4) plant response to penetration, 5) redirection of the plant's metabolism, 6) production of phytoalexins (often flavonoids), and 7) the appearance of specific symptoms related to the infection. As the legume-rhizobia relationship likely began as an interaction between a host and a parasite (Vance 1983; Djordjevic *et al.* 1987), how rhizobia manage to infect the legume without provoking defensive responses in the plant is of particular interest.

Much of the research of the rhizobia-legume interaction has focused on the symbiosis in the context of N_2 fixation, and has only recently begun to explore it in the context of the foliar application of a rhizobial signaling molecule. That a foliar application of a molecule normally restricted to the rhizosphere would nevertheless induce various effects in the plant is very interesting. It is possible that the LCOs are perceived there by a receptor similar to the one used in the symbiosis, and that this

receptor is involved in broader responses to potential invading organisms. Nearly all of the plant nodulin genes are expressed elsewhere in the plant, with either the same or similar jobs, but at levels of activity appropriate to the tissues wherein they reside (Journet *et al.* 2001). The LCO receptor might therefore have been co-opted from its original task, perhaps as a detector of pathogens, and is now utilized by the plants as a way to detect the symbiont, allowing initiation of a somewhat altered set of responses, leading to nodulation.

Of the various changes and effects in plants brought on by infection, one of the best studied is the defense-response 'systemic acquired resistance' (SAR), which is characterized by 1) increased disease resistance in plant tissues distal to actual infection, and 2) increased resistance to subsequent infections by a variety of pathogens (Ryals *et al.* 1994). Disease resistance is brought about through a series of steps including the production of pathogenesis-related (PR) proteins, such as chitinases, peroxidases, and cellulases. Salicylic acid (SA), a plant hormone, has been implicated as one of the key signaling-components of SAR. Some studies have shown that specific rhizobia can affect legume SA levels (Martinez-Abarca *et al.* 1998; van Spronsen *et al.* 2003). How rhizobia and their LCO signaling-molecules affect SA, is of great interest, as it seems that rhizobia must interact with at least some aspects of the plant's defenses, such as SA levels, for successful infection to occur.

The biochemical pathway responsible for SA synthesis is thus a good target for study of the effects of LCO on plant tissue SA levels. Of the two distinct pathways suggested for the biosynthesis of SA in plants, the better-characterized phenylalanine pathway (itself a branch of the phenylpropanoid pathway) synthesizes SA from L-phenylalanine (Phe) via *trans*-cinnamic acid (CA) and benzoic acid (BA). The conversion of Phe to CA, also the first step of flavonoid and isoflavonoid biosynthesis, is the rate-limiting step of SA biosynthesis (Coquoz *et al.* 1998) and is catalyzed by the enzyme phenylalanine ammonia-lyase (PAL). The first step of the branch of the phenylpropanoid pathway leading to isoflavone biosynthesis is the conversion of the flavones liquiritigenin and naringenin (derived from CA) into the isoflavonoids daidzein or genistein, by the enzyme isoflavone synthase (IFS).

Another good target for study of this defense pathway are the PR proteins themselves, such as chitinase, β-1,3-glucanase and guaiacol peroxidase (GPOX). As the LCO molecule has a chitin component, it is of interest to know whether the plant perceives these as it might perceive similar components of a fungal cell wall, and induce the chitinase and β-1,3-glucanase defenses. GPOX is a good candidate because it has been implicated in plant growth and development (Campa, 1991, Polle *et al.* 1994), and LCO has recently been shown to have growth-promoting effects on plants (Prithiviraj, *et al.* 2003, Olah *et al.* 2005).

Given that: 1) LCOs have been shown to affect legume SA levels (Martinez-Abarca *et al.* 1998), and that 2) the spraying of LCO from *Bradyrhizobium japonicum* on soybean leaves increases the level of seed isoflavonoids (Al-Tawaha *et al.* 2005), which are formed by a separate branch of a key pathway leading to SA biosynthesis: the effects of the foliar application of LCO on local and systemic soybean SA levels, PR protein activity and soybean gene expression – particularly those genes related to stress – are therefore of interest. Studying the effects of LCO on a soybean mutant that is unable to nodulate is also potentially revealing. These effects may offer insight into what biochemical conditions are required for successful infection of the root by the symbiont, and how the symbiotic relationship may have evolved from a parasitic one. Furthermore, these effects may offer some insight into the mechanisms by which LCO can mediate increases in plant resistance to pathogenic infection and in plant growth.

Hypotheses:

1) Foliar application of LCO changes SA levels.
2) Foliar application of LCO will change *IFS2* and *PAL1* gene expression.
3) Foliar application of LCO results in the differential expression of stress-related soybean genes.
4) Foliar application of LCO results in a change in local and systemic PR protein levels.

Objectives:

1) Determine the effect of the foliar application of LCO on soybean SA levels.

2) Measure the abundance of *IFS2* and *PAL1* gene transcripts, relative to the *actin* housekeeping gene, in the first trifoliolate leaf of two soybean genotypes (nodulating and non-nodulating) after foliar LCO application.
3) Examine, at the transcript level, the response of soybean genes to foliar application of LCO.
4) Determine the effect of the foliar application of LCO on soybean PR protein activity.

2. Literature Review

2.1 The Legume-Rhizobia Interaction

The classic plant-microbe symbiotic interaction consists of a partnership between plants of the *Leguminosae* family (eg. soybean, alfalfa, and pea) and bacteria of the family *Rhizobiaceae*, generally referred to as 'rhizobia'. The term 'rhizobia' designates root-colonizing bacteria in the *Rhizobiaceae*, often referring specifically to members of genera such as *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Bradyrhizobium*, and *Azorhizobium*. Some species of rhizobia are specialists and colonize only a few legume species, whereas others are generalists and have a wide range of host-specificity. It should also be noted that not all members of the *Rhizobiaceae* are root-colonizers (Djordjevic *et al.* 1987).

2.1.1 The Plant Signals: Flavonoids and Isoflavonoids

The first step in the colonization of the plant roots by rhizobia is an exchange of molecular signals between the host plant and the colonizing bacteria. The plant host initiates this exchange, by the constitutive production and secretion of either flavonoids or isoflavonoids (Schultze and Kondorosi, 1998), specific plant signals found in root exudates that often vary in regard to relative amount and kind, and from plant to plant (Kosslak *et al.* 1987). Regions of the root that have emerging root hairs are especially susceptible to infection by rhizobia and flavonoid and isoflavonoid signal compounds are often secreted most abundantly from this location (Verma, 1992).

Flavonoids are a broad class of organic compounds and are the major product of the phenylpropanoid pathway as well as the most ubiquitous group of polyphenolic secondary metabolites in plants and have many functions, including disease resistance, enhancing fertility, flower pigmentation, and UV-scavenging (Schiljen *et al.* 2004).

Isoflavones, such as the three most abundant in soybean (genistein, glycitein and daidzein), are derived from flavanones (a derivative of chalcone) and have been found to occur almost exclusively in the *Fabaceae* family of plants. Isoflavones are used by leguminous plants to attract rhizobia and to induce rhizobial *nod* genes. The first step in the formation of isoflavones from flavanones such as naringenin is a hydroxylation

accompanied by a P450-dependent oxidative aryl migration. This conversion is catalyzed by the cytochrome monooxygenase, 2-hydroxyisoflavanone synthase (isoflavone synthase, or IFS; Kochs and Grisebach, 1986; Hakamatsuka *et al.* 1991).

2.1.2 The Bacterial Signals: Nod Factors (Lipo-chitooligosaccharides)

The plant-to-rhizobia signals are recognized by the cellular membrane-bound NodD protein of rhizobia: in the presence of flavonoids, NodD protein, a Lys R-type gene regulator, will induce the expression of *nod* genes common to all rhizobia (Kosslak *et al.* 1987; Gottfert *et al.* 1992). Multiple isoforms of NodD exist in different rhizobia species, and these respond to different flavonoids, thereby allowing specific sets of rhizobia to infect specific sets of legumes. For example, in the soybean (*Glycine max*) – *B. japonicum* interaction, the primary soybean signaling molecules are the isoflavones genistein and diadzein (Ip *et al.* 2001). Having recognized and bound the extracellular plant molecular signals, the NodD protein binds to the 'nod box' sequence upstream of the *nod* genes (Loh and Stacey, 2003), which induces the DNA to bend and thereby activate transcription of the common nodulation genes, *nodA*, *nodB*, and *nodC*. These genes are responsible for the production and secretion of extracellular rhizobia-to-plant signal-response molecules, known as 'Nod factors' (Boone *et al.* 1999; Downie and Walker, 1999).

An alternate pathway for *nod* gene induction exists, and consists of two other proteins, NodV (a sensor kinase) and NodW (a cognate response regulator protein). NodV and NodW act together as a two-component regulator for *nod* gene induction (Sanjuan *et al.* 1994). While not essential in soybean, NodV and NodW are necessary for *nod* gene activation in cowpea, siratro, and mungbean (Gottfert *et al.* 1990). Detection of an environmental stimulus (eg. an isoflavonoid) triggers the autophosphorylation of NodV and the subsequent transfer of a phosphoryl group to NodW, that, in turn, activates the *nod*A, *nod*B, and *nod*C genes responsible for the production of the basic Nod factor structure (Loh and Stacey, 2003).

Nod factors are lipo-chitooligosaccharides (LCO), comprised of a chitin oligomer backbone of chains of three to five 1-4 β-linked acetylglucosamine residues with an acyl chain at the terminal non-reducing sugar in place of an N-acetyl group (Sanjuan *et al.*

1992). LCO biosynthesis begins with the incorporation of N-acetyl glucosamine into cell-wall polysaccharides, a process catalyzed by β-glucosyl transferase, the product of the *nod*C gene. A secondary product of the *nod*C gene (NodC protein - UDP-N-acetyl glucosaminyltransferase), is responsible for the elongation of the non-reducing end of the LCO molecule (Hirsch *et al.* 2000; Perret *et al.* 2000). The N-acetyl group of the terminal non-reducing sugar is first removed by the NodB protein (deacetylase) and is then replaced by a fatty acyl chain linked to the acetyl-free C-2 carbon. The linking of the fatty acyl chain is catalyzed by the product of the *nod*A gene, NodA (acyltransferase) (Perret *et al.* 2000). Host-specificity is determined by both LCO 'decorations' – substitutions on the chitin backbone – and by the lipid group (Spaink *et al.* 1991; Schultze and Kondorosi, 1998). Once assembled, LCO are ultimately secreted into the rhizosphere by the bacteria (Scheres *et al.* 1990; Perret *et al.* 2000).

2.1.3 Nodule Organogenesis

The plant is able to sense the secreted bacterial LCO(s), produced in response to flavonoid compounds released by the plant. Lipo-chitooligosaccharides induce expression of plant *nodulin* genes (Sanchez *et al.* 1991; Horvath *et al.* 1993), the products of which are required for the successful infection of the roots by the rhizobia. *Nodulin* genes turn on the plant processes required for 'nodulation' – the formation of specialized root-organs known as 'nodules', that house the rhizobia and are the site of nitrogen fixation (van Kammen, 1984).

In general, the formation of specialized infection threads and nodules occurs as the rhizobia enter the root via the root hairs, and infection thread membranes bud-off the end of the infection thread, surrounding the modified bacteria (bacteroids) with plant membranous material, during later stages of nodule development. These elements collectively form the 'symbiosome' (Stougaard, 2000). Once the cortical cells have been penetrated by the infection thread, bacteria morph into bacteroids and begin reducing atmospheric nitrogen into ammonia (Broughton *et al.* 2003).

In legumes, there are two common types of nodulation. Some legumes, such as bean (*Phaseolus vulgaris*), soybean, and *Lotus japonicus*, form determinate nodules. The formation of most determinate nodules occurs in the outer root cortex and nodule

development occurs via cell expansion and export nitrogen to the plant as ureides (Hirsch, 1992). Other legumes, such as pea (*Pisum sativum*) or alfalfa (*Medicago sativa*), form indeterminate nodules. These nodules start at the inner cortex and contain a root apical meristem. Nodule development in indeterminate nodules occurs via cell division, and nitrogen exported to the host is in the form of the amino acids glutamine and asparagine (Hirsch, 1992; Stougaard, 2000).

2.1.4 Plant LCO-Response

Lipo-chitooligosaccharides appear to trigger a number of physiological effects within the plant, including root hair deformation, enhancing medicarpin content (Savoure *et al.* 1997), increases in particular flavonoids in root exudates (Schmidt *et al.* 1994), infection-thread formation (the channel by which rhizobia gain access to the root) in root hairs, as well as enhancing plant growth (Prithiviraj *et al.* 2003; Olah *et al.* 2005). The latter is evidenced by seedlings' increased demand on seed reserves, by mature plants' increased rate of photosynthesis and by changes in root branching. As LCO have mitogenic properties concerning cortical cells (Schmidt *et al.* 1988; Schlaman *et al.* 1997), they may be causing an increase in plant meristematic activity (Dyachok *et al.* 2000, 2002).

Non-legumes that do not form symbiotic relationships with rhizobia have also been shown to respond to the application of LCO. In temperature-sensitive carrot mutants (de Jong *et al.* 1993) and in Norway spruce somatic embryo cultures (Egertsdotter and von Arnold, 1998), cell division is restored after treatment with LCO. Lipo-chitooligosaccharides induce alkalination in tomato and tobacco cell cultures (Staehelin *et al.* 1994; Baier *et al.* 1999). Lipo-chitooligosaccharides can also enhance the colonization of both nodulating and non-nodulating plants by a different kind of plant-symbiote: mycorrhizae (Xie *et al.* 1995). Recently, it has been found that a particular LCO (Nod Bj V (C18:1, MeFuc) enhances seed germination and seedling growth in a variety of non-host plants, as well as in the host-plant soybean (Souleimanov *et al.* 2002). Chitin oligomers induce responses in plants similar to those of LCO, including the expression of the early nodulin gene enod40, involved in nodule-development (Minami *et al.* 1996). Increases in cytosolic calcium [Ca^{2+}] levels have been observed in transgenic

soybean cells treated with either LCO or chitin oligomers, with greater increases in calcium levels observed for LCO with a longer, pentameric, chitin backbone versus LCO with a smaller, trimeric, backbone. It is conceivable then, that calcium is acting as a secondary messenger in the plant LCO-response signal transduction (Muller *et al.* 2000).

While the main purpose of isoflavone exudation by legume roots is thought to be the attraction of rhizobia and the induction of rhizobial *nod* genes, it appears that the plant continues to produce and exude flavonoids even after nodulation, and can even increase in the amount exuded. When vetch is inoculated with *R. leguminosarum* biovar *viciae*, an increase in the exudation of flavanones and chalcones is observed (Recourt *et al.* 1991). It has also been shown that LCO, even in the absence of rhizobia, are sufficient to induce an increase in plant phytoalexin biosynthesis and even elicit a defense response in alfalfa (Savoure *et al.* 1994; Savoure *et al.* 1995).

Many plant growth responses induced by LCO are similar to plant-responses to elevated levels of the plant hormone SA (Schmidt *et al.* 1988; Schlaman *et al.* 1997; Zhang and Klessig, 1997) – which would make it candidate for study, as a possible signaling molecule following treatment with LCO.

2.2 Salicylic Acid

Although SA, initially isolated from willow bark, has been known for millennia to ease pain (an acetylated derivative of SA, which retains some of the potency but is less acidic, is the active ingredient in aspirin), the function of SA in plants has only relatively recently been identified (Malamy *et al.* 1990). Salicylic acid, a phenolic hormone and plant growth regulator, has been found to play an integral role in various plant physiological and biochemical processes, and is best known as a key component in plant defense responses.

In barley (*Hordeum vulgare* L. cv. Karlsberg), SA was found to inhibit potassium and phosphate uptake through reversible alterations in membrane permeability (Glass 1973, 1974). SA has also been identified as a compound responsible for inducing flowering in the plant *Lemna gibba* G3 (Cleland and Ajami, 1974). Other functions of interest are its ability, in pear cell suspension cultures, to inhibit ethylene biosynthesis by blocking the conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene (Leslie

and Romani, 1986) and the ability to inhibit jasmonic acid biosynthesis (Schaller, 2000). In addition, the ability of SA to cause the a substantial increase in oxygen consumption, coupled with the generation of heat up to 12 °C over ambient temperature in the inflorescene during blooming has been found in certain thermogenic species of *Arum* lilies (Raskin *et al.* 1987, 1989). More recently, the role of SA in plant defense has been of particular interest, especially with respect to SAR (Dempsey *et al.* 1999; Durrant and Dong, 2004).

2.2.1 Salicylic Acid and Systemic Acquired Resistance

Systemic acquired resistance is a mechanism of plant defense that causes resistance to general or specific pathogens of various types (fungal, viral, and bacterial) in response to infection by a pathogen; SAR was recently reviewed in Grant and Lamb (2006). Resistance can be induced by the pathogen in parts of the plant distant from the site of infection. In addition, resistance stemming from SAR can be enduring, lasting long after infection has been removed, and providing resistance to a variety of potential pathogens.

Salicylic acid has been implicated in various studies as a key component of the SAR mechanism in many plants. Metraux *et al.* (1990) first found that when inoculated with either the tobacco necrosis virus (TNV) or the fungal pathogen *Colletotrichum lagenarium*, a transient increase in SA was observed in the phloem sap of cucumber (*Cucumis sativus* L.), with a peak in SA levels observed just prior to the onset of SAR. Another study found that, following the inoculation of cucumber leaves with the pathogen *Pseudomonas syringae* pathovar *syringae*, SA accumulated in the phloem exudate of the petioles above, below, and at the site of infection (Rasmussen *et al.* 1991).

The endogenous SA levels in rice seedlings (*Oryza sativa* L.) remained unchanged following exposure to the pathogens *P. syringae* D20, *Magnaporthe grisea* (which causes rice blast), or *Rhizoctonia solani*. However, general resistance of 28 different rice varieties to rice blast was associated with high basal levels of SA. Among a number of plants tested for leaf SA content, rice was found to have the highest (Raskin *et al.* 1990), which may explain why rice does not respond to pathogenic attack with an increase in SA synthesis (Silverman *et al.* 1995).

In tobacco mosaic virus (TMV)-infected tobacco (*Nicotiana tabacum* L.), a plant that does not contain large, conjugated pools of SA, it was shown that when SA was applied to excised leaves, it induced the accumulation of PR proteins (the accumulation of PR proteins is one of the key indicators of the onset of SAR), and that when sprayed on inoculated or uninoculated leaves, SA alone was sufficient for the induction of PR proteins (Yalpani *et al.* 1991). SA, as an exogenous treatment, has also been shown to induce the same defensive genes in tobacco as when the plant is infected with TMV (Ward *et al.* 1991). Experiments designed to affect SA accumulation in tobacco, for example: by transforming tobacco plants with the bacterial gene salicylate hydroxylase that converts SA to catechol, have shown that when infected with TMV, these plants are unable to accumulate SA and were unable to induce the PR proteins essential to SAR, thereby revealing the potential critical importance of SA in tobacco defense responses (Gaffney *et al.* 1993).

A later study, however; showed that under similar conditions, tobacco transformed with bacterial salicylate hydroxylase was still able to induce resistance to further infection, thus, while SA may be crucial to some of the outcomes of SAR, it is not the primary signaling molecule, transduced throughout the plant and initiating systemic activation of resistance (Vernooij *et al.* 1994). Further studies have supported the hypothesis that SA, while playing a role in SAR, is not the mobile signal responsible for the transduction of resistance throughout the plant, and that methyl salicylate may in fact be the transduced signal (Park *et al.* 2007).

Research has since shown that the 'nonexpressor of *PR* genes' protein (NPR1) is potentially the transduced signal of SAR, although SA is nevertheless critical to this response; without SA SAR cannot occur (Gaffney *et al.* 1993). Cao *et al.* (1994) showed that *Arapidopsis* with a mutation in the *npr1* gene were unresponsive to SAR inducers, and hypothesized that the product of the *NPR1* gene might be a key signal in the transduction of SAR. Delaney *et al.* (1995) showed that NPR1 occurs downstream of SA accumulation in the SAR signal pathway, and Cao *et al.* (1997) found that *PR* gene expression is reliant upon the NPR1 protein when *npr1* mutants were transformed with wildtype NPR1 and regained the SAR response to various elicitors; these plants produced

PR proteins. In another study, Chini *et al.* (2004) determined that some elements of stress-response, specifically, dehydration, are SA dependent but NPR1 independent.

2.2.2 Salicylic Acid and Pathogenesis-Related Proteins

Within the broad class of PR proteins are enzymes such as the anti-fungal chitinases and glucanases, peroxidases, proteinase-inhibitors and lipid-transfer proteins. White (1979) first showed that injecting tobacco plants with SA caused production of PR proteins leading to development of resistance to TMV. Some PR proteins can be produced in response to environmental factors, as well as pathogenic attack. Yalpani *et al.* (1994) found that ultraviolet light and ozone treatments, which cause the production of potentially destructive reactive oxygen species in plant, resulted in higher levels of leaf SA and PR protein.

Recent research on PR proteins has focused on the effects of various stimuli of PR protein activity, and how PR proteins can be utilized for various benefits. Polle *et al.* (2004) found that when Norway spruce tissue was stained with guaiacol, high GPOX activity was found in lignifying cell walls, thus GPOX is a likely participant in lignin formation (which results in, among other things, greater resistance to pathogenic attack). Jung *et al.* (2005) showed that treatment of the commercially-valuable pepper plant with a biocontrol, chitinase-producing bacterium activates PR proteins such as chitinase, cellulase and β-1,3-glucanase activity in the leaves of infected plants, thereby increasing resistance to the pathogen *Phytophthora capsici.* Similarly, *Phytophthora colocasiae*-resistant varieties of taro have greater GPOX induction and/or activity (Sahoo *et al.* 2007).

Priming seeds of soybean, tomato and corn with fish protein hydrolysates resulted in increased GPOX activity and seedling vigor, with soybean seeds showing the greatest response (Horii *et al.* 2006b). A similar experiment, involving soybean, pea, and corn, found that seedling vigor was increased by two elicitors that also increased GPOX activity, and that plant height and growth were correlated to GPOX activity (Horii *et al.* 2006a). The developmental role of GPOX was studied by Ghamsari *et al.* (2006), who found that at least one form of GPOX was expressed during the development of *Crocus sativus* L. corm.

The symbiotic relationship between legumes and rhizobia may have evolved from a parasitic relationship as it shares many characteristics of a prolonged, pathogenic infection (Baron and Zambriski, 1995; Djordjevic *et al.* 1987; Vance, 1983); it is of great interest as to how rhizobia interact with SAR and, more specifically, how rhizobia and their signaling molecules affect SA, a hormone that has been shown to be crucial to plant defense, and PR proteins, which are one of the end-products of SA accumulation.

2.2.3 Salicylic Acid and Nodulation in Legumes

While the relationship between legumes and their bacterial symbionts is clearly symbiotic in nature, similarities between this relationship and those of pathogens and their hosts have been demonstrated. For example, in both cases, microbes attach to the surface of the plant, penetrate into the host, and redirect the host plant's metabolism while causing a noticeable effect on host physiology Djordjevic *et al.* 1987). In addition to these similarities, in many ways the plant responds to both pathogenic and symbiotic events as if they were indistinguishable, despite one resulting in benefits to the plant whereas the other results in disease (Vance, 1983; Djordjevic *et al.* 1987). The biochemical responses of legumes, to the presence of rhizobia, and how rhizobia are able to successfully colonize their host without triggering a number of varied and potent defense mechanisms is therefore of great interest. Given the importance of SA in plants suffering from a pathogenic attack, or as an elicitor of a defense response in plants, SA has become a model compound in the study of the plant response to nodulation (van Spronsen *et al.* 2003; Blilou *et al.* 1999; Martinez-Abarca *et al.* 1998).

Initially, it was found that some rhizobia, such as *Sinorhizobium meliloti*, suppress the biosynthesis of SA in alfalfa, prior to nodulation. Inoculating alfalfa plants with wild type *S.meliloti* resulted in much less SA being produced than inoculation with a NodC$^-$ mutant unable to successfully infect and nodulate. However; inoculation with another type of rhizobia, *Rhizobium leguminosarum* bv. *trifolii*, (which is unable to nodulate alfalfa) resulted in an increase in alfalfa SA levels, with a spike at 24 h after exposure, relative to the control and to alfalfa inoculated with *S. meliloti*. Furthermore, the exogenous application of SA prior to treatment with LCO resulted in fewer nodules per plant (Martinez-Abarca *et al.* 1998).

Similar results were reported for a study on pea roots, where resistance to infection by the mycorrhizal fungus *Glomus mosseae* or *R. leguminosarum* was correlated to endogenous SA levels. For example, the inoculation of pea with the fungus caused SA accumulation in the wildtype and in the symbiosis-resistant (Nod^-) genotype P2, however, in wildtype alfalfa, SA accumulated only when the plant was inoculated with mutant rhizobia for which LCO biosynthesis had been blocked. When inoculated with wildtype rhizobia, the wildtype alfalfa did not accumulate SA. When the Nod^- P2 alfalfa was inoculated, both types of rhizobia caused an accumulation in SA (Blilou *et al.* 1999).

A later study (van Spronsen *et al.* 2003) concluded, however; that an increase in SA inhibits only nodule formation in plants that form indeterminate nodules (such as pea or alfalfa), and that it does not inhibit nodulation in plants that form determinate nodules, such as soybean (*Glycine max*). Adding 10^{-4} M SA to vetch (*Vicia sativa* subsp. *nigra*), an indeterminate-type nodulating plant, completely blocked nodule development when it was inoculated with the symbiont, *R. leguminosarum* biovar (bv.) *viciae*, and inhibited the mitogenic effects of the LCO produced by the rhizobia. However; a similar treatment of SA on *L. japonicus* (a determinate-type nodulating plant), did not inhibit nodulation when inoculated with its symbiont, *Mesorhizobium loti*. Work on other determinate and indeterminate-nodulating plants showed the same result (van Spronsen *et al.* 2003).

That rhizobia, and by extension, LCO mediated effects, are capable of interacting with SA, and nodulation can be affected as a result, suggests that interplay is occurring between rhizobia via their signaling molecules and SA biosynthesis within the host plant. Understanding the numerous steps and components of SA biosynthesis is therefore required before the effects of rhizobia and their LCO on plant biochemistry can be fully understood.

2.2.4 The Phenylalanine Pathway and Salicylic Acid Biosynthesis

The phenylalanine pathway, a component of the phenylpropanoid pathway, is one of two separate plant pathways that synthesize SA (Figure 2.1). In addition to the formation of SA, the phenylpropanoid pathway also leads to the biosynthesis of many other compounds such as phytoalexins, coumarins, anthocyanins lignins and the

flavonoids and isoflavonoids that act as signaling molecules between plants and rhizobia, prior to nodulation (Schultze and Kondorosi, 1998).

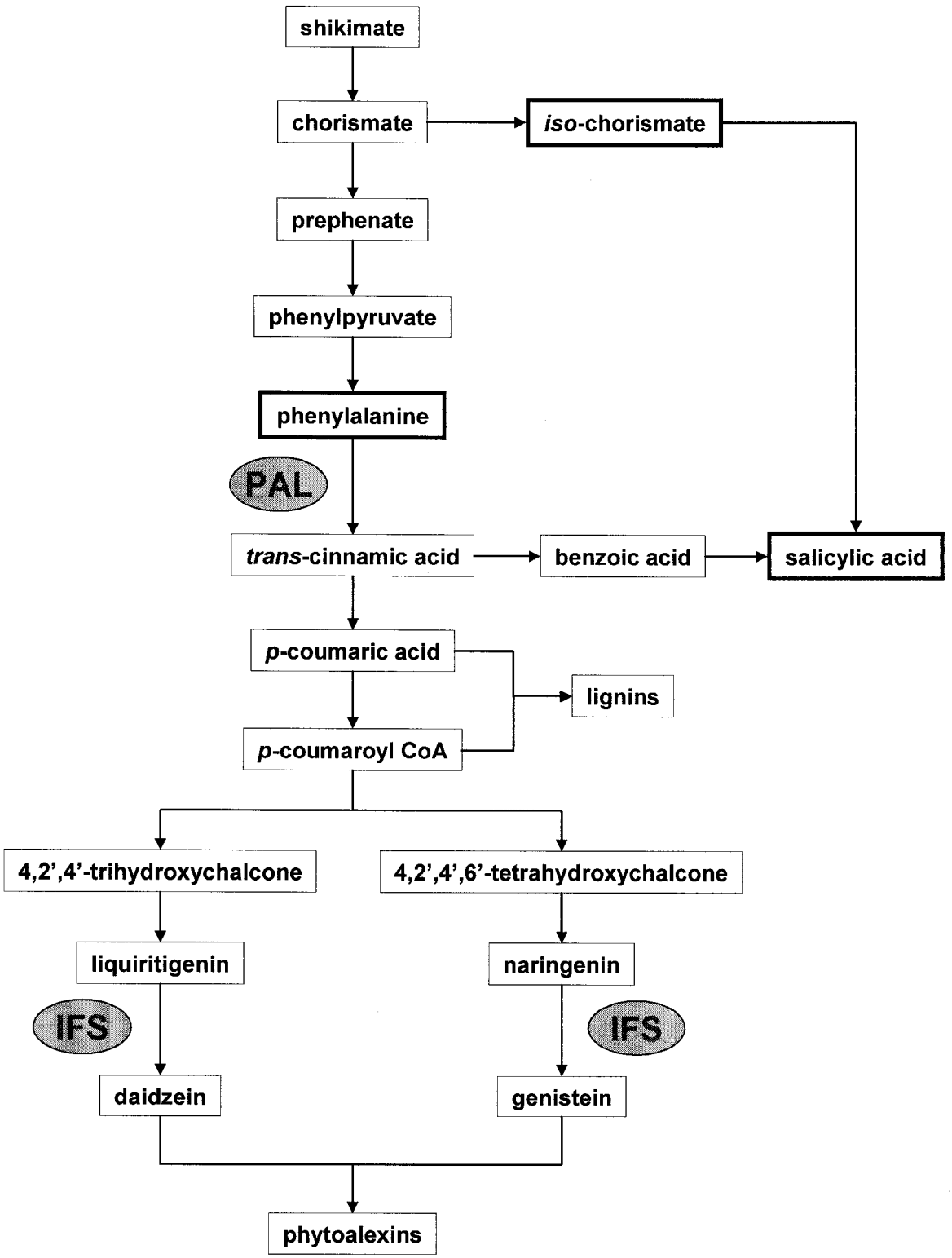
In the phenylalanine pathway, SA synthesis proceeds from the conversion of Phe (itself synthesized from shikimate via chorismate) into CA, which is then decarboxylated into BA, followed by a 2-hydroxylation step, resulting in SA (also known as 2-hydroxybenzoic acid; Leon *et al.* 1993, Meuwly *et al.* 1995). Some evidence suggests that in some plants the first step from CA is a 2-hydroxylation step leading to *o*-coumaric acid, followed by a decarboxylation to form SA (El-Basyouni *et al.* 1964; Chadha and Brown, 1974)

The first evidence that SA is formed from BA was found in a study on the conversion of SA to gentisic acid. Plants fed radiolabeled [^{14}C]BA formed [^{14}C]SA (Ibrahim, 1959). A later experiment (Klambt, 1962), found that when fed radiolabeled [^{14}C]BA, sunflower (*Helianthus annuus*) hypocotyls, pea internodes and roots and potato (*Solanum tuberosum*) tubers all formed radiolabeled [^{14}C]SA. This was supported by another study that showed that young leaves of *Gaultheria procumbens*, fed radiolabeled [^{14}C]CA or [^{14}C]BA, produced [^{14}C]SA, confirming that the main route of SA synthesis in the phenylalanine pathway occurs via Phe→CA→BA→SA (Ellis, 1971). A similar conversion was observed in rice when fed radiolabeled [^{14}C]CA or [^{14}C]BA, which produced [^{14}C]SA (Silverman *et al.* 1995).

In tobacco, it was shown that SA is synthesized strictly through BA and not *o*-coumaric acid. Radiolabeled [^{14}C]CA was fed to tobacco cell suspension cultures or tobacco leaves that were mock or TMV inoculated. The leaves were harvested 48 h after inoculation and labeled [^{14}C]BA and [^{14}C]SA was detected, but not labeled *o*-coumaric acid. In addition, when the healthy or TMV inoculated plants were fed [^{14}C]BA, a significant accumulation of [^{14}C]SA and PR1 proteins was observed. In a similar trial with *o*-coumaric acid, neither an accumulation of [^{14}C]SA nor PR1 proteins was detected (Leon *et al.* 1993). These results were supported by a separate experiment, although with similar conditions, involving tobacco. It was found that floating TMV-inoculated plant leaf discs that had been vacuum infiltrated with [^{14}C]BA led to total [^{14}C]SA levels that were 2-fold greater than the control, thus showing that BA levels in infected tobacco tissue affect SA accumulation. Furthermore, infected leaf discs exposed to [^{14}C]*o*-

coumaric acid did not accumulate [^{14}C]SA. Interestingly, whereas in healthy tobacco the specific activity of [^{14}C]SA was approximately the same as that of the supplied [^{14}C]BA, [^{14}C]SA, infected tobacco had a lower specific activity than that of the supplied [^{14}C]BA, perhaps evidence of SA synthesis via an alternative pathway (Yalpani *et al.* 1993).

Figure 2.1. A simple diagram of the two SA biosynthesis pathways: 1) the isochorismate pathway and 2) the phenylalanine pathway. Two key enzymes of the phenylpropanoid pathway are represented as ovals: 1) Phenylalanine ammonia-lyase and 2) Isoflavone synthase (modified from Yu *et al.* 2000; Wildermuth *et al.* 2001).


shikimate
chorismate
iso-chorismate
prephenate
phenylpyruvate
phenylalanine
PAL
trans-cinnamic acid
benzoic acid
salicylic acid
p-coumaric acid
lignins
p-coumaroyl CoA
4,2',4'-trihydroxychalcone
4,2',4',6'-tetrahydroxychalcone
liquiritigenin
naringenin
IFS
IFS
daidzein
genistein
phytoalexins

In a study of SA biosynthesis in potato, potato leaves were sprayed with the polyunsaturated fatty acid arachidonic acid (AA), previously shown to induce SAR. When the treated plants were fed radiolabeled [^{14}C]Phe, [^{14}C]CA and [^{14}C]BA [^{14}C]SA was produced, and when the potato plants were fed radiolabeled CA or BA, [^{14}C]SA was produced, whereas no labeled *o*-coumaric acid was generated, demonstrating that, at least in potato, SA is synthesized from Phe via CA and BA (Coquoz *et al.* 1998).

The majority of the enzymological work on SA biosynthesis via the phenylpropanoid has focused on PAL (EC 4.3.1.5), the enzyme responsible for catalyzing Phe into CA. It was shown that treating cucumber with 2-aminoindan-2-phosphonic acid (AIP), a specific inhibitor of PAL, caused complete inhibition of SA biosynthesis from radiolabeled [^{14}C]Phe, whereas plants treated with AIP and fed [^{14}C]BA produced [^{14}C]SA as normal, indicating that PAL is essential for SA synthesis and that in healthy and infected cucumber, SA is synthesized from CA, via BA (Meuwly *et al.* 1995). Interestingly, in another enzyme-inhibition study, when *Arabidopsis* (*A. thaliana*) was transformed with a *PAL1* promoter-β-glucuronidase (GUS) reporter construct and treated with AIP, *PAL1* was said to be inhibited. When SA was supplied to the plants treated with AIP, *PAL1* promoter activity was restored (Mauch-Mani and Slusarenko, 1996). In addition, the use of AIP in an inhibition study showed that the conversion of Phe to CA by PAL is the rate-limiting step of the phenylpropanoid pathway in SA biosynthesis (Coquoz *et al.* 1998).

2.2.5 The Isochorismate Pathway and Salicylic Acid Biosynthesis

Three experiments studying SA biosynthesis in the phenylalanine pathway yielded results that at least partly suggested that SA may be produced via an alternate pathway. It was found that tobacco infected with TMV and fed with radiolabeled BA resulted in [^{14}C]SA of a much lower specific activity than had been anticipated (Yalpani *et al.* 1993). In an experiment involving enzyme-inhibition of PAL, the enzyme responsible for the conversion of Phe to CA (a precursor of SA), it was found that SA was produced even when SA biosynthesis via the phenylpropanoid pathway had been blocked (Mauch-Mani and Slusarenko, 1996). Furthermore, in a separate study, it was also found that AIP did not result in complete inhibition of SA synthesis, and when the AIP-treated plants were

fed [^{14}C]CA or [^{14}C]BA, the free SA level recouped 40%, but that conjugated SA was unaffected by the addition of either BA or CA (Coquoz *et al.* 1998), suggesting that the plants were able to synthesize SA via an alternate route.

SA biosynthesis has also been found to occur also from chorismate but via isochorismate rather than Phe. Isochorismate was first isolated from the bacterium *Aerobacter aerogenes* (Young *et al.* 1969), and the biosynthesis of SA from shikimate (a precursor of chorismate) was first identified in the bacteria *Mycobacterium smegmatis* using radiolabeled shikimate that was incorporated into radioactive SA (Hudson and Bentley, 1970).

The isomerization of chorismate to form isochorismate is catalyzed by isochorismate synthase (ICS, EC 5.4.99.6). ICS activity was first detected in plants in the family *Rubiaceae* (Leduc *et al.* 1991). Wildermuth *et al.* (2001) found that biosynthesis of SA via isochorismate is required in *Arabidopsis* for both systemic and local acquired resistance. When *Arabidopsis sid2* mutants (with a blocked ICS1 gene) were inoculated with fungal biotroph *Erysiphe orontii* or the bacterial necrotroph *P. syringae* pv. *maculicola*, the mutants accumulated only 5-10 % of the level of SA accumulated by the wildtype and only 1-10 % the amount of PR1 protein accumulated by the wildtype inoculated with the same pathogens. As PR1 is an indicator of SAR and downstream from SA synthesis, it would appear that the *Arabidopsis* SAR defense response requires ICS. Given that low levels of SA were produced despite ICS being blocked, it may be that some of the biosynthesis of SA in *Arabidopsis* occurs via PAL, or that the mutation of the ICS1 gene was leaky, enabling some chorismate to be isomerized into isochorismate. The authors hypothesized that SA accumulation resulting in programmed cell-death, as a defensive response to pathogen attack, may occur via PAL given that the *sid2* mutant still exhibits cell death (Nawrath and Metraux 1999; Dewdney *et al.* 2000).

While our understanding of SA in plant responses such as SAR is expanding, little is known regarding the effects of LCO application, particularly when sprayed on the leaves of soybean plants, especially with regard to SA levels and plant defense responses. Research has suggested that LCO can increase gene expression, and the various outputs, of the phenylpropanoid pathway (Inui *et al.* 1996; Savoure *et al.* 1994; Savoure *et al.* 1997). The LCO and rhizobial effect on SA in legume roots has been studied (Martinez-

Abarca *et al.* 1997; Blilou *et al.* 1999; van Spronsen *et al.* 2003), but no work has yet been done to compare LCO effect on different soybean genotypes, and to study the LCO effect on soybean leaf SA content, or SA content in other soybean tissues. Tailoring legume biosynthetic outputs to our own specifications and, indeed, introducing the nitrogen-fixation symbiotic relationship to non-legumes, are worthy goals requiring that we first study how legumes perceive the rhizobial signaling molecules, and how the legume's defenses respond to symbiotic infection.

2.3 Research into Soybean Gene Expression

Recent research on soybean gene expression has been largely focused in three areas: 1) the soybean response, at the genetic level, to various biotic and abiotic stress factors, and 2) the elucidation of soybean gene function, and 3) the metabolic engineering of biochemical pathways in order to increase beneficial pathway products, such as isoflavones.

2.3.1 Biotic and Abiotic Factors

The biotic and abiotic factors examined in soybean gene expression studies are usually those that exert some form of stress. Abiotic factors are generally environmental stresses, such as high salinity or temperature. A recent study examined the effects of nitric oxide (NO; a plant signaling molecule implicated in combating oxidative damage and known to mediate stress responses to both abiotic and biotic factors) on soybean heme oxygenase gene expression by plants under oxidative stress (Noriega *et al.* 2007). Heme oxygenase (HO) catalyzes the oxidation of heme to biliverdin IXα, and is thought to play a role in protecting soybean from oxidative damage. Noriega *et al.* (2007) examined the abundance of HO transcript in the presence of oxidative stress and in conjunction with an NO donor. They found that HO mRNA levels increased significantly, as detected by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR), and that the damaging effects of the oxidative stress was nullified by the NO donor treatment. Furthermore, they determined that NO's function is concentration dependent and that the protective function of NO may be occurring via an increase in HO mRNA levels.

In another study, a low-temperature inducible cDNA sequence encoding L-asparaginase (which catalyzes the hydrolysis of the amide group of L-asparagine) was isolated (Cho *et al.* 2007). Fluorescence *in situ* hybridization and gDNA blotting were used to determine that soybean has two copies of the L-asparaginase cDNA and that the mRNA of those copies was induced by low temperature, abscisic acid, and salinity.

Biotic factors that affect soybean gene expression are generally some type of soybean pathogen or pest, such as Asian soybean rust (ASR), or soybean cyst nematodes (SCN). For example, van de Mortel *et al.* (2007) used microarrays to measure changes in mRNA abundance over a 7-day period for rust-infected leaves of resistant and susceptible soybean lines. They detected changes in mRNA abundance in the susceptible genotype after 96 h, the time of rapid fungal growth onset, whereas in the resistant genotype, which does not experience rapid fungal growth, changes in mRNA abundance were detected only 72 h following inoculation with the pathogen, long before the susceptible type.

In a similar experiment, Tucker *et al.* (2007) used microarrays and RT-PCR to examine changes in transcript abundance of soybean cell-wall modifying proteins in root pieces that had been colonized by nematodes. They identified novel cellulases and polygalacturonases, and found that many soybean genes responsible for cell-wall modifications are strongly up-regulated when the host plant was colonized by SCN. This may result from a signal secreted by the nematode, used to stimulate cell-wall degrading proteins in the host plant, for the nematode's benefit. Another experiment, involving soybean gene expression and SCN, utilized comparative microarray analysis (Klink *et al.* 2007), and compared soybean gene response to compatible and incompatible SCNs during infection of soybean roots. They found a differential response with respect to transcript abundance in the presence of compatible or incompatible SCNs, and concluded that the soybean genes must respond very differently to the two types of pathogen, prior to feeding site selection by the nematodes.

Subramanian *et al.* (2004) cloned the 5' upstream regions of the soybean *IFS1* and *IFS2* genes, and determined the tissue-specificity of the *IFS1* and *IFS2* gene expression patterns using RT-PCR. Inoculation of transgenic soybean roots with *B. japonicum* (a biological factor) induced the expression of the IFS1:GUS (β-glucuronidase) construct in root hairs and xylem poles of the root. Similarly, the exogenous application of SA

(another biotic factor) induced the expression of the IFS1:GUS construct in all root cell types.

2.3.2 Soybean Gene Function

Gene function elucidation has been, as with other organisms, a key area of research regarding soybean gene expression. A few examples are given from the two primary areas of soybean genetic research: pathway elucidation and stress-tolerance. Chronis and Krishnan (2004) screened a soybean cDNA library with ESTs and isolated a full-length cDNA encoding a serine acetyltransferase (SATase), which was found to have great similarity to other plant SATases. In combination with an *Escherichia coli* cysteine-auxotrophic mutant, the protein identity was confirmed when the mutant *E.coli*, expressing the soybean SATase, was able to grow on minimal medium lacking cysteine.

Bilyeu *et al.* (2003) identified and characterized three omega-3 fatty-acid desaturase genes (which contribute to seed linolenic acid levels) in soybean, using database homology searches and gene cloning. The relative expression of the genes was quantified using real-time RT-PCR; one of the genes was expressed primarily in developing seeds. A low linolenic acid soybean breeding line was found to contain 2 of the 3 genes, thus molecular markers for defects in these omega-3 fatty-acid desaturase genes will facilitate attempts to breed soybean lines with low levels of linolenic acid.

Byfield *et al.* (2006) determined that there are two Δ9 stearoyl-ACP desaturases (which convert stearic to oleic acid) in soybean, and detected unique amino acid variability between the genes. Using RT-PCR on the unique regions, quantifiable differences in transcript abundance were detected among soybean tissues, though transcript accumulation for the three stages of seed development examined was found to be approximately equal.

Zhu *et al.* (2006) identified and characterized a heat stress/shock transcription factor (HSF) from a soybean EST database using sequence comparisons from known HSFs of other species and rapid amplification of cDNA ends. Overexpression of the HSF in a transgenic soybean line resulted in the accumulation of a soybean heat shock protein at normal temperatures and overexpression of the heat shock protein at high temperatures.

The transgenic line showed greater tolerance to high temperature stress than non-transgenic plants, confirming the identity of the transcription factor as an HSF.

2.3.3 Metabolic Engineering

Given that certain soybean products, such as isoflavones, provide numerous health benefits for humans and animals (Watanabe *et al.* 2002), that diverse products stemming from the phenylpropanoid pathway have been shown to have anti-microbial properties (Kramer *et al.* 1984, Rivera-Vargas *et al.* 1993) and that the inherent importance of flavonoids and isoflavonoids in legume-rhizobial symbioses (recently reviewed in Broughton *et al.* 2003), metabolic engineering of soybean biochemical pathways has been a primary area of soybean gene expression research.

Of particular interest has been the transformation of non-legumes with legume genes responsible for the expression of isoflavonoid biosynthesis proteins, with the aim of producing isoflavonoids in non-legumes. Jung *et al.* (2000) used a yeast expression assay to screen soybean ESTs encoding cytochrome P450 proteins and identified two soybean genes (*IFS1* and *IFS2*), that encode for isoflavone synthase. They expressed the *IFS1* gene in transgenic *Arabidopsis* and found that transformation led to accumulation of the isoflavone genistein. In a similar experiment Yu *et al.* (2000) transformed tobacco and *Arabidopsis* with *IFS1*. Genistein was produced in the tobacco floral tissue, and enhanced genistein accumulation occurred in the transgenic *Arabidopsis* when flavonoid/anthocyanin production was induced via treatment with UV-B. In maize cells transformed with *IFS1*, *CHR*, and an anthocyanin pathway transcription factor, both genistein and daidzein were synthesized, demonstrating that *IFS1* can successfully compete with anthocyanins for the shared precursor, naringenin (Yu *et al.* 2000). In another metabolic engineering experiment, rice was transformed with soybean *IFS1*. Genistein was detected using high performance liquid chromatography (HPLC), and was found to induce *nod* gene expression in rhizobia (Sreevidya *et al.* 2006).

Transforming soybean with maize transcription factors that turn on phenylpropanoid pathway genes responsible for anthocyanin production resulted in a small increase in overall isoflavone accumulation (Yu *et al.* 2003). When the anthocyanin branch of the transformed soybean was blocked via suppression of

flavanone-3-hydroxylase (the enzyme that catalyzes the first committed step of the anthocyanin pathway), even higher levels of isoflavones accumulated in soybean seeds (Yu *et al.* 2003). Lozovaya *et al.* (2007) used gene silencing in soybean hairy roots transformed with *CHS* or *IFS2* to determine the effects on fungal pathogen susceptibility. The low isoflavone transformed soybean lines did not accumulate glyceollin, a pterocarpin phytoalexin derivative of isoflavones produced after infection. These same lines were more susceptible to the fungal pathogen *Fusarium solani* f. sp. *glycines*, demonstrating the importance of phytoalexin synthesis on soybean root resistance to pathogen attack – something that had been suspected for a long time, but has been difficult to prove.

2.4 Gene Expression Methodologies

Recent research on soybean gene expression utilized two powerful techniques that have been particularly effective at elucidating differences in gene expression: 1) Real-time reverse transcription polymerase chain reaction (QRT-PCR), and 2) DNA microarrays.

2.4.1 Real-time Reverse Transcription Polymerase Chain Reaction (QRT-PCR)

In fluorescence-based QRT-PCR, extracted RNA is reverse-transcribed into cDNA, the transcribed cDNA strand is amplified, and the level of amplification is quantified. QRT-PCR proceeds via either a one or two-step procedure: in the one-step method both the reverse-transcription and the cDNA amplification occur in one tube, whereas in the two-step method, these steps are spatially seperated. The one-step method is simpler and reduces the risk of contamination, whereas the two-step method is more sensitive and reproducible (Vandesompele *et al.* 2002).

There are two types of detection chemistries available for use in QRT-PCR: specific and non-specific. Specific chemistries utilize a probe consisting of a fluorophore/quencher complex that hybridizes to a user-selected sequence on the cDNA strand. Once the polymerase cleaves the probe, the fluorophore and quencher are separated and fluorescence can be detected. Non-specific chemistries utilize an intercalating fluorescent dye, such as SYBR Green dye (Stratagene, San Diego, USA),

that emits little fluorescence unless bound to double-stranded DNA (Morrison *et al.* 1998). This method relies upon the use of DNA primers that have been designed to hybridize to only a specific region of one gene in the genome of the species in question. Only the segments of DNA that hybridize to the designed primers will amplify with each cycle, thus as more strands of the selected region of DNA are amplified, more fluorescent dye is able to intercalate into the DNA, thereby increasing the fluorescence with every cycle.

The instruments used in QRT-PCR consist of a specific light source that causes the fluorescent molecules to emit light at a wavelength other than the source, a detector of fluorophore emissions, which are proportional to the quantity of amplified DNA, and software that analyzes the data. Software determines a baseline of fluorescence, and the cycle at which a product's fluorescence crosses the threshold is defined as its *C*t value, which can be used to determine the amount of initial template DNA. If a product has more copies of first-strand cDNA (and thus more copies of extracted mRNA), it reaches the *C*t faster than does a product with fewer starting copies (Higuchi *et al.* 1993; Bustin, 2005a).

Two methods exist for quantifying mRNA levels from QRT-PCR data: absolute and relative quantification. Absolute quantification expresses changes in mRNA levels relative to a standard curve consisting of a dilution series of known concentrations of initial target DNA. The slope of the curve is the efficiency of the amplification, and a linear regression of that curve can be used to determine the quantity of first-strand cDNA in each sample. The principle drawback to absolute quantification is that any PCR or RT inhibitors in the sample alter the results; a fundamental assumption of QRT-PCR is that the sample and the standard are amplified at the same level of efficiency (Bustin, 2000; Livak and Schmittgen, 2001). Relative quantification compares the *C*t of an unknown sample to the *C*t of an amplified internal control mRNA, producing a ratio of the two *C*t values. The primary drawback of the relative quantification method is that differences between the amplification efficiencies of the sample and the internal control will affect the accuracy of the result (Liu and Saint, 2002; Pfaffl, 2005).

Samples between individuals can be compared following the normalization of values against an internal reference, as there can be a great deal of variation between

individuals with regard to the amount of initial material, based on inherent differences between the basal levels of expression of the individuals, rather than the result of the treatments of the experiment (Karge *et al.* 1998). In addition to compensating for variation between samples not due to treatment differences, variation in PCR runs (efficiency, sample loading) or in RNA integrity can be accounted for via the normalization of the target gene expression by using the expression of an unregulated, constitutively-expressed reference gene. A program has been designed for the comparison of multiple genes, called Relative Expression Software Tool (REST; Pfaffl *et al.* 2002); it includes correction for PCR efficiency and the normalization of target genes against a reference gene. The program utilizes an equation (Pfaffl, 2001) that takes efficiency and the use of a reference gene into account:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta CP\text{target(control-sample)}}}{(E_{\text{ref}})^{\Delta CP\text{ref(control-sample)}}}$$

"Ratio" represents the relative expression ratio of a target gene, which is calculated via the efficiencies (E) of the target gene and the reference gene, and the difference (Δ) between the crossing point (CP) of a treated sample and a control sample.

2.4.2 DNA Microarrays

Similar to QRT-PCR, DNA microarrays rely on the fluorescence produced by hybridization being proportional to transcript abundance. The primary advantage of the DNA microarray technology is that it allows researchers to examine the entire genome of an organism and observe interactions between thousands of genes on a single chip. Microarray technology is based on the hybridization of complementary nucleic acids: nucleic acid probes of a known sequence are each attached to a specific location on a glass or nylon chip, and then the probes are exposed to the sample nucleic acid 'targets', the identity and abundance of which are to be determined (Lipshutz *et al.* 1999; Weeraratna and Taub 2007).

There are two types of DNA microarray chips. These types differ primarily based on the type of probe used. In the first method, for each gene or expressed sequence tag (EST), the probe consists of a single cDNA double-strand around 1000 base pairs (bp) in length that has either been immobilized on a chip using robotic spotting or has been

sprayed onto the chip by inkjet printing. In the second method, oligonucleotides less than 100 bases in length are synthesized and immobilized on the chip (Lockhart and Winzeler, 2000; Wiltgen and Tilz, 2007). Arrays from both methods are used in the same way: 1) they are exposed to fluorescently-labeled sample DNA that hybridizes based on sequence complementarity, and 2) the arrays are scanned and the fluorescence of the image is used to determine the relative abundance of each gene or EST in each sample. The signal intensity of each probe is proportional to the amount of molecules bound at that site, and also indicates, based on the principles of hybridization, the gene's probable identity (Schena *et al.* 1995; Lockhart and Winzeler, 2000).

3. Materials and Methods

3.1 General Methodology

3.1.1 Plant Material and Growth Conditions

Soybean cultivar (cv.) Ontario Agricultural College (OAC) Bayfield seeds or non-nodulating soybean seeds of the Evans-derived genotype were surface-sterilized in 0.25% v/v sodium hypochlorite (Javex) for 2 min and then washed five times with distilled water (dH_2O). The seeds were then spread evenly on a 5 cm thick bed of autoclaved vermiculite (Holiday®, Normiska Co., Montreal, Canada) in a large plastic tray (54 x 28 x 6.5 cm) with holes at the bottom, to allow drainage of excess water, and then covered with 1 cm of vermiculite.

The tray, with seeds, was placed in a growth chamber kept at 25 °C with a 16 h photoperiod and an 8 h period of darkness, at a constant 20 °C. The germination/rooting medium were kept moist through regular additions of dH_2O. When the cotyledons had emerged and separated (V1 growth stage; Fehr *et al.* 1971), the seedlings were transferred to 5 cm pots containing a mixture of Pro-Mix (sphagnum peat moss) and perlite (Holiday®, Normiska Co., Montreal, Canada) as growth media. Each seedling was transferred to an individual pot and returned to the growth chamber under the conditions described above. The seedlings were watered with ½ strength Hoagland's solution (Hoagland and Arnon, 1950) every day.

3.1.2 Bacterial Culture and LCO Induction

The rhizobial strain used for LCO production in this study was *B. japonicum* strain 532C, obtained from Liphatech Inc. (Milwalkee, USA). Cells were grown in the dark in Yeast Extract Mannitol (YEM) broth medium (10 g mannitol, 0.5 g K_2HPO_4, 0.1 g $MgSO_4$, 0.2 g $MgSO_4 \bullet 7H_2O$, 0.1 g NaCl, 0.4 g yeast extract, 1 L dH_2O, 15 g agar), pH 6.8, at 28 °C and shaken at 150 revolutions per minute (rpm) until the cell density at OD_{600} was 4 x 10^8 cells per mL. A subculture was created by adding 20 mL of inoculum from the above culture to 2 L of YEM medium. The growth conditions of the subculture were the same as above. By 4 days after subculture initiation, the rhizobia had reached

the exponential growth phase. A 1 mM stock solution of genistein (Sigma, St. Louis, USA) in 100% methanol was aliquoted into the 2 L of medium, for a final concentration of 5 μM, to induce rhizobium to synthesize LCOs.

3.1.3 LCO Extraction and Purification

The culture was incubated for 2 more days, and at the end of the incubation the culture was pooled with other 2 L cultures to make 12 L of medium, which were extracted by phase partitioning against 40 % HPLC-grade n-butanol while shaking for 30 min at 150 rpm. The organic fraction was collected and evaporated in a rotary evaporator (Yamato, NJ, USA) under vacuum at 50 °C. The brown, viscous material that remained was redissolved in 18% acetonitrile, and loaded onto a C18 column (PRESEP™ Fisher Scientific, Montreal, Canada) and eluted three times with 10 mL of 30 % acetonitrile. The second elution was carried out with 10 mL of 60 % acetonitrile and this eluent contained the LCO. This eluent was further fractionated using HPLC, with Waters 501 pumps, a Waters 401 detector set at 214 nm, and a WISP712 autosampler using a C18 reverse phase column (4.6 mm × 250 mm, 5 μm packing; Vydac, CA, USA).
The chromatography was conducted for 60 min using an acetonitrile linear gradient from 18 to 60 %. The LCO peak was identified by comparing its retention time to that of a standard (NodBj-V ($C_{18:1}$, MeFuc)) from strain *B. japonium* strain 532C. The eluent containing the corresponding peak was collected, freeze-dried, rechromatographed and the sample collected. The LCO concentration was calculated by taking the area under the HPLC peak of a standard, at a known concentration, and comparing it to the peak of the purified LCO sample.

3.1.4 Foliar Application of LCO

Once the soybean plants reached the vegetative V1 stage of development (when the cotyledons are no longer touching each other; Fehr *et al.* 1971) the plants were divided into groups at random, with each group representing a treatment and time-point. The first trifoliolate leaf of each plant was sprayed with 2 mL of sterilized dH_2O containing one of the following treatments:

1. **Control**: 0.02 % Tween® 20 (Fisher Scientific, Ottawa, Canada)

2. **10^{-7} M LCO**: 10^{-7} M LCO and 0.02 % Tween® 20

3.1.5 Plant Leaf and Root Sampling

The first trifoliolate leaf and the roots of each plant were harvested with a scalpel a certain number of hours following the spray application (see 3.2 Specific Methods and Experiments for the duration of each experiment). The leaves were weighed and then individually wrapped in aluminum foil following harvest and immediately submerged in liquid N_2 and stored at -80 °C until extraction.

The roots of each plant were harvested with a scalpel a certain number of hours following the spray application (see 3.2 Specific Methods and Experiments for the duration of each experiment) following spray application of LCO or control solution to the first trifoliolate leaf. The roots were cut from the shoot using a scalpel and carefully separated from the rooting medium by soaking and washing the medium off the roots with dH_2O. The roots were gently dried with a paper-towel, weighed, individually wrapped in aluminum foil and immediately submerged in liquid N_2, and then stored at -80 °C until extraction.

3.1.6 Salicylic Acid Extraction and Quantification

Frozen leaf or root tissue was rapidly ground with a chilled (in liquid N_2) mortar and pestle set. The finely ground tissue was extracted with 1.5 mL 100 % methanol g^{-1} tissue (protocol modified from Raskin *et al.* 1989). The samples were spun for 20 min at 41,000 g, while being held at 4 °C. The supernatant was collected, without disturbing the pellet, and used for HPLC analysis. HPLC was performed with the Breeze system: Waters 1525 pumps, a Waters 2487 detector set at 214 nm, and a Waters 717 plus autosampler, using a C18 reverse phase column with the appropriate guard column (4.6 mm × 250 mm, 5 µm packing; Vydac, CA, USA). The chromatography was conducted for 45 min using 2 solvents, methanol and 0.1 % H_3PO_4, with a linear gradient of methanol from 25 to 100 % over 35 min. Salicylic acid was detected photometrically at 214 nm. The SA peak was identified by comparing its retention time with that of a SA standard (Sigma, St. Louis, USA). The concentration (µg·g FW^{-1}) of SA in the sample was calculated by taking the area under the HPLC peak of the standard, at a known

concentration, for each sample. When the standard was added to the soybean plant extract, the same peak at the same retention time as in the original sample was greatly increased in magnitude (Figure 3.1). Each sample was also run twice, and the average was taken to be the concentration of SA in the sample.

3.1.7 Extraction and Quantification of RNA

Frozen leaf tissue was rapidly ground with a mortar and pestle chilled with liquid N_2 that had been placed in a -20 °C freezer for 4 h prior to RNA extraction. Total RNA was extracted from ~100 mg of finely-ground, frozen tissue using the Qiagen RNeasy® Plant Mini Kit (Qiagen, Missisauga, Canada) for total RNA isolation, with an on-column DNase digestion via the RNase-free DNase Set (Qiagen, Missisauga, Canada), according to the manufacturer's instructions. Following RNA extraction, the concentration of total isolated RNA was measured using a NanoDrop® ND-1000 spectrophotometer at 260 nm (NanoDrop Technologies, Wilmington, USA). Samples were stored at -80 °C until use.

3.1.8 cDNA Synthesis

From each sample, 1 μg of RNA was used with nonspecific primers to construct complementary DNA (cDNA) via polymerase chain reaction (PCR) using the QuantiTect® Reverse Transcription Kit, according to the manufacturer's instructions (Qiagen, Missisauga, Canada). The 20 μL PCR reactions contained gDNA wipeout buffer for the removal of any DNA extracted during RNA isolation, and all components required for first-strand cDNA synthesis. Standard PCR conditions were used, in a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Streetsville, Canada). All samples were diluted 20-fold and stored at -80 °C until further use.

3.1.9 Quantitative Real-Time Polymerase Chain Reaction

All tools and surfaces were sprayed with DNA ZAP® solution prior to each QRT-PCR run to prevent DNA contamination (Ambion, Inc., Austin, USA). Quantitative real-time PCR (Winer *et al.*, 1998; Bustin, 2002) was performed using the Stratagene Mx3000P Real-Time PCR System (Stratagene, San Diego, USA). Each reaction contained 12.5 μL of 2X Stratagene Brilliant® SYBR® Green QPCR Master Mix

(Stratagene, San Diego, USA), 6 µL of nuclease-free water, 0.1 µg of sample cDNA and the forward and reverse primers of one of three genes (*actin*, *IFS2*, *PAL1*), for a final volume of 25 µL. A study of IFS transcripts found that there was a much higher abundance of *IFS2* transcript than *IFS1* in leaves (Subramanian *et al.* 2004). In another comparison of *IFS1* and *IFS2*, a search of soybean ESTs originating from cDNA libraries found that the most numerous *IFS* ESTs were those of *IFS2* corresponding to cDNA libraries constructed from stressed, pathogen-infected or elicitor-treated soybean tissues (Dhaubhadel *et al.* 2003). As this experiment is concerned with soybean leaf tissue and the use of LCO as an elicitor, the *IFS2* gene was selected as a candidate for the study of leaf IFS expression. For the enzyme phenylalanine ammonia-lyase, the gene *PAL1* was selected for study based on results from the above experiments pertaining to salicylic acid: PAL is the rate-limiting step in the biosynthesis of salicylic acid via the phenylpropanoid pathway (Coquoz *et al.* 1998) and therefore a good candidate for the study of salicylic acid accumulation. Although there have been primers for the *IFS2* and *PAL1* genes previously published in the literature, most resulted in relatively large product sizes (>800 base pairs - bp), and were therefore not selected. Primers for *IFS2* and *PAL1* were designed using the Primer3 software (Rozen and Skaletsky, 2000), available online. The primers were selected for an optimal GC content of ~50%, a primer length of ~20 bp, and a product size of ~200 bp. The *actin* forward and reverse primers were chosen from previously published literature (Byfield *et al.* 2006; Moniz de Sa and Drouin, 1996). A preliminary assay was performed (data not shown) to determine the optimal concentrations for each primer used in this study. The characteristics of the selected primers are given in Table 3.1.

The reactions were performed in a 0.2 mL volume tubes and sealed with flat top caps. Quantitative real-time PCR amplification for the *actin* gene consisted of 45 cycles of 30 s at 95°C, 45 s at 55°C, and 45 s at 72°C followed by dissociation curve analysis over a 40°C temperature gradient at 0.066°C s^{-1} from 55°C to 95°C. The amplification of the *IFS2* and *PAL1* genes was identical to the above procedure, but performed for 55 cycles for *IFS2* and 50 cycles for *PAL1*. Two technical replicates were performed for each sample. Sequencing of the amplified products was performed, and confirmed that the fragments amplified in this experiment are actually part of the genes studied and not

the amplified regions of some other soybean gene. Sequencing was performed by Genome Quebec. Transcript levels for the *PAL1* and *IFS2* genes were determined by an automatic comparison of individual cycle threshold (*Ct*) values with a serial dilution QRT-PCR standard curve for each gene run in conjunction with each set of reactions. The QRT-PCR efficiency ranged from 85 to 95 %.

3.1.10 Microarray Labeling, Hybridization and Scanning

Total RNA was isolated as described above. All RNA samples were adjusted to a concentration of 0.7 $\mu g\ \mu l^{-1}$, and mRNA transcript abundance was measured using GeneChip Soybean Genome Arrays (Affymetrix, Santa Clara, CA, USA). RNA concentration and quality were determined using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The quality of a sample was considered "acceptable" when the 28S/18S ribosomal RNA ratio is close to 2.0, when the Electropherogram baseline of the profile is close to zero (representing the state of degradation of the RNA) and when the baseline between the 18S and 28S peaks is close to zero as well. Furthermore the RNA Integrity Number (RIN), indicative of how intact the RNA is, should be between 8.6 and 10 to be considered acceptable. The sample RINs were all 8.8 or above. All samples were of good quality and 3.5 μg was used to synthesize labeled target cRNA using the GeneChip HT One-Cycle Target Labeling and Controls kit (Affymetrix). All steps of labeling, hybridization, and scanning were performed at the Genome Quebec Innovation Centre. The cRNA quality was determined using the 2100 BioAnalyzer (Agilent, (Santa Clara, CA, USA) equipped with an RNA Nano LabChip, overnight hybridization of the chip utilized 15 μg of fragmented cRNA and the staining and washes were performed using the GeneChip Fluidics Station 450 robot (Affymetrix). The microarray chips were scanned with a GeneChip Scanner 3000 7G (Affymetrix).

3.1.11 Gene Annotation

The 10^{-7} M LCO-regulated and control soybean genes were annotated using the Affymetrix GeneChip Soybean Genome Array Annotation. For soybean, this is based on

the UniGene database, which is EST-based, and uses non-redundant gene-oriented clusters of mRNA

3.1.12 Enzyme Extraction

Crude extract preparation followed a modified version of the soybean crude extract protocol of Chernikova *et al.* (2000). The first and second trifoliolate leaf samples were ground to a fine powder using a mortar and pestle, with liquid N_2 and 150 mg of each sample was extracted with 1 mL of extraction buffer (50 mM Tris-HCl buffer, 3 mM $MgCl_2 \bullet 6H_2O$, 1 mM ethylenediamine tetraacetic acid, 1% polyvinylpyrrolidone, pH 7.0). The samples were spun for 20 min at 10,000 g at 4 °C. The samples were stored at -20 °C.

3.1.13 Chitinase Activity Assay

Chitinase activity (EC 3.2.1.14) was measured as described by Lingappa *et al.* (1962). The reaction mixture consisted of 20 µL of enzyme solution, 500 µL of 0.5% colloidal chitin, and 480 µL of 50 mM sodium acetate buffer (pH 5.5). After incubation at 37 °C for 1 h, 200 µL of 1 N NaOH was added and the mixture was vortexed. The samples were spun for 5 min at 10,000 g, and then 500 µL of supernatant was mixed with 1 mL of Schales' Reagent (0.5 M sodium carbonate, 1.5 mM potassium ferricynide), and heated in boiling water for 15 min. The samples were cooled on ice and then absorbance was measured at 420 nm with an Ultrospec 4300 pro spectrophotometer (GE Healthcare, Canada). The activity was calculated from a standard curve obtained from known concentrations of chitin (N-Acetylglucosamine; GlcNAc). One unit of chitinase activity was defined as the amount of enzyme that liberated 1 µmol of GlcNAc per hour.

3.1.14 β-1,3-Glucanase Activity Assay

β-1,3-glucanase activity (EC 3.2.1.6) was measured as described by Yedidia *et al.* (2000). The reaction mixture consisted of 50 µL of enzyme solution, 50 µL of 1% laminarin and 400 µL of 50 mM sodium acetate buffer (pH 5.0), and vortexed. After incubation at 37 °C for 1 h, 1.5 mL of dinitrosalicylic acid (DNS) reagent was added. The reaction was stopped by heating in boiling water for 5 min. The absorbance was

immediately measured at 550 nm. The reducing sugar was calculated from a standard curve obtained from known concentration of glucose. One unit of β-1,3-glucanase activity was defined as the amount of enzyme that liberated 1 μmol of glucose per hour.

3.1.15 Guaiacol Peroxidase Activity Assay

Guaiacol peroxidase activity (EC 1.11.1.7) was measured following a modification of the protocol of Maehly and Chance (1959). The reaction mixture contained 25 μl of 200 mM guaiacol, 1.350 mL of 50 mM Tris-HCl buffer (pH 8.0), and 100 μL of enzyme extract. The reaction was started by the addition of 10 μL of 40 mM H_2O_2. The production of tetraguaiacol via the oxidation of guaiacol was measured as an increase in absorbance at 470 nm for 1 min. The activity was calculated as μmol of tetraguaiacol production per minute using the mM extinction coefficient for tetraguaiacol of 26.6 mM^{-1} cm^{-1}. The mixture without the enzyme extract was used as a control.

3.2 Experimental Design and Data Analysis

3.2.1 Experiment 1: LCO Concentration and Leaf and Root SA Levels Following Foliar Application

The level of first trifoliolate leaf and root SA was quantified at 0, 48, 72 and 96 h following treatment of the first trifoliolate leaf with one of two concentrations of LCO (10^{-7} M and 10^{-8} M). The experiment was organized following a Completely Randomized Design (CRD) with 5 replicates per treatment per sampling time (20 plants per treatment, 60 plants total). After treatment application, the position of each plant was randomly assigned within the growth chamber. The general linear model (GLM) procedure and the Student-Newman-Keuls (SNK) nonparametric statistical test were conducted with the SAS System, to determine the significance of treatment on total SA of soybean tissue, at $p \leq 0.05$.

3.2.2 Experiment 2: Foliar Application of 10^{-7} M LCO and SA Levels

Based on results of experiment 1, and the published literature (Martinez-Abarca *et al.* 1998; Savoure *et al.* 1994; Al-Tawaha *et al.* 2005), we selected 10^{-7} M as the most

effective concentration of LCO. This concentration was tested on two different soybean genotypes (nodulating and non-nodulating) and the level of SA in the first and second trifoliolate leaf was quantified for each type of soybean plant at 0, 24 and 48 h following treatment.

To determine the response of soybean types to LCO treatment, two types were selected that are most likely to have different responses. The first was OAC Bayfield, a soybean cultivar (cv.) produced commercially in southwestern Quebec and able to nodulate and fix nitrogen. The second type was the progeny of an F4 selection from cv. Evans x L66-2470 which carries the rj_1 gene for non-nodulation (Evans-derived line). As the LCO compound tested is a rhizobial signaling molecule that is normally perceived by soybean plants and induces specific changes in and responses from the plant, a soybean type that lacks the ability to nodulate may be physiologically deficient in aspects of its response to infection by a symbiont and also to exposure to signal compounds from a symbiont (LCO treatment), when compared to the cultivated, nodulating type. One possibility is that the non-nodulating line perceives the LCO treatment as the prelude to a pathogenic attack, whereas the nodulating cultivar perceives the treatment as a symbiotic signal. Alternatively, the non-nodulating line may simply be unable to respond positively to the treatment.

The experimental design was a CRD with 4 replicates per treatment sampling time (12 plants per treatment, 24 plants total). After treatment application, the position of each plant was randomly assigned within the growth chamber. The GLM procedure and the SNK nonparametric statistical tests were used, with the SAS System, to determine the significance of LCO concentration or the total SA of soybean tissue $p \leq 0.05$.

3.2.3 Experiment 3: Foliar Application of LCO and PAL1 and IFS2 Genes

The relative gene expression level of two soybean phenylpropanoid pathway genes (*PAL1* and *IFS2*) in the first and second trifoliolate leaf for two different soybean genotypes (cv. OAC Bayfield and the Evans-derived line) was quantified using QRT-PCR, at 0, 24 and 48 h following foliar application of 10^{-7} M LCO.

The experiment was structed following a CRD with 8 replicates per treatment per sampling time. The 8 replicates were divided into 4 pooled groups to create 4 biological

replicates per treatment per sampling time. After treatment application, the position of each plant was randomly assigned within the growth chambers. The crossing point for each gene of each sample was normalized against the soybean *actin* gene expression level of each sample. Relative gene expression was calculated using the Relative Expression Software Tool (REST; Pfaffl, 2001; Pfaffl *et al.* 2002), which used the following equation to calculate the ratio of gene expression:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta CP\text{target(control-sample)}}}{(E_{\text{ref}})^{\Delta CP\text{ref(control-sample)}}}$$

A gene was determined to be significantly upregulated or downregulated, relative to the control treatment, at $p \leq 0.05$.

3.2.4 Experiment 4: Foliar Application of LCO and Leaf Transcript Abundance

The transcript abundance was measured for the first trifoliolate leaf of cv. OAC Bayfield soybean plants, at 48 h following the application of 10^{-7} M LCO onto the same leaf. The experiment was organized following a CRD with 3 replicates per treatment. After treatment application, the position of each plant was randomly assigned within the growth chamber.

The base 2 logarithm of robust multi-array average (RMA; Bolstad *et al.* 2003) signals were median-centered so that the median log-scale expression measure for each GeneChip was zero. RMA uses background correction estimates derived from the lowest signals on the array and subtracted out. The data was normalized to enforce a common probe distribution across all 6 arrays used. All calculations were done by FlexArray software (Blazejczyk *et al.* 2007), provided by the Genome Quebec Innovation Centre, using Bioconductor libraries. Three separate algorithms were used to compute p-values: Cyber-T, Empirical Bayes (EB; Rocke) and Local Pooled Error (LPE). Cyber-T is a statistical algorithm that is suited to analyses of DNA microarray data to identify differentially expressed genes. The Cyber-T uses regularized t-tests with a Bayesian estimate of variance between measurements to conclude significant differences in gene expression (Baldi and Long, 2001). The EB (Rocke, 2004)) statistical algorithm is frequently used for the analysis of microarray data. This method utilizes prior distribution of the probability of positive expression or the mean expression across genes,

via multiplicity-adjusted F-tests. The LPE test is a statistical algorithm designed to overcome the problems that arise from analyses of data sets derived from a limited sample size. The algorithm assumes that genes with similar signals will demonstrate similar levels of inter-array variability (Jain *et al.* 2003; Lee and O'Connell, 2003; Jain *et al.* 2005).

The GeneChip Soybean Genome Array contains in excess of 37500 probe sets, each of which is comprised of 11 oligonucleotide probe pairs, and as the majority of these hybridize to unique sequences in the soybean genome, most mRNA hybridizations represent the mRNA abundance of specific soybean genes.

For each method, the False Discovery Rate (FDR) controlling procedure approach to multiple hypothesis testing was used, with the Benjamini Hochberg algorithm, to determine the expected proportion of true null hypotheses rejected out of the total number of null hypotheses that were tested and rejected, and an adjusted p-value (q-value) was produced for each comparison. The q-values were used to produce lists of genes differentially expressed at fold-changes of ≥ 1.6, with FDRs of ≤ 0.05. A comparison of the lists created by each statistical algorithm produced list of genes that were differentially expressed, at a statistically-significant level, and common to all 3 algorithms. Genes from that list were examined for biological relevance, given the treatment conditions.

3.2.5 Experiment 5: Foliar Application of LCO and Leaf PR Protein Activity

The total enzyme activities of 3 PR proteins: chitinase, β-1,3-glucanase and GPOX were measured for two soybean genotypes (cv. OAC Bayfield and Evans-derived line), for the first trifoliolate leaf at 0, 24, and 48 h following the application of 10^{-7} M LCO onto the first trifoliolate leaf, and at 48 h for the untreated second trifoliolate leaf.

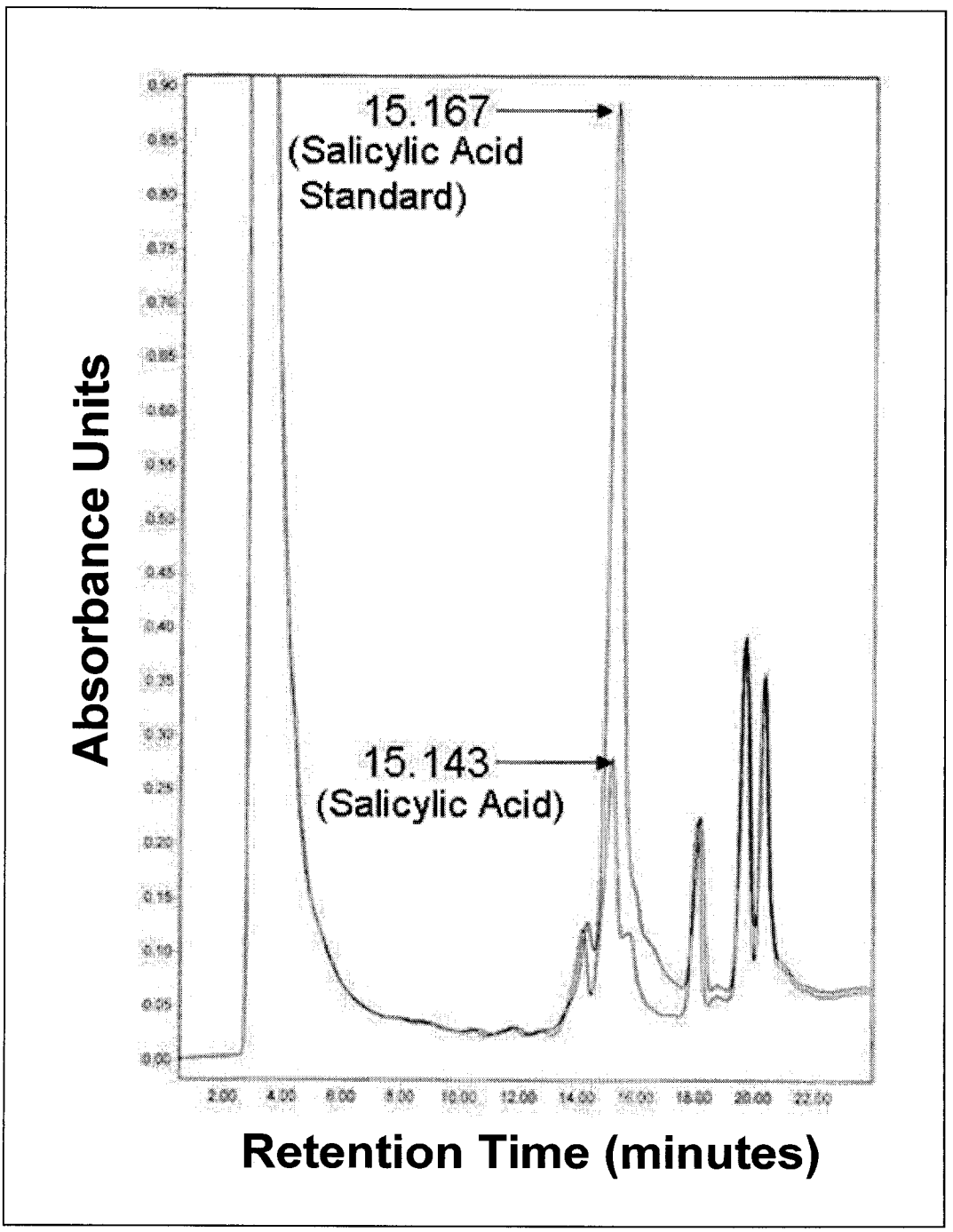
The experiment was structured following a RCBD with 8 replicates per treatment per sampling time divided equally between 2 blocks. Each block consisted of a separate growth chamber operating under similar lighting and temperature conditions. For each block, the 4 replicates were divided, into 2 and pooled to create 2 biological replicates per block, and 4 replicates per treatment per sampling time, overall, with the position of each plant was randomly assigned within the growth chambers. The GLM procedure and the

SNK nonparametric statistical tests were conducted, under the SAS System, to determine the significance of LCO treatments on total enzyme activity expression of chitinase, β-1,3-glucanase and GPOX.

Table 3.1. Primer characteristics for each primer for the genes *actin*, *IFS2*, and *PAL1*, used to amplify cDNA from soybean.

Primer Characteristics	Target Gene		
	Actin	***Isoflavone synthase 2***	***Phenylalanine ammonia-lyase 1***
Forward Primer Sequence	5′ GAGCTATGAATTGCCTGATGG 3′	5′ CGTGAGGAGGAGAAAGAACG3′	5′ TAACTGGCAGACCCAACTCC3′
Reverse Primer Sequence	5′ CGTTTCATGA-ATTCCAGTAGC 3′	5′ AGTCTGTTCCTG-CCGAGAAA 3′	5′ CCTGCATCAC-TTCAGCAAAA 3′
Forward Primer Tm (°C)	60.0	60.0	60.1
Reverse Primer Tm (°C)	57.0	60.0	60.0
Forward Primer %GC	47.6	55.0	55.0
Reverse Primer %GC	42.9	50.0	45.0
Forward Primer Reaction Concentration (nM)	450	350	250
Reverse Primer Reaction Concentration (nM)	450	450	450
Product Size (bp)	118	161	240
Gene Accession Number	U60500	AF195819	X52953
Reference	Moniz de Sa and Drouin, 1996	Jung *et al*. 2000	Frank *et al*. 1991

Figure 3.1. Two overlain HPLC chromatograms of the same sample (cv. OAC Bayfield, first trifoliolate leaf) with and without an added SA standard.


15.167
(Salicylic Acid
Standard)
15.143
(Salicylic Acid)
Absorbance Units
Retention Time (minutes)

4. Results

4.1 Effect of Foliar Application LCO on SA Levels

How the LCO signaling-molecules of rhizobia affect SA is of great interest, as it is likely that during signal transmission, rhizobia must interact with aspects of the plant's defenses, such as SA levels, for successful infection to occur. The biosynthesis of SA is thus a good target for study of the effects of LCO on soybean defenses.

4.1.1 Effect of different concentrations of LCO

The spray application on the first trifoliolate leaf of soybean plants (cv. OAC Bayfield) at either concentration of LCO (10^{-7} or 10^{-8} M) increased ($p \leq 0.05$) soybean leaf SA levels, relative to the control, at 48 h after treatment application. The increase was greatest with the 10^{-7} M treatment. No difference ($p \leq 0.05$) was found among the treatments at 0, 72, and 96 h after foliar application. Basal SA levels were observed to increase over time from 0 h to a maximum at 96 h (Figure 4.1A).

Neither LCO treatment was found to effect on root SA levels, at 0, 48, 72, or 96 h after foliar application of LCO. The root basal SA levels were observed to be substantially lower than that of the leaves, at all measurement times, and root SA levels remained relatively unchanged over the 96 h following leaf treatment (Figure 4.1B).

4.1.2 Effect of foliar application of LCO on SA levels of two soybean types

Given the result of 4.1.1, the treatment '10^{-7} M LCO' was chosen as the best concentration for use in further studies. As the effect on leaf SA levels was not seen past 48 h after foliar application, it was decided to limit the duration of further experiments to 48 h, and to expand the range of study to include a 24 h time-point. In addition, it was decided that further experiments would focus on the shoot and not include the roots, given the lack of effect on root SA levels in 4.1.1.

4.1.2A Effect of LCO on nodulating soybean

The application of 10^{-7} M LCO on the first trifoliolate leaf of cv. OAC Bayfield plants was found to induce an increase in the SA levels of the same leaf at 24 h, relative to the control. No difference ($p \leq 0.05$) was found between treatments at 0 or 48 h after

treatment, following the foliar application. Basal SA levels were observed to increase slightly over time, reaching a maximum at 48 h (Figure 4.2A).

The application of 10^{-7} M LCO on the first trifoliolate leaf of cv. OAC Bayfield plants did not increase ($p \leq 0.05$) the SA levels of the second trifoliolate leaf at 48 h after treatment. The concentration of SA in the second trifoliolate leaf was actually substantially higher than the concentration of SA in the sprayed first trifoliolate leaf of the same plant (Figure 4.2B).

4.1.2B Effect of LCO on non-nodulating soybean

The application of 10^{-7} M LCO to the first trifoliolate leaf of Evans-derived line plants was found to induce an increase ($p \leq 0.05$) in the SA levels of the same leaf at 24 h, relative to the control. No difference ($p \leq 0.05$) was found between the treatments at 0 or 48 h after the foliar application. Basal SA levels were observed to increase substantially over time, reaching a maximum at 48 h (Figure 4.3A).

The application of 10^{-7} M LCO to the first trifoliolate leaf of Evans-derived line plants was not found to induce an increase ($p \leq 0.05$) in the SA levels of the second trifoliolate leaf (Figure 4.3B).

The concentration of SA in the second trifoliolate leaf was found to be substantially lower than the concentration in the first trifoliolate leaf of the same plant at 48 h after treatment (Figure 4.3B).

The concentration of SA in both the first and second trifoliolate leaves of the Evans-derived line plants was observed to be substantially lower than the concentration in the same leaves of cv. OAC Bayfield plants in 4.1.2A (Figure 4.2).

4.2 Effect of Foliar Application of LCO on *PAL1* and *IFS2* Genes

Given the effects of the LCO treatment on soybean leaf SA levels in 4.1, it was decided to study the expression levels of two genes that encode two phenylpropanoid pathway enzymes: PAL and IFS, using QRT-PCR. The amplification plots of each gene (Appendix 1), the gene melting temperatures (Appendix 2) and the QRT-PCR efficiencies (Appendix 3) were determined during each run with the MxPro QPCR software (Stratagene, San Diego, USA). cDNA synthesized from the extracted RNA of each gene of each soybean variety was visualized on agarose gels (Appendix 4).

4.2.1 Nodulating soybean

4.2.1A Isoflavone synthase

The spray application of 10^{-7} M LCO on the first trifoliolate leaf of cv. OAC Bayfield plants resulted in an increase in the relative abundance of the *IFS2* transcript, relative to the control plants, at 0 h, and a decrease at 24 h and 48 h, following the spray treatment. However, as the results of all 3 time-points had a p-value > 0.05, these numerical increases were not statistically significant (Table 4.1A).

4.2.1B Phenylalanine ammonia-lyase

The spray application of 10^{-7} M LCO on the first trifoliolate leaf of soybean cv. OAC Bayfield plants resulted in an increase in the relative abundance of the *PAL1* transcript, relative to the control plants, at 0 h, and a decrease at 24 h and 48 h, following the spray treatment. However, as the results of all 3 time-points had a p-value > 0.05, these numerical increases were not statistically significant (Table 4.2A).

4.2.2 Non-nodulating soybean

4.2.2A Isoflavone synthase

The spray application of 10^{-7} M LCO on the first trifoliolate leaf of plants of the soybean non-nodulating line, derived from cv. Evans resulted in a decrease in the relative abundance of the *IFS2* transcript, relative to the control plants, at 0 h, a slight increase at 24 h and a slight decrease at 48 h, following the spray treatment. However, as the results of all 3 time-points had a p-value > 0.05, these numerical differences were not statistically significant (Table 4.1B).

4.2.2B Phenylalanine ammonia-lyase

The spray application of 10^{-7} M LCO on the first trifoliolate leaf of Evans-derived line plants resulted in an increase in the relative abundance of the *PAL1* transcript, relative to the control plants, at 0 h and 24 h, and a decrease at 48 h, following the spray treatment. However, as the results of all 3 time-points had a p-value > 0.05, these numerical changes were not statistically significant (Table 4.2B).

4.3 Effect of Foliar Application of LCO on Leaf Transcript Abundance

Given the effects of the LCO treatment on soybean leaf SA levels in 4.1, I decided to study the abundance of the soybean gene mRNA transcripts when soybean was exposed to 10^{-7} M LCO, using the GeneChip Soybean Genome Array technology.

4.3.1 Effect of LCO on soybean mRNA abundance

The foliar application of 10^{-7} M LCO was found to induce gene expression changes in soybean cv. OAC Bayfield at 48 h following treatment. Microarray data of 10^{-7} M LCO-treated soybean was compared with that of soybean treated with a water control. A comparison was made of the lists created by each statistical algorithm, of genes whose level of expression satisfies the two conditions of being statistically-significant at $q \leq 0.05$ and having a fold-change ≥ 1.6 ($\log_2$ 0.68). The comparison produced a list of 639 differentially expressed genes common to all 3 algorithms. All data from this experiment were deposited in the Gene Expression Omnibus gene expression/molecular abundance repository (accession number GSE9730). Of the 639 genes, 259 are unknown and share no similarity to other soybean genes or to genes of other sequenced species available for comparison. There were 322 genes whose function was unknown in soybean, but that were similar to genes of other species. The function of the remaining 58 soybean genes were either known or suggested based on similarity to other soybean genes. Genes from that list of 639 were examined for biological relevance given the treatment conditions. Based on satisfying at least 1 of 4 criteria: relation to stress, relation to salicylic acid, relation to nodulation, or similarity of function between genes, 38 genes were selected from the 639. A heatmap was constructed to graphically-depict the changes in gene expression between the two treatments for the 38 selected genes (Figure 4.4). Of the selected genes, 11+2 were grouped as relating to abiotic stress, 12+2 were grouped as relating to biotic stress (2 are universal stress proteins, shared between both the biotic and abiotic groups), 3 were related to salicylic acid, 7 were cytochrome P450s and 3 were designated as 'other'.

The $\log_2$ ratios of the expression results of the 38 genes are listed along with gene function (Table 4.3). In the abiotic stress category, 7 were upregulated in the 10^{-7} M LCO-treated soybean, and 6 genes were downregulated, including 3 distinct glutathione

S-transferase genes. In the biotic stress category, 12 genes were upregulated after treatment with 10^{-7} M LCO, and 2 were downregulated. In the salicylic acid-related category, for one gene of unknown function found to be upregulated, EST annotation noted that this EST is expressed when soybean is exposed to salicylic acid. A second gene of that category, also upregulated after treatment with 10^{-7} M LCO, was the gene encoding cinnamic acid 4-hydroxylase, an enzyme is crucial to SA biosynthesis, that catalyzes an intermediate step between Phe and SA. A third gene, downregulated in the 10^{-7} M LCO-treated soybean, is similar to an *Oryza sativa* isochorismatase hydrolase-like protein. Of the cytochrome P450 category and the last category, all 10 were upregulated after treatment with 10^{-7} M LCO. Of the 39 genes, 5 are peroxidases, 4 of which were upregulated after treatment with 10^{-7} M LCO. Genes similar to an *Arabidopsis* gene encoding a β-1,3-glucanase-like protein and a gene encoding an endo-1,4-β-glucanase precursor were found to be upregulated after treatment with 10^{-7} M LCO. Furthermore, a chitinase gene was found to be downregulated after treatment with 10^{-7} M LCO.

4.4 Effect of Foliar Application of LCO on Leaf PR Protein Activity

Given the effects of the LCO treatment on soybean leaf SA levels in 4.1, and the changes in soybean gene regulation of: 5 peroxidases, a gene similar to an *Arabidopsis* gene encoding a β-1,3-glucanase-like protein, and a chitinase gene, I decided to study soybean total enzyme activity for three PR proteins: chitinase, β-1,3-glucanase and GPOX. Enzyme activity was studied using separate colourimetric assays for each enzyme, for two different soybean genotypes (cv. OAC Bayfield and the Evans-derived line), and in two different tissue types (first trifoliolate leaf and the untreated second trifoliolate leaf) following the application of either 10^{-7} M LCO or the control treatment onto the first trifoliolate leaf.

4.4.1 Nodulating soybean

4.4.1A Chitinase

The spray application of 10^{-7} M LCO to the first trifoliolate leaf of cv. OAC Bayfield plants resulted in no difference ($p \leq 0.05$) in total chitinase activity between the 10^{-7} M LCO treatment and the control, at 0, 24, or 48 h (Figure 4.5A).

The application of 10^{-7} M LCO to the first trifoliolate leaf of cv. OAC Bayfield plants did not increase ($p \leq 0.05$) the total chitinase activity of the second trifoliolate leaf at 48 h (Table 4.4).

4.4.1B β-1,3-Glucanase

The spray application of 10^{-7} M LCO to the first trifoliolate leaf of cv. OAC Bayfield plants resulted in no difference ($p \leq 0.05$) in total β-1,3-glucanase activity between the 10^{-7} M LCO treatment and the control, at 0, 24, or 48 h (Figure 4.6A).

The application of 10^{-7} M LCO to the first trifoliolate leaf of the cv. OAC Bayfield plants did not increase ($p \leq 0.05$) the total β-1,3-glucanase activity of the second trifoliolate leaf at 48 h after treatment (Table 4.4).

4.4.1C Guaiacol Peroxidase

The spray application of 10^{-7} M LCO to the first trifoliolate leaf of cv. OAC Bayfield plants increased ($p \leq 0.05$) total GPOX activity over the control, at 48 h (Figure 4.7A). There was no difference ($p \leq 0.05$) in activity at 0 or 24 h following the spray treatment.

The application of 10^{-7} M LCO on the first trifoliolate leaf of the cv. OAC Bayfield plants increased ($p \leq 0.05$) total GPOX activity of the second, untreated trifoliolate leaf at 48 h after treatment (Table 4.4).

4.4.2 Non-nodulating soybean

4.4.2A Chitinase

The spray application of 10^{-7} M LCO to the first trifoliolate leaf of the Evans-derived line plants resulted in no difference ($p \leq 0.05$) in total chitinase activity between the 10^{-7} M LCO treatment and the control, at 0, 24, or 48 h (Figure 4.5B). The basal level of chitinase activity of the Evans-derived line plants was observed to be approximately the same as that of cv. OAC Bayfield plants (Figure 4.5A).

The application of 10^{-7} M LCO to the first trifoliolate leaf of the Evans-derived line plants did not increase ($p \leq 0.05$) the total chitinase activity of the second trifoliolate leaf at 48 h after treatment (Table 4.4).

4.4.2B β-1,3-Glucanase

The spray application of 10^{-7} M LCO to the first trifoliolate leaf of the Evans-derived line plants resulted in no difference ($p \leq 0.05$) in total β-1,3-glucanase activity between the 10^{-7} M LCO treatment and the control, at 0, 24, or 48 h (Figure 4.6B). The basal level of β-1,3-glucanase activity of the Evans-derived line plants was observed to be approximately the same as that of cv. OAC Bayfield plants (Figure 4.6A).

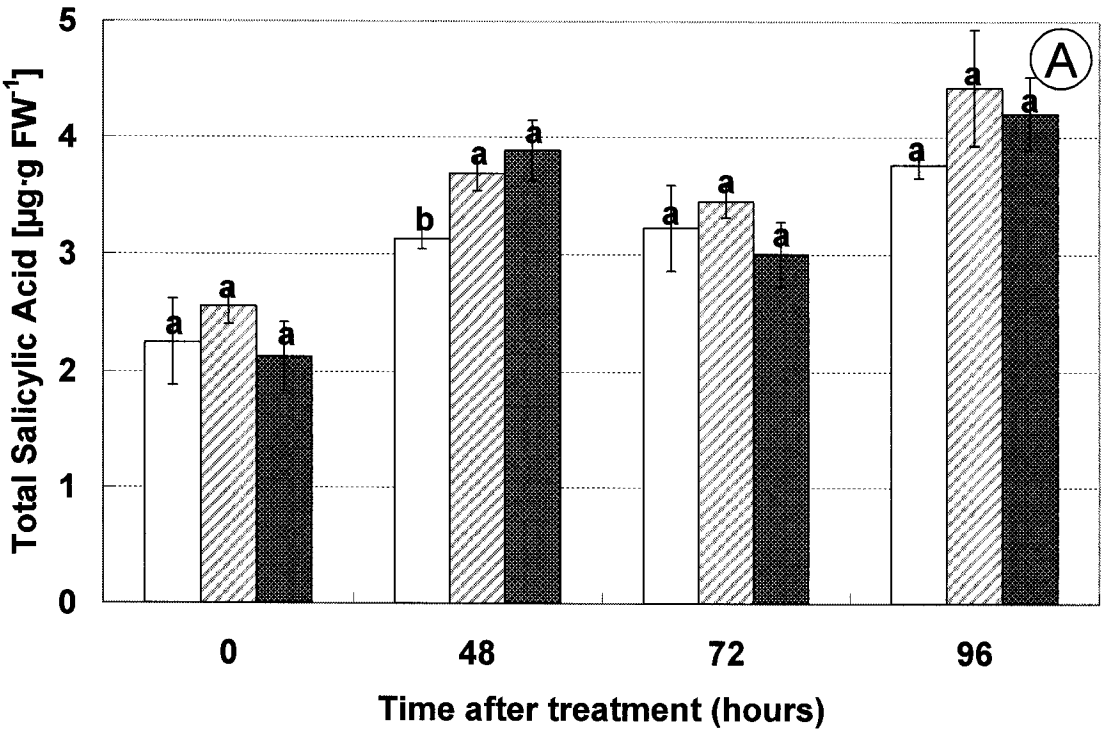
The application of 10^{-7} M LCO to the first trifoliolate leaf of the Evans-derived line plants increased ($p \leq 0.05$) total β-1,3-glucanase activity of the second trifoliolate leaf at 48 h after treatment (Table 4.4).

4.4.2C Guaiacol Peroxidase

The spray application of 10^{-7} M LCO to the first trifoliolate leaf of the Evans-derived line increased ($p \leq 0.05$) total GPOX activity over the control, at 48 h (Figure 4.7 B). There was no difference ($p \leq 0.05$) in activity at 0 or 24 h following the spray treatment. The basal level of GPOX activity of the Evans-derived line was observed to be more than 50% greater than that of cv. OAC Bayfield plants (Figure 4.7A).

The application of 10^{-7} M LCO to the first trifoliolate leaf of the non-nodulating soybean (Evans x L66-2470) increased ($p \leq 0.05$) total GPOX activity of the second, untreated trifoliolate leaf at 48 h after treatment (Table 4.4).

Figure 4.1. The concentration of total SA in the A) sprayed, first trifoliolate leaf, and B) the roots, of cv. OAC Bayfield plants. In both cases, samples were collected at 0, 48, 72, 96 h following treatment. The first trifoliolate leaf of each plant was sprayed with either a water control (white bars), or LCO 1 x 10^{-8} M (hatched bars) or LCO 1x10^{-7} M (dark bars). This experiment was repeated three times with five replicates each time and analyzed separately. Each experiment gave the same trend of significance; data from the second experiment is shown. Each time-point was analyzed separately to compensate for changes in basal levels over time. Bars followed by different letters are different at $p \leq 0.05$ by the Student-Newman Keuls (SNK) test.


A
Total Salicylic Acid [µg·g FW-1]
5
4
3
2
1
0
a
a
a
b
a
a
a
a
a
a
a
a
0
48
72
96
Time after treatment (hours)

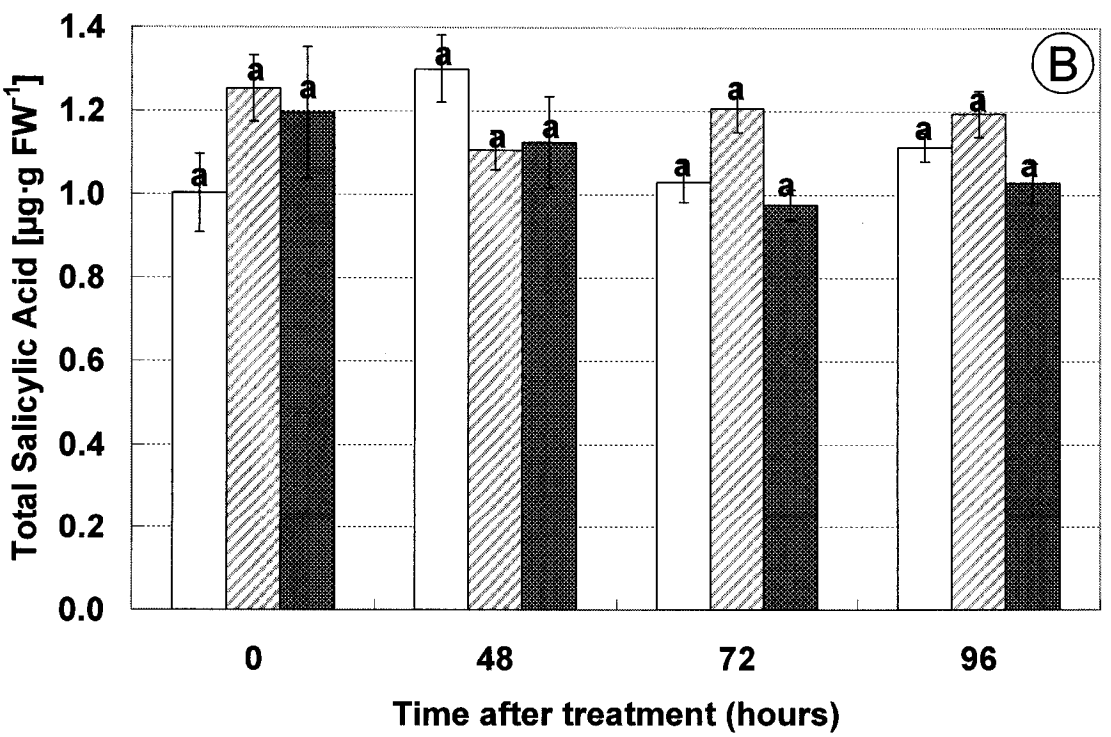
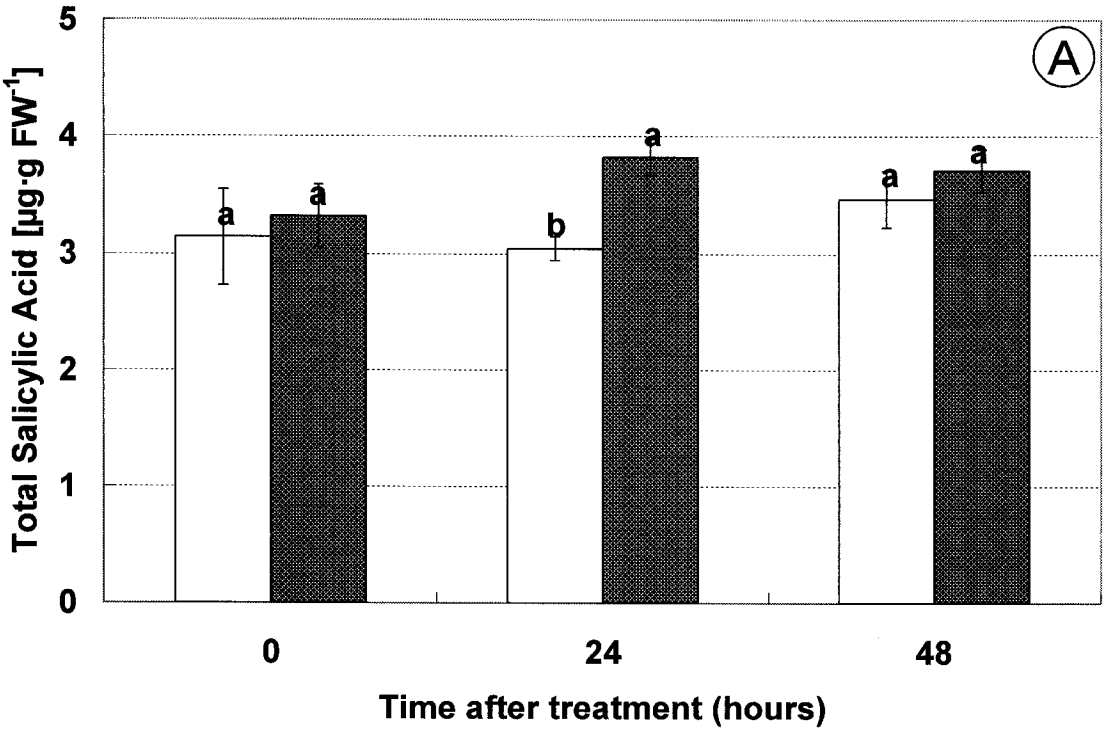

B
Total Salicylic Acid [µg·g FW-1]
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
a
a
a
a
a
a
a
a
a
a
0
48
72
96
Time after treatment (hours)

Figure 4.2. The concentration of total SA in A) the first trifoliolate leaf, and B) the second trifoliolate leaf, of cv. OAC Bayfield plants. Samples were collected at 0, 24, and 48 h following treatment for the first trifoliolate leaf, and for the second trifoliolate leaf, samples were collected at 48 h. The first trifoliolate leaf of each plant was sprayed with either: a water control (white bars), or 10^{-7} M LCO (dark bars). The second trifoliolate leaf of each plant was unsprayed. This experiment was repeated three times with four replicates measures, and data from all experiments were analyzed separately. Each experiment gave the same trend of significance; data from the second experiment is shown. Each time-point was analyzed separately to compensate for changes in basal levels over time. Bars followed by different letters are significantly different at $p \leq 0.05$ by the Student-Newman Keuls (SNK) test.


A
Total Salicylic Acid [µg·g FW-1]
5
4
3
2
1
0
a
a
b
a
a
a
0
24
48
Time after treatment (hours)

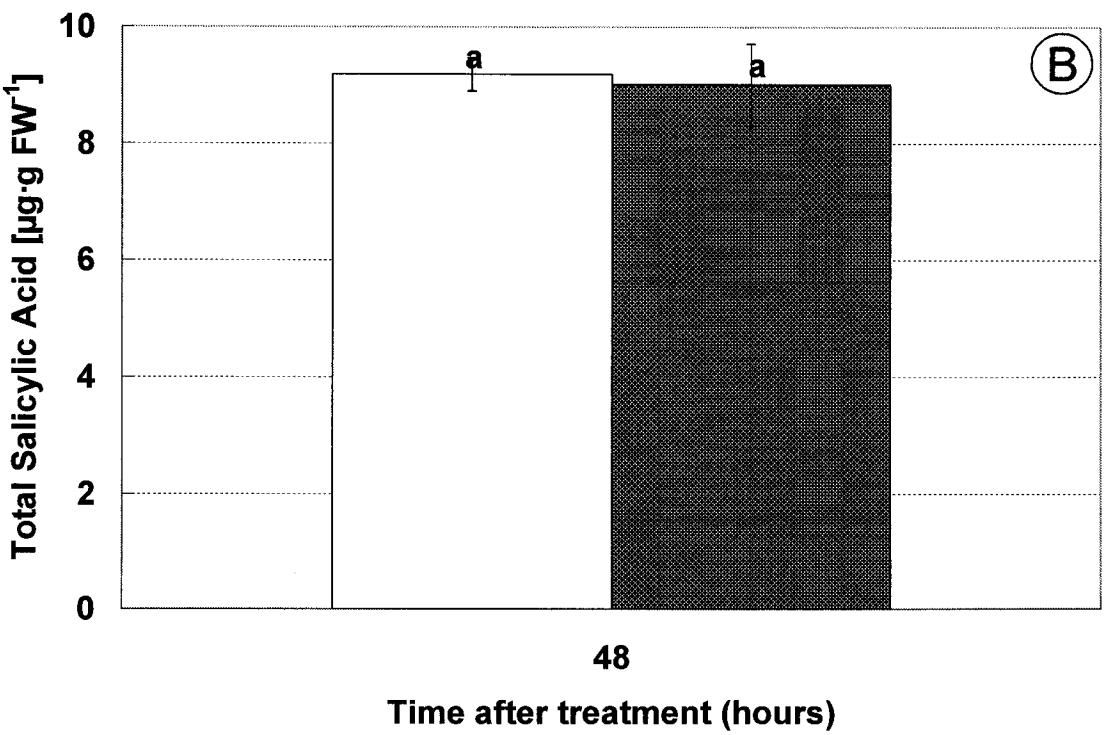
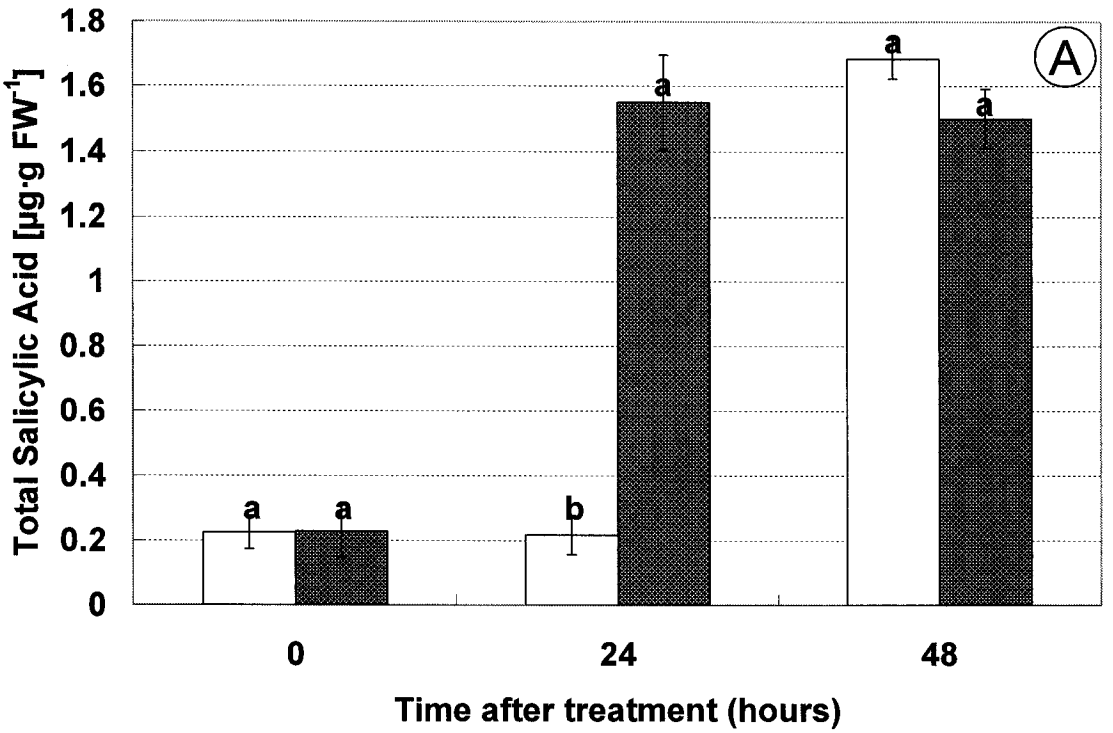

B
Total Salicylic Acid [µg·g FW-1]
10
8
6
4
2
0
a
a
48
Time after treatment (hours)

Figure 4.3. The concentration of total SA in A) the first trifoliolate leaf, and B) the second trifoliolate leaf, for plants of the non-nodulating line derived from cv. Evans, at 0, 24, and 48 h following treatment for the first trifoliolate leaf, and for the second trifoliolate leaf, samples were harvested at 48 h. The first trifoliolate leaf of each plant was sprayed with either: a water control (white bars), or 10^{-7} M LCO (dark bars). The second trifoliolate leaf of each plant was unsprayed. This experiment was repeated three times with four replicates and each experiment was analyzed separately. Each experiment gave the same trend of significance; data from the second experiment is shown. Each time-point was analyzed separately to compensate for changes in basal levels over time. Bars followed by different letters are significantly different at $p \leq 0.05$ by the Student-Newman Keuls (SNK) test.


A
Total Salicylic Acid [µg·g FW-1]
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a
b
a
a
0
24
48
Time after treatment (hours)

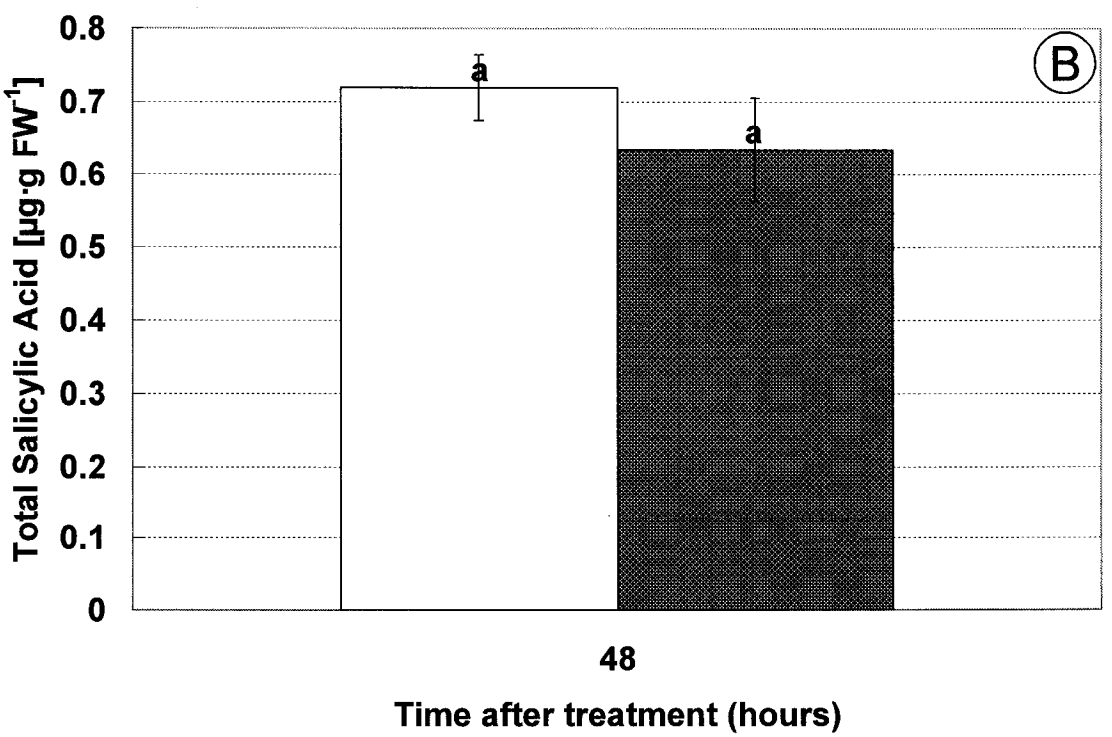

B
Total Salicylic Acid [µg·g FW-1]
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
a
48
Time after treatment (hours)

Table 4.1. The effect of LCO foliar application on the expression of the *IFS2* gene transcript relative to the water sprayed control treatment. Data are the absolute gene regulation, the $\log_2$ ratios of gene expression, and p-values for the first trifoliolate leaf of A) cv. OAC Bayfield or B) plants of the non-nodulating line derived from cv. Evans, at 0, 24, and 48 h after being sprayed with 10^{-7} M LCO. This experiment was repeated twice and analyzed separately using REST (Pfaffl, 2001; Pfaffl *et al.* 2002). Each experiment gave the same trend of significance, data from the first experiment is shown. The *IFS2* gene was determined to be significantly upregulated or downregulated relative to the control treatment at $p \leq 0.05$.

		Time after treatment (hours)		
		0	24	48
A	Absolute gene regulation	1.69	0.70	0.37
	Log_2	0.76	-0.52	-1.44
	p-Value	0.53	0.93	0.11
B	Absolute gene regulation	1.45	1.78	0.80
	Log_2	0.54	0.84	-0.33
	p-Value	0.58	0.09	0.47

Table 4.2. The effect of LCO foliar application on the expression of the *PAL1* gene transcript relative to the water sprayed control treatment. Data are the absolute gene regulation, the $\log_2$ ratios of gene expression, and p-values for the first trifoliolate leaf of A) cv. OAC Bayfield or B) plants of the non-nodulating line derived from cv. Evans, at 0, 24, and 48 h after being sprayed with 10^{-7} M LCO. This experiment was repeated twice and analyzed separately using REST (Pfaffl, 2001; Pfaffl *et al.* 2002). Both experiments gave the same trend of significance; data from the first experiment is shown. The *PAL1* gene was determined to be significantly upregulated or downregulated relative to the control treatment at $p \leq 0.05$.

		Time after treatment (hours)		
		0	24	48
A	Absolute gene regulation	1.93	0.42	0.34
	Log_2	0.95	-1.27	-1.57
	p-Value	0.54	0.52	0.13
B	Absolute gene regulation	0.62	1.02	0.96
	Log_2	-0.70	0.03	-0.06
	p-Value	0.50	0.85	0.94

Figure 4.4. A heatmap display of the $\log_2$ ratios for the expression profiles of abiotic, biotic stress-related and other probe sets of the Affymetrix GeneChip hybridizations of cDNA from the first trifoliolate leaf of cv. OAC Bayfield plants, 48 h after being sprayed with either (A) a water control, or (B) 10^{-7} M LCO. Probe sets shown in the heat map were clustered by hand to group genes according to the similarity of their reported or hypothesized function. Each row represents a probe set on the Soybean Genome and each column represents a replicate of one of the two treatments. White boxes represent upregulated gene expression of a particular sample in comparison to the samples of the other treatment, black boxes represent downregulated gene expression of a particular sample in comparison to the samples of the other treatment and gray boxes represent no change in gene regulation. Intense colours represent a greater fold change. This heatmap was constructed using the Heatmap Builder (King *et al.* 2005).

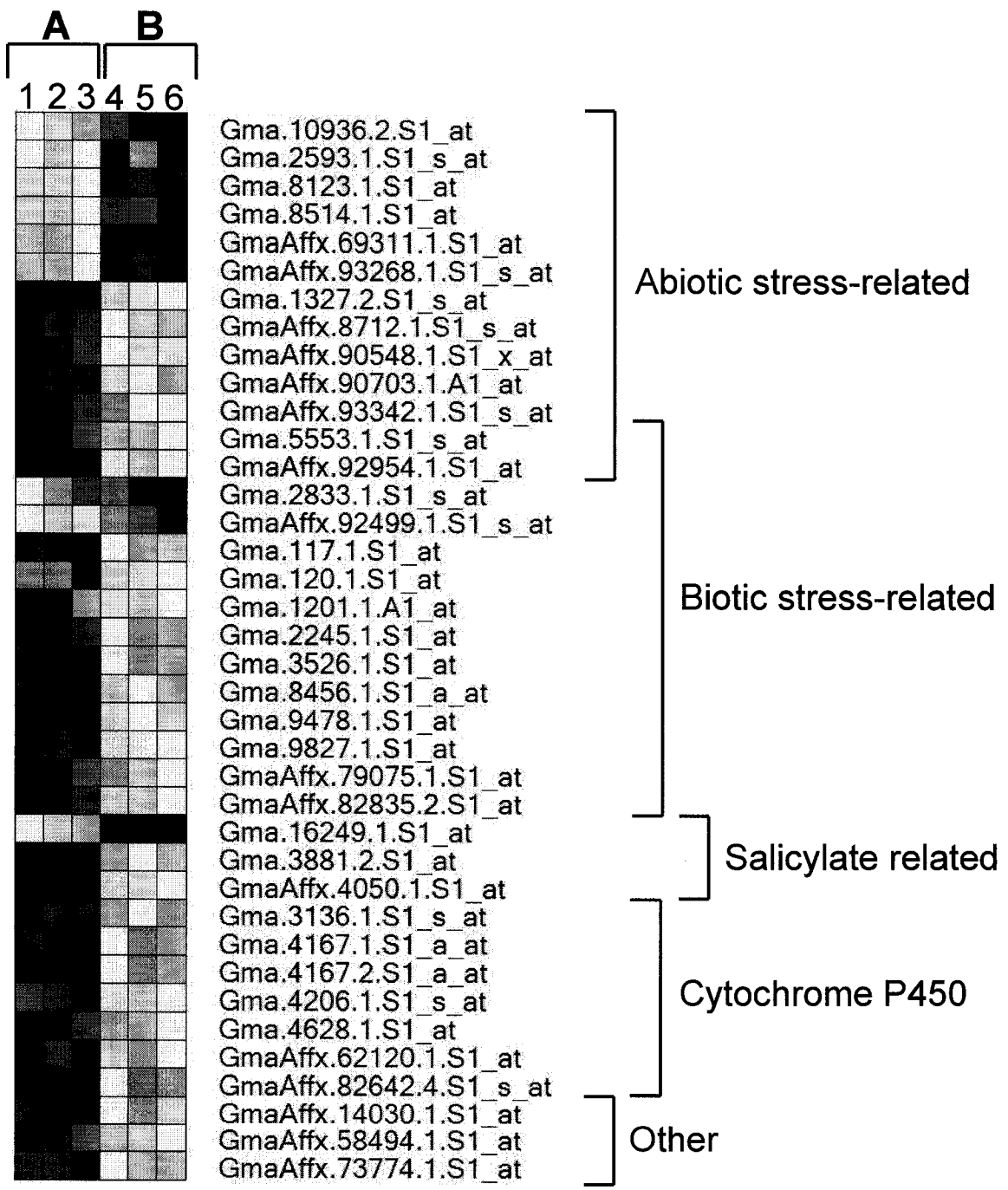

A
B
1 2 3 4 5 6
Gma.10936.2.S1_at
Gma.2593.1.S1_s_at
Gma.8123.1.S1_at
Gma.8514.1.S1_at
GmaAffx.69311.1.S1_at
GmaAffx.93268.1.S1_s_at
Gma.1327.2.S1_s_at
GmaAffx.8712.1.S1_s_at
GmaAffx.90548.1.S1_x_at
GmaAffx.90703.1.A1_at
GmaAffx.93342.1.S1_s_at
Gma.5553.1.S1_s_at
GmaAffx.92954.1.S1_at
Gma.2833.1.S1_s_at
GmaAffx.92499.1.S1_s_at
Gma.117.1.S1_at
Gma.120.1.S1_at
Gma.1201.1.A1_at
Gma.2245.1.S1_at
Gma.3526.1.S1_at
Gma.8456.1.S1_a_at
Gma.9478.1.S1_at
Gma.9827.1.S1_at
GmaAffx.79075.1.S1_at
GmaAffx.82835.2.S1_at
Gma.16249.1.S1_at
Gma.3881.2.S1_at
GmaAffx.4050.1.S1_at
Gma.3136.1.S1_s_at
Gma.4167.1.S1_a_at
Gma.4167.2.S1_a_at
Gma.4206.1.S1_s_at
Gma.4628.1.S1_at
GmaAffx.62120.1.S1_at
GmaAffx.82642.4.S1_s_at
GmaAffx.14030.1.S1_at
GmaAffx.58494.1.S1_at
GmaAffx.73774.1.S1_at
Abiotic stress-related
Biotic stress-related
Salicylate related
Cytochrome P450
Other

Table 4.3. Gene expression results of the log_2 fold change ratios of the probe sets for the first trifoliolate leaf of cv. OAC Bayfield plants, 48 h after being sprayed with 10^{-7} M LCO or water, for abiotic and biotic stress-related, and other genes. Log_2 ratios are for 10^{-7} M LCO-treated soybean, relative to water control-treated soybean. *Q*-values are the adjusted *p*-values determined using False Discovery Rates. A *q*-value ≤ 0.05 was considered significantly different.

Probeset tested	Log2 Ratio	*Q*-value	Gene Function	Soybean sequence similar to:	Notes
Gma.10936.2.S1_at	-2.30	0.02	-	NP_181674.1[a]	ERD15 (Early responsive to dehydration) 15, peroxidase-like protein
Gma.2593.1.S1_s_at	-1.59	0.04	glutathione S-transferase 15	n/a	-
Gma.8123.1.S1_at	-2.03	0.00	glutathione S-transferase 25	n/a	-
Gma.8514.1.S1_at	-1.83	0.02	glutathione S-transferase 18	n/a	-
GmaAffx.69311.1.S1_at	-1.13	0.01	-	NP_912354.1[b]	putative class I low-molecular-weight heat shock protein
GmaAffx.93268.1.S1_s_	-1.24	0.01	-	NP_912354.1[b]	putative class I low-molecular-weight heat shock protein
Gma.1327.2.S1_s_at	9.25	0.00	-	Q08298[a]	RD22_ARATH Dehydration-responsive protein RD22 precursor
GmaAffx.8712.1.S1_s_a	1.40	0.02	-	NP_196153.1[a]	peroxidase
GmaAffx.90548.1.S1_x_	1.54	0.00	-	NP_200076.1[a]	HSP81-1 (Heat Shock Protein 81-1)
GmaAffx.90703.1.A1_at	1.15	0.02	-	NP_196153.1[a]	peroxidase
GmaAffx.93342.1.S1_s_	2.06	0.04	-	NP_002076.2[c]	glutathione peroxidase 4 isoform A precursor
Gma.5553.1.S1_s_at	0.97	0.04	-	XP_475357.1[b]	putative universal stress protein (USP)
GmaAffx.92954.1.S1_at	1.44	0.00	-	XP_479478.1[b]	universal stress protein USP1-like protein
Gma.2833.1.S1_s_at	-1.07	0.03	chitinase class I	n/a	-
GmaAffx.92499.1.S1_s_	-1.79	0.05	Win gene encoding wound-induced protein	n/a	-
Gma.117.1.S1_at	1.79	0.01	syringolide-induced protein 19-1-5	n/a	-
Gma.120.1.S1_at	3.29	0.05	syringolide-induced protein B13-1-9	n/a	-
Gma.1201.1.A1_at	1.80	0.05	function unknown	n/a	differentially expressed in soybean - soybean cyst nematode interactions
Gma.2245.1.S1_at	1.14	0.04	-	T47682[a]	beta-1,3-glucanase-like protein
Gma.3526.1.S1_at	3.33	0.01	-	NP_194311.1[a]	xyloglucan endo-1,4-beta-D-glucanase (XTR-6)
Gma.8456.1.S1_a_at	2.18	0.01	resistance protein KR3	n/a	-
Gma.9478.1.S1_at	1.36	0.00	-	NP_568634.1[a]	peptidase/ subtilase
Gma.9827.1.S1_at	3.25	0.00	resistance protein KR4	n/a	-
GmaAffx.79075.1.S1_at	1.48	0.05	endo-1,4-beta-glucanase precursor	n/a	Cel2
GmaAffx.82835.2.S1_at	1.57	0.00	-	NP_197045.1[a]	DND1 (Defense No Death) 1
Gma.16249.1.S1_at	-1.33	0.00	-	XP_466634.1[b]	isochorismatase hydrolase-like protein
Gma.3881.2.S1_at	1.04	0.02	cinnamic acid 4-hydroxylase	n/a	CYP73
GmaAffx.4050.1.S1_at	0.82	0.00	function unknown	n/a	EST expressed when soybean is exposed to 2.0M salicylic acid
Gma.3136.1.S1_s_at	2.24	0.02	cytochrome P450-like protein	n/a	-
Gma.4167.1.S1_a_at	3.81	0.03	cytochrome P450 monooxygenase	n/a	CYP707A16
Gma.4167.2.S1_a_at	3.96	0.03	cytochrome P450 monooxygenase	n/a	CYP707A16
Gma.4206.1.S1_s_at	2.09	0.00	cytochrome P450 peroxidase	n/a	H2O2-dependent urate-degrading
Gma.4628.1.S1_at	0.87	0.05	cytochrome P450 monooxygenase	n/a	CYP76O2
GmaAffx.62120.1.S1_at	1.04	0.03	cytochrome P450 monooxygenase	NP_180997.1[a]	CYP710A1
GmaAffx.82642.4.S1_s_	3.04	0.04	cytochrome P450 monooxygenase	n/a	CYP707A16
GmaAffx.14030.1.S1_at	1.24	0.03	-	XP_482640.1[b]	putative glycine-rich cell wall structural protein
GmaAffx.58494.1.S1_at	1.72	0.04	-	XP_481036.1[b]	nodulin-like protein
GmaAffx.73774.1.S1_at	1.11	0.02	-	NP_705452.1[d]	cell division control protein 2 homolog

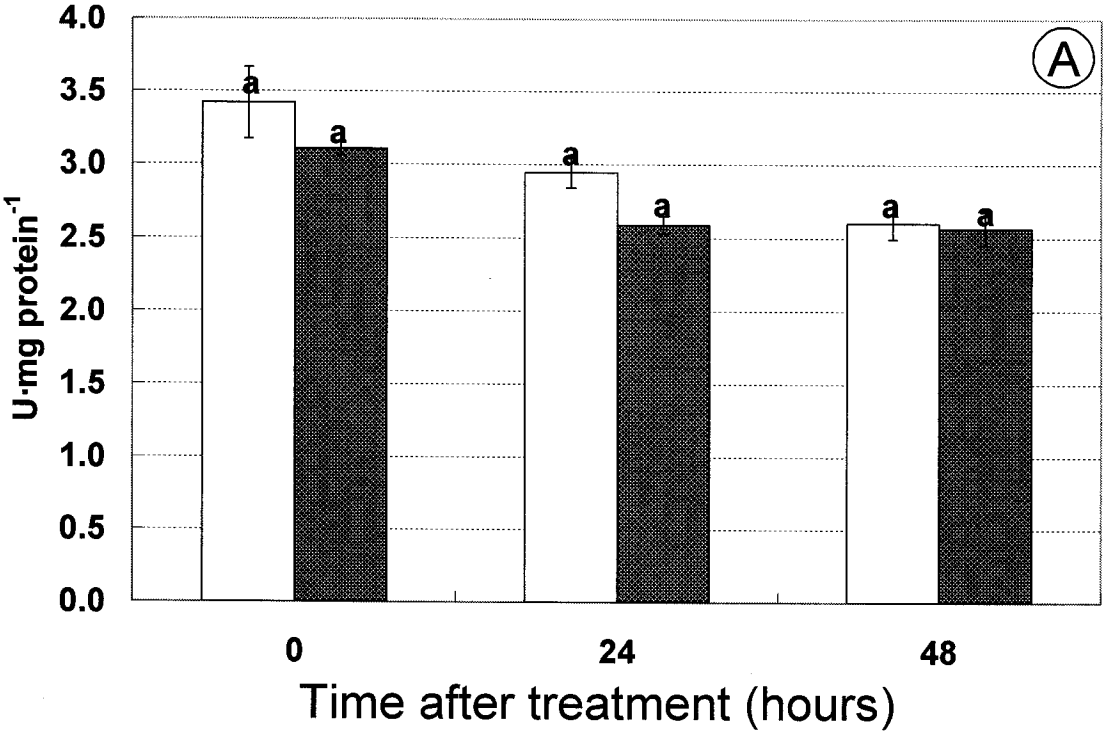
[a] protein sequence from *Arabidopsis thaliana*

[b] protein sequence from *Oryza sativa* (*japonica* cultivar-group)

[c] protein sequence from *Homo sapiens*

[d] protein sequence from *Plasmodium falciparum* 3D7

Figure 4.5. The total chitinase activity of the first trifoliolate leaf of A) cv. OAC Bayfield plants and of B) Evans-derived line plants. In both cases, samples were collected at 0, 24, and 48 h following treatment. The first trifoliolate leaf of each plant was sprayed with either: a water control (white bars), or 10^{-7} M LCO (dark bars). This experiment was repeated twice with four replicates, and data from both experiments were analyzed separately. Both experiments gave the same trend of significance; data from the first experiment is shown. Each time-point was analyzed separately to compensate for changes in basal levels over time. Bars followed by different letters are significantly different at $p \leq 0.05$ by the Student-Newman Keuls (SNK) test. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 µmol of GlcNAc per hour.


A
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
U·mg protein-1
a
a
a
a
a
a
0
24
48
Time after treatment (hours)

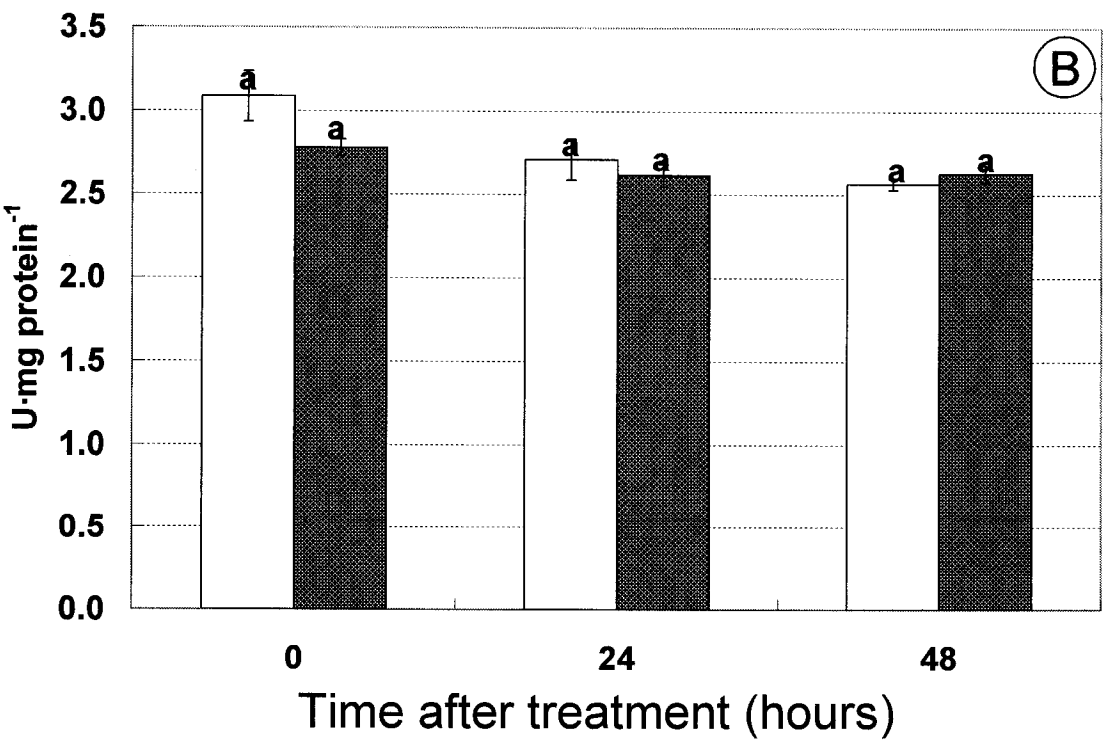
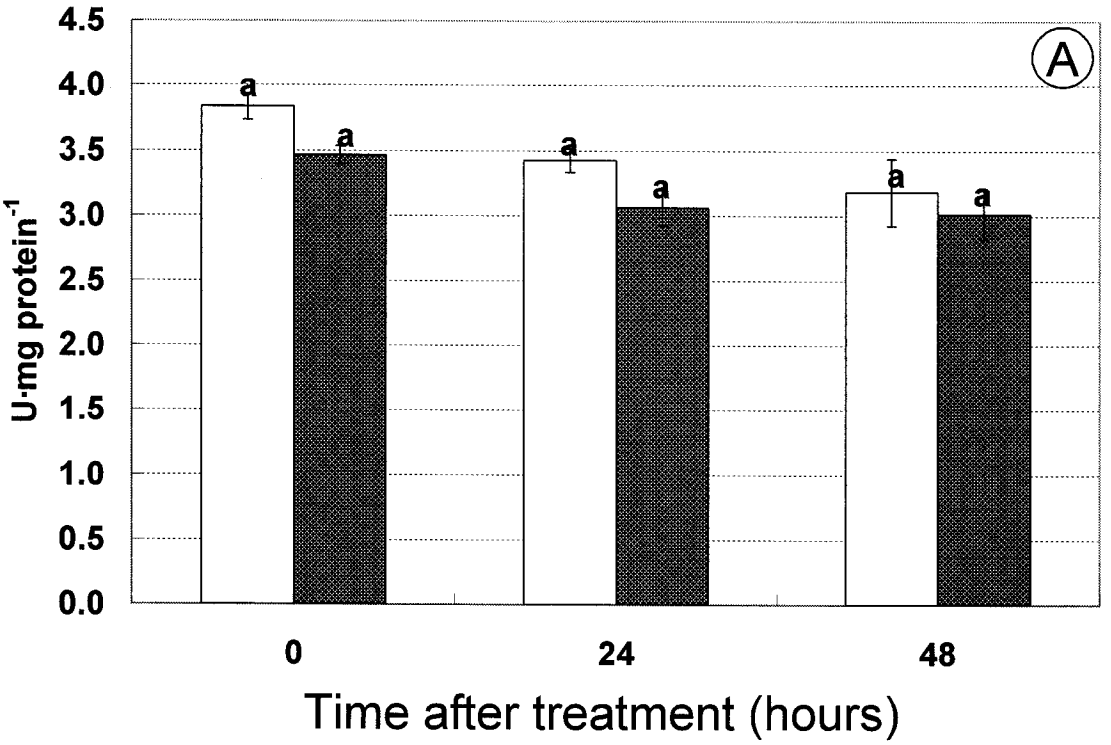

B
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
U·mg protein-1
a
a
a
a
a
a
0
24
48
Time after treatment (hours)

Figure 4.6. The total β-1,3-glucanase activity of the first trifoliolate leaf of A) cv. OAC Bayfield plants and of B) Evans-derived line plants. In both cases, samples were collected at 0, 24, and 48 h following treatment. The first trifoliolate leaf of each plant was sprayed with either: a water control (white bars), or 10^{-7} M LCO (dark bars). This experiment was repeated twice with four replicates, and data from both experiments were analyzed separately. Both experiments gave the same trend of significance; data from the first experiment is shown. Each time-point was analyzed separately to compensate for changes in basal levels over time. Bars followed by different letters are significantly different at $p \leq 0.05$ by the Student-Newman Keuls (SNK) test. One unit of β-1,3-glucanase activity was defined as the amount of enzyme that liberated 1 μmol of glucose per hour.


A
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4.0
3.5
3.0
2.5
2.0
1.5
1.0
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0.0
U·mg protein-1
a
a
a
a
a
a
0
24
48
Time after treatment (hours)

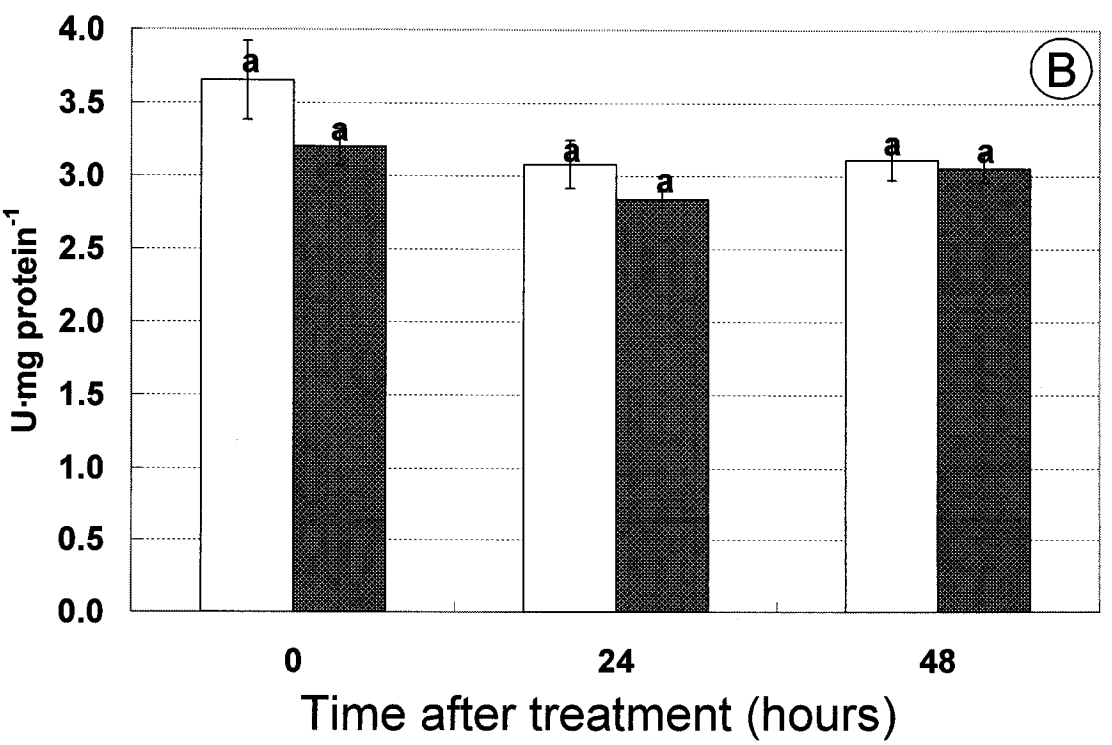
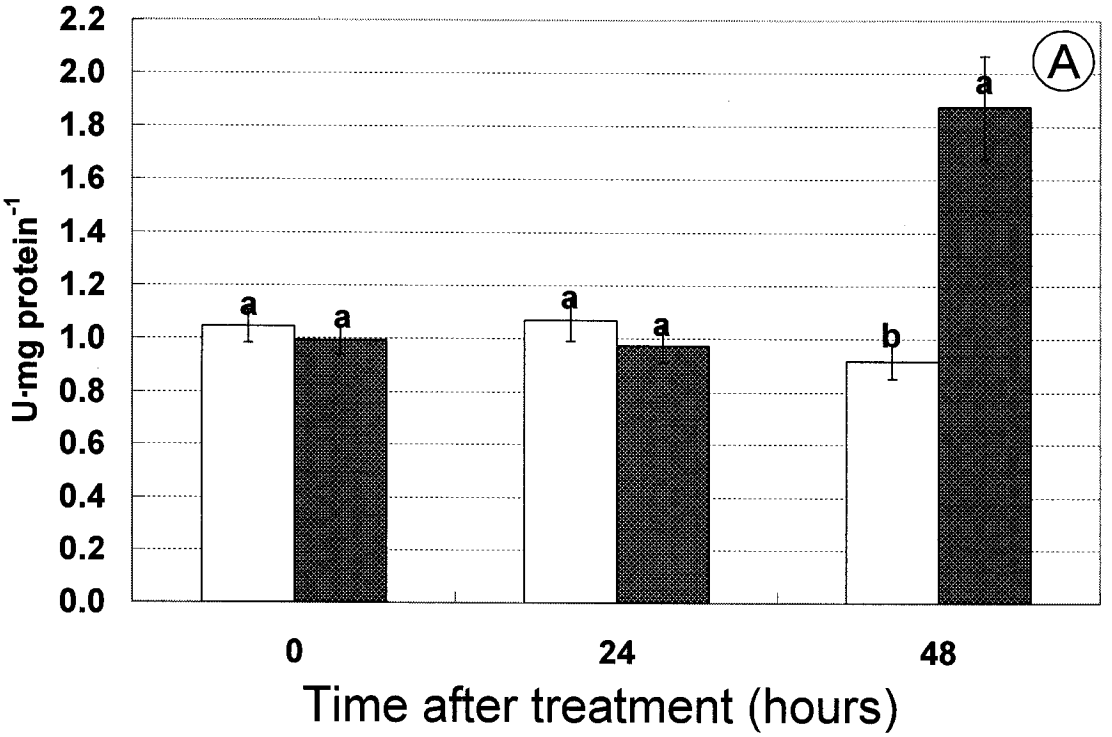

B
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
U·mg protein-1
a
a
a
a
a
a
0
24
48
Time after treatment (hours)

Figure 4.7. The total GPOX activity of the first trifoliolate leaf of A) cv. OAC Bayfield plants, and of B) Evans-derived line plants. In both cases, samples were collected at 0, 24, and 48 h following treatment. The first trifoliolate leaf of each plant was sprayed with either: a water control (white bars), or 10^{-7} M LCO (dark bars). This experiment was repeated twice with four replicates, and data from both experiments were analyzed separately. Both experiments gave the same trend of significance; data from the first experiment is shown. Each time-point was analyzed separately to compensate for changes in basal levels over time. Bars followed by different letters are significantly different at $p \leq 0.05$ by the Student-Newman Keuls (SNK) test. One unit of GPOX activity was defined as the amount of enzyme that produced 1 µmol of tetraguaiacol per minute.


A
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
U·mg protein-1
a
a
a
a
b
a
0
24
48
Time after treatment (hours)

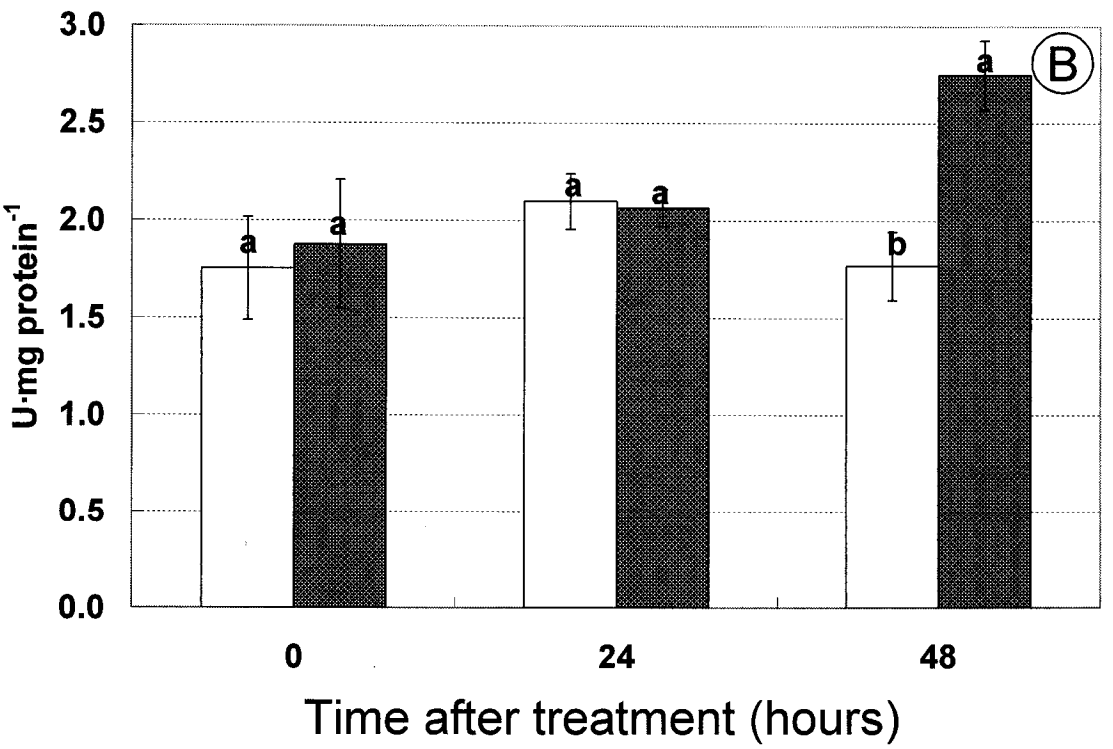

B
3.0
2.5
2.0
1.5
1.0
0.5
0.0
U·mg protein-1
a
a
a
a
b
a
0
24
48
Time after treatment (hours)

Table 4.4. The total enzyme activities of chitinase, β-1,3-glucanase, and GPOX of the second trifoliolate leaf of cv. OAC Bayfield plants and of Evans-derived line plants. Samples were collected at 48 h following treatment of the first trifoliolate leaf. The first trifoliolate leaf of each plant was sprayed with either: a water control (white bars), or 10^{-7} M LCO (dark bars). This experiment was repeated twice and data from both experiments were analyzed separately. Both experiments gave the same trend of significance; data from the first experiment is shown. Each time-point was analyzed separately to compensate for changes in basal levels over time. Different letters within rows indicate significant differences ($p \leq 0.05$) according to the Student-Newman Keuls (SNK) test. Enzyme activity for all the three enzymes is expressed as U·mg protein^{-1}.

One unit of chitinase activity was defined as the amount of enzyme that liberated 1 µmol of GlcNAc per hour.

One unit of β-1,3-glucanase activity was defined as the amount of enzyme that liberated 1 µmol of glucose per hour.

One unit of GPOX activity was defined as the mount of enzyme that produced 1 µmol of tetraguaiacol per minute.

Soybean Type / Enzyme Activity *	Treatments	
	Control	LCO 10^{-7}M
cv. OAC Bayfield		
Chitinase	**6.17**[a] ± 0.47	**6.28**[a] ± 0.24
ß-1,3-Glucanase	**6.74**[a] ± 0.33	**6.53**[a] ± 0.51
GPOX	**3.56**[b] ± 0.60	**6.31**[a] ± 0.47
Evans x L66-2470		
Chitinase	**6.01**[a] ± 0.44	**6.09**[a] ± 0.59
ß-1,3-Glucanase	**6.54**[a] ± 0.43	**6.41**[a] ± 0.32
GPOX	**3.17**[b] ± 0.35	**4.99**[a] ± 0.51

* Activity for all enzymes is expressed as U•mg protein^{-1}

5. Discussion

I have studied the changes induced by LCO produced by the rhizobial soybean symbiont *B. japonicum* strain 532C on the soybean defense-response hormone, SA and some of the genes that could code for enzymes involved in its synthesis, as well as the activity of some PR proteins that can be altered by SA. Evidence is provided that LCO, at both 10^{-7} and 10^{-8} M concentrations, has caused a rapid, transient increase in SA levels in the treated leaf 24-48 h after treatment, when applied to the first trifoliolate leaf. This effect was limited to the treated leaf and was not observed to be transduced to either roots or the untreated second trifoliolate of plants, despite SA being a key compound in the plant defense response phenomena generally known as SAR. The relative gene expression of the *IFS2* gene, involved in a branch of the phenylpropanoid pathway leading to isoflavonoid synthesis, was found to be unaffected by LCO treatment. A second gene, *PAL1*, involved in a second branch of the same pathway, and known to play a key role in the synthesis of SA, was also unaffected. Using microarrays, a number of nodulating soybean genes were found to be differentially expressed as a result of the LCO treatment, including a number of abiotic and biotic stress-related genes, as well as some salicylic acid biosynthesis-related genes. Of the three PR proteins that were studied, chitinase and β-1,3-glucanase were unaffected by the treatment, but GPOX activity was found to be strongly increased both locally and distally to the site of the LCO treatment, revealing that LCO has a systemic effect on the plant. GPOX is known to play a role in plant growth and development, and it may play a part in the growth-promoting effects observed for LCO.

It has been shown that *Medicago sativa* microcallus suspensions treated with *S. meliloti* LCO have an elevated number of S (synthesis) phase cells and an increase in kinase activity (Savoure *et al.* 1994). They proposed that LCO may lead to the production of certain biochemical effectors that disturb the hormonal balance of the plant by interfering with endogenous plant hormones that may be required for mitosis. SA has since been shown to induce mitogen-activated protein kinases (MAPK), known to have various mitogenic and cytokinetic properties in tobacco cell suspension cultures (Zhang and Klessig, 1997). It is likely that the increase in S phase cells and kinase activity

observed by Savoure *et al.* (1994) was due to the phenomenon studied by Zhang and Klessig (1997), given that we have now shown that soybean leaves, when treated with LCO, have an increased level of the plant hormone SA, and it is possible that the mitogenic properties of LCO described previously (Schmidt *et al.* 1988; Schlaman *et al.* 1997) are due to increases in SA caused by LCO.

5.1 Salicylic Acid

The role of SA in the major plant defense response SAR has been studied extensively, and increases in SA have been linked to plant resistance to a number of bacterial and fungal pathogens (Malamy *et al.* 1990; Metraux *et al.* 1990; Narusaka *et al.* 1999; Kubota and Nishi, 2006). Additional cell suspension experiments with *Medicago* have shown that LCO elicit a defense response, including the biosynthesis of the plant phytoalexin medicarpin and increased PR gene expression (Savoure *et al.* 1997), and a study on soybean seedling roots infected with powdery mildew and treated with LCO showed increased disease resistance (Duzan *et al.* 2005). Thus it is possible that the increase in SA observed in this study is also a form of defense response by the plant. While the increases due to LCO are transient, it is possible that the increase is a temporary 'pulse', and is all that is required by the plant to reorganize certain aspects of the metabolism, at least for a short period. Had a pathogen been present, the increase may have been more prolonged, given that a pathogenic attack results in chronic exposure to signal compounds, such as chitin fragments, rather than an acute one, as with the LCO application used in this work. The presence of a pulse in leaf SA at 48 h in the first experiment, and a shift to 24 h in the second experiment can be attributed to refining of the techniques used to apply the treatment. Greater efficiency and speed in the application of the treatment could have exposed the leaves to a greater concentration of LCO earlier on, which would have resulted in the pulse being detected at an earlier time-point.

Arachidonic acid has been shown to induce SAR in potato plants: following foliar application to the lower leaves, potato plants developed a resistance to *P. infestans* that was also conferred on higher, untreated leaves (Cohen *et al.* 1991). A subsequent study found that, for potato plants under similar conditions, treated leaves showed an increase

in SA, and that the untreated leaves, though resistant to a subsequent pathogenic attack, showed no increase in SA, suggesting that AA induces SAR systemically, whereas SA synthesis is induced only at the site of application (Coquoz *et al.* 1995). LCO may induce SA synthesis in a way similar to AA, given that in this study the foliar application of LCO resulted in only the local accumulation of SA and not a systemic accumulation. The SA of higher, untreated leaves in soybean was unaffected by the foliar application of LCO on lower, treated leaves (Figures 4.2; 4.3). Similarly, there was no change in soybean root SA levels when the first trifoliolate leaf of the plants was treated with either 10^{-7} or 10^{-8} M LCO (Figure 4.1). This is consistent with other studies involving the accumulation of SA, which found that root application of compatible rhizobia did not alter root SA levels (Blilou *et al.* 1999). Kubota and Nishi (2006) found that, for cucumber, when lower leaves were infected with *Colletotrichum lagenarium*, the treated leaves accumulated far more SA than did higher, uninfected leaves. Furthermore, a study of SA effects on nodule formation in determinate and indeterminate nodulating plants found that the inhibitory effects of SA on indeterminate nodule formation were not systemic, while nodulation in legumes that form determinate nodules was unaffected by the SA (van Spronsen *et al.* 2003). Narusaka *et al.* (1999) found that, when applied exogenously on the leaves of cucumber plants, SA induces resistance to *Cladosporium cucumerinum*, but only for the treated leaves and not untreated leaves. If increased SA levels in treated leaves do not lead to the accumulation of SA elsewhere in the plant, as found by Narusaka *et al.* (1999), it is therefore possible that the effect of LCO would not result in the accumulation of SA in plant tissue aside from in the treated leaf. The SA content of the roots in nodulating soybean was only 25-50% that of the first trifoliolate leaf, and was relatively unchanged over time. This is consistent with pea, where the SA levels of the roots were relatively unchanged at 72 h after inoculation with rhizobia (Blilou *et al.* 1999).

Although in this study the application of LCO was foliar, rather than at the rhizosphere, given previous findings in foliar application of LCO, and that many plant *nodulin* genes are expressed in tissues other than the roots (Journet *et al.* 2001), it is not unreasonable to suspect that the LCO receptor in the roots might have a homologous receptor in the rest of the plant. The rhizobial symbiote might have suborned the receptor

in the root tissue at some point in the evolution of the symbiotic interaction, diverting the receptor from its original task for the express purpose of initiating plant responses to LCOs. In the rest of the plant the receptor may be performing its original purpose, for example, initiating a broad response to a potential invading organism.

In this study, it was found that the SA levels of younger leaves (the second trifoliolate) of cv. OAC Bayfield plants, while unaffected by LCO foliar application to older leaves, had higher basal levels of SA than did the older leaves. The SA level of the younger leaf was more than 2-fold higher than the SA level of the older, treated leaf, even after SA accumulation is induced by the foliar LCO treatment. This is consistent with the results of Coquoz *et al.* (1995), where older potato leaves accumulated SA in response to an elicitor, while younger, untreated leaves were unaffected. In that study, the younger leaves were found to contain, overall, higher levels of SA than older leaves, both prior to, and following, the elicitation of SA accumulation in lower leaves. Coquoz *et al.* (1995) correlated resistance in potato leaves to the *P. infestans* pathogen with the level of SA present in those leaves, thus it would seem that younger leaves were either naturally resistant to the pathogen, or that the younger leaves would not benefit from an increase in SA, given the already high levels. This could be an explanation for the similar pattern of SA levels observed in this study.

In this study it was found that over 96 h, the SA level in the first trifoliolate leaf doubles in the control plants, indicating that SA might play a role in leaf development, as during that time the first trifoliolate leaf continues to expand (data not shown). As both LCO and SA have been shown to have mitogenic properties and, at least in the case of LCO, growth promoting effects in legumes (Schmidt *et al.* 1988; Schlaman *et al.* 1997; Prithiviraj *et al.* 2003), it is possible that the increase in growth is because of a transient increase in SA that precedes the natural increases in SA in growing leaf tissue. Given that younger, immature tissue had higher levels of SA (this study; Coquoz *et al.* 1995), it is plausible that SA is involved in leaf maturation, possibly by inducing mitosis and cytokinesis via MAP kinase proteins.

It was also observed in this study that the pattern in SA levels of older and younger leaves of nodulating soybean is reversed in the non-nodulating soybean. The level of SA in the younger, untreated leaf of the non-nodulating line was only 50% that of

the level in the older, treated leaf below, whereas for the nodulating cultivar the younger leaf had double the SA as that of the older, treated leaf. While these two genotypes are not isolines, and I was not able to sample a population of each soybean type (nodulating and non-nodulating) so that I cannot draw conclusions regarding nodulating and non-noduling genotypes, these results are intriguing and merit further investigation. In addition, throughout the time-course experiment, the non-nodulating line had substantially lower basal levels of SA than the nodulating cv. OAC Bayfield, although this may be due to other genetic differences between the two soybean types, and not related to the mutant line's inability to nodulate. It is worth noting, however; that the first trifoliolate leaf of the non-nodulating line underwent an 800% increase in SA, relative to the control, in response to the 10^{-7} M LCO treatment, compared to an increase of only 30% in the nodulating cultivar. As these two types of soybean are not isolines of the same genotype, it is difficult to draw conclusions based on comparisons between the two, but the magnitude of the difference in response to the rhizobial LCO signaling molecule could indicate why one type can nodulate and the other cannot. The relatively greater increase in leaf SA in the non-nodulating soybean may indicate that it perceives the LCO signal as a pathogenic attack rather than as the signal of a symbiont. If so, this could mean that the metabolic machinery still exists to nodulate, but that the mutant is unable to discriminate between friend and foe, blocking entry of both. Certainly, at some point in the evolution of legumes, rhizobia would have had to overcome the defenses of the host, potentially after a mutation that prevented the host from recognizing the symbiont as a pathogen, or a mutation might have occurred that enabled the host to control the infection and utilize the bacteria for its own purposes.

5.2 Isoflavone Synthase

The enzyme IFS catalyzes one step of a branch of the phenylpropanoid pathway that leads to the biosynthesis of flavonoids such as isoflavones, some of which are converted into phytoalexins. This branch of the pathway is different from the one that leads to SA biosynthesis, and is comprised of many steps. The biochemical step catalyzed by IFS is between the steps catalyzed by the enzymes chalcone synthase (CHS) and chalcone reductase (CHR) and the step catalyzed by isoflavone reductase (IFR).

CHS and CHR catalyze the conversion of p-coumaroyl-CoA to flavones, and IFS catalyzes the conversion of flavones into compounds leading to the biosynthesis of isoflavones. IFR catalyzes a step down-stream from the step catalyzed by IFS, and uses a derivative of isoflavones as its substrate. *Medicago sativa* microcallus suspensions treated with *S. meliloti* LCO have been shown to have increased expression of the *IFR* gene (Savoure *et al.* 1994) and the *CHS* and *CHR* genes (Savoure *et al.* 1997), suggesting the possible involvement of IFS in the plant response to LCO treatment.

Kneer *et al.* (1999) found that treating *Lupinus luteus* with SA resulted in the rhizosecretion of the isoflavonoid genistein, and Al-Tawaha *et al.* (2005) found that spraying various LCOs on the leaves of soybean resulted in an increase in isoflavone concentration of soybean seeds (including genistein concentration), and Savoure *et al.* (1997) found an increased synthesis of the phytoalexin medicarpin (a derivative of isoflavonoids) in *Medicago* cells treated with LCO. Collectively, these three studies suggest that either LCO or the effects of LCO (such as increased SA accumulation) lead to increased biosynthesis of isoflavonoids. However, in this study we were unable to find a biologically significant effect on relative expression of the *IFS2* gene as a result of foliar LCO treatment, for either nodulating or non-nodulating soybean. It is therefore possible that the rate-limiting step of the biosynthesis of isoflavonoids is not the one catalyzed by IFS2, which would explain the effects of LCO and/or SA on isoflavonoid biosynthesis without observing an accompanied increase in the gene expression of *IFS2*. Another explanation is that as at least two separate IFS genes exist; LCO application may have effected the isoform not examined in this study (*IFS1*), although given that *IFS2* is much more abundant in the leaf than *IFS2* (Subramanian *et al.* 2004), and that of the 2 genes, *IFS2* appears to be more responsive to stress (Dhaubhadel *et al.* 2003), a change in *IFS1* rather than *IFS2* is unlikely. Given the increases observed by Savoure *et al.* (1997) in the *CHS* and *CHR* gene expression, and that no effect was found on *IFS2* gene expression in my work, it is possible that the rate-limiting step of this branch of the flavonoid biosynthesis pathway is, instead, the conversion of coumaroyl-CoA to trihydroxychalcone, catalyzed by CHS and CHR. Alternatively, given that Al-Tawaha *et al.* (2005) used a higher concentration of LCO (10^{-6} M) than was used in my work (10^{-7} M), it is possible that a 10^{-7} M concentration of LCO is sufficient to induce a transient

increase in SA, but that it is not sufficient to induce *IFS2* gene expression. Had a higher concentration been used, it is possible that it would have induced *IFS2* gene expression. It would also be worth assessing the quantity and activity of the IFS2 enzyme after treatment with LCO, as the lack of a change at the transcription level does not necessarily exclude changes at the enzyme level.

Transforming rice with the *IFS* gene allows it to produce genistein, and genistein produced in this way has the potential to induce *nod* genes in several types of rhizobia (Sreevidya *et al.* 2006). We found that the non-nodulating soybean used in this study has the *IFS2* gene, and that this gene was expressed at levels comparable to that of the nodulating soybean, showing that at least part, if not all, of the metabolic machinery required for the biosynthesis of isoflavonoids exists in the non-nodulating mutant.

5.3 Phenylalanine Ammonia-Lyase

For SA synthesized by the phenylalanine pathway, the rate-limiting step is the conversion of Phe to CA, catalyzed by the enzyme PAL (Coquoz *et al.* 1998). In this study, we examined relative expression of the *PAL1* gene following foliar application of LCO, after having found that the same treatment results in an increase in soybean leaf SA. Although an increase was observed in the *PAL1* gene transcript at 0 h, and a decrease was observed at 24 and 48 h, these differences were not statistically significant. The lack of an effect on *PAL1* gene expression may be due to the transient nature of the LCO-driven SA increase (seen 24 h after treatment). A significant effect of LCO treatment on the *PAL1* gene may be occurring between 0 and 24 h after treatment, between 24 h and 48 h, or after 48 h. Alternatively, whereas *PAL1* gene transcript abundance was not affected, PAL1 enzyme activity or quantity may be affected, and bears further study.

Non-nodulating soybean plants were found to have the *PAL1* gene transcript, which is expressed at a level, relative to the *actin* gene, similar to that of nodulating soybean. This indicates that this part of the phenylpropanoid pathway is intact and that a mutation in the *PAL1* gene resulting in little or no expression of PAL can most likely be ruled out as the cause for the inability of this line to nodulate.

An alternative possibility is that the SA accumulation following LCO treatement that I have demonstrated in this study may, instead, be due to SA biosynthesis via a pathway that does not use Phe as an intermediate, and thus does not require PAL activity. If SA that accumulates following LCO treatment was produced via the isochorismate pathway, recently shown to be required for SA biosynthesis under certain conditions (Wildermuth *et al.* 2001), then a lack of effect on *PAL1* gene expression would be expected. Wildermuth *et al.* (2001) found that biosynthesis of SA via isochorismate (a derivative of the shikimate pathway) is required in *Arabidopsis* for both systemic and local acquired resistance. In a study of the induction of SA accumulation via the elicitor AA, Coquoz *et al.* (1998) were unable to account for the accumulation of SA, as it occurred even when the phenylpropanoid pathway for SA biosynthesis was inhibited. Wildermuth *et al.* (2001) suggested that the SA could be synthesized from shikimate via isochorismate. AA has been shown to induce SAR in cucumber (Cohen *et al.* 2001), but generates only local SA accumulation (Coquoz *et al.* 1995). Given that LCO has been shown to elicit a defense response in treated plants, including the biosynthesis of antibiotic phytoalexins in *Medicago* cell suspension cultures (Savoure *et al.* 1997), and that SA accumulation from LCO occurs only at the site of treatment, it is possible that LCO elicits a plant response similar to that of AA. As SA accumulation from AA has been shown to occur even when PAL is inhibited, biosynthesis of SA could be from isochorismate; it is also possible that LCO causes SA to accumulate via biosynthesis from isochorismate. It has also been found that while transforming tobacco or corn with IFS can result in the marginal production of isoflavonoids (Yu *et al.* 2000), only by inhibiting the branch of the phenylpropanoid pathway responsible for the biosynthesis of anthocyanins (this branch and the one leading to the biosynthesis of the isoflavone genistein both utilize naringenin as a precursor), can biosynthesis of isoflavonoids be substantially increased (Yu *et al.* 2003). This suggests that it may not be possible for two branches of the phenylpropanoid pathway to be up-regulated at the same time, possibly because they would compete for the same precursors. Similarly, it is plausible that, given the increases observed in the levels of isoflavonoids and phytoalexins following LCO or SA treatment (Savoure *et al.* 1997; Kneer *et al.* 1999; Al-Tawaha *et al.* 2005), a simultaneous increase in SA accumulation, as a result of the treatment of a legume with

LCO (as observed in this study), could not be produced via the phenylalanine pathway, and that SA that accumulates in this case may be produced by an alternate pathway from an alternative precursor, such as isochorismate.

We had attempted to quantify expression of the isochorismate synthase gene (*ICS1*), responsible for the conversion of chorismate to isochorismate, but found that at least one other gene was amplified at the same time. This may have been the second ICS gene (soybean is known to have at least two), but even when a) the annealing temperature was raised, b) new primers were selected, and c) the number of cycles of amplification were increased, both the ICS1 gene and the other gene were amplified (data not shown).

5.4 Soybean Transcrupt Abundance

Given the connection in plants between SA and stress, investigating soybean gene expression in the context of stress-related genes I believed that this area was likely to yield interesting results. Using microarrays, I was able to quantify soybean mRNA transcript abundance in nodulating soybean first trifoliolate leaf tissue, 48 h after being sprayed with 10^{-7} M LCO. Thirty-eight of the genes that were significantly upregulated or downregulated were selected based on relation to either: stress, SA, or nodulation. That the majority of the stress-related genes were upregulated after exposure to LCO is interesting and consistent with the observed increase in SA. This supports thinking that LCO treatment induces a stress-response in the plant. As the division between abiotic or biotic-related genes upregulated by the LCO treatment is approximately equal, the stress-response induced by LCO is probably a general one, rather than a response targeted, for example, against a specific type of pathogen.

Of the stress-related genes that were affected, five encoded peroxidases, one was a gene encoding a β-1,3-glucanase-like protein, one was a gene for an endo-1,4-β-glucanase precursor and one was a chitinase class 1 gene. This is interesting as a number of PR proteins are peroxidases (such as guaiacol peroxidase), and β-1,3-glucanase, endo-1,4-β-glucanase and chitinase are also PR proteins. That they are all affected by the treatment is consistent with the observed increase in SA, as there is a close connection between SA and PR protein activity. It is therefore likely that at least some of the genes affected by the LCO treatment are being upregulated or downregulated as a direct result

of the transient increase in SA. Interestingly, 3 separate glutathione S-transferase genes were significantly downregulated in the 10^{-7} M LCO-treated nodulating soybean, and are thus, as a class, a good candidate for further study on the effects of LCO on soybean, given their known function as a detoxification mechanism in plants in the event of oxidative stress (Dixon *et al.* 2002). That so many stress-related genes are differentially-expressed as a result of the LCO treatment, with the majority being upregulated, lends support to the idea that LCOs might confer upon soybean some sort of resistance to various stresses, if applied prior to the onset of stress, and therefore bears further study.

In addition to various stress-related genes that, as a result of the LCO treatment, were differentially expressed, three genes related to SA biosynthesis or accumulation, were also observed to be differentially expressed. A gene with an unknown function, but known to be expressed when soybean is exposed to an exogenous application of salicylic acid, was found to be upregulated in the 10^{-7} M LCO-treated plants. This supports our data showing an increase in SA, following the LCO treatment, and suggests that the plant perceives in a similar way SA that is either synthesized internally, or SA that is applied exogenously.

After treatment with 10^{-7} M LCO, the mRNA transcripts of the gene encoding the enzyme cinnamic acid 4-hydroxylase were significantly more abundant relative to the control treatment. As cinnamic acid 4-hydroxylase is responsible for catalyzing the conversion of CA to SA, this suggests that, despite the lack of effect on *PAL1* gene expression, SA accumulation is nevertheless occurring, at least in part, via the phenylpropanoid pathway. A second gene, similar to one encoding an isochorismatase hydrolase-like protein, was found to be significantly downregulated following LCO treatment. This is relevant as isochorismatase hydrolase converts isochorismate into 2,3-dihydro-2,3-dihydroxybenzoic acid, thereby competing directly with the enzyme (currently unknown in plants) responsible for converting isochorismate into SA. A decrease in isochorismatase hydrolase would make more isochorismate available to the plant, thus this may be indicative of an increase in conversion of isochorismate to SA. If this were true, it is possible that the SA increase observed in this study may be due to biosynthesis from both pathways known to produce SA.

Another pattern that emerged from the microarray data was the upregulation in the LCO-treated plants of 7 cytochrome P450 genes. Cytochrome P450s are monooxygenases involved in a wide array of plant reactions and functions, including peroxidation, apoptosis, and deamination. Why genes encoding this diverse class of protein might be upregulated is unclear, but it suggests that either cytochrome P450s, as a class, are susceptible to modulation via exposure to LCO, or that the 7 genes encode proteins of similar function, and these are somehow related to the plant's response to exposure to LCO.

Three other genes of interest were found to be upregulated after treatment with LCO. The first was a gene similar to an *Oryza sativa* gene that encodes a nodulin-like protein, suggesting that the plant perceives the LCO as the signaling molecule of its symbiote, and may be turning-on nodulin genes in preparation for expected root colonization. The second was a gene similar to an *Arabidopsis* gene that encodes a cell division protein. This is interesting as LCOs have been shown to possess certain mitotic abilities (Schlaman *et al.* 1997), and the upregulation of this gene in soybean could be indicative of the potential for occurance of such a phenomenon in treated soybean. The third gene, a putative cell wall structural protein, supports the notion that LCO treatment may induce cell division in plants, as this gene may be upregulated during cell division.

5.5 Pathogenesis-Related Proteins

SA is a major component of SAR, which is characterized by local and systemic activation of a large number of diverse PR proteins. It has been shown that the exogenous application of SA induces PR genes (White, 1979), and that an increase in SA is coupled with an increase in certain PR proteins (Malamy *et al.* 1990). In addition, the results of the microarray experiment found genes encoding the PR proteins β-1,3-glucanase, endo-1,4-β-glucanase, chitinase, and 5 different peroxidases to be differentially expressed as a result of the LCO treatment. For this reason and because I found, in this study, that the exogenous application of LCO to soybean leaves increased endogenous SA levels, it was decided to investigate the potential effects of this increase on the activity of three different PR proteins: chitinase, β-1,3-glucanase and GPOX. Chitinase and β-1,3-glucanase were selected because the LCO molecule contains both a chitin and an

oligosaccharide component, made of simple sugars, and research has shown that chitinases can be elicited in rice by treatment with chitooligosaccharide molecules (Inui *et al.* 1996). As plants can detect the chitin and β-1,3-glucan components of fungal cell walls, it is of interest to know whether soybean leaves would perceive, and respond to, the LCO molecule as it might to the structural components of the cell wall of a potentially pathogenic fungus.

As LCO have been shown to affect the biosynthesis of various products of the phenylpropanoid pathway, such as isoflavonoids (Al-Tawaha *et al.* 2005) and SA (this study), a good target for further study of the effects of LCO on SA-mediated or pathogen-related responses would be the PR protein GPOX, as it is connected to the lignification of plant cell walls and lignins are another end-product of the phenylpropanoid pathway. Peroxidases of this type have been implicated in cross-linking of glycoproteins in cell walls, wherein it is the enzyme most directly involved, and in the plant response to pathogenic attack, especially with regard to lignification (the cause of plant cell rigidity). This slows pathogen infiltration by making the cell walls of infected cells more rigid (Lewis and Yamamoto, 1990; Polle *et al.* 1994; Sahoo *et al.* 2007).

The activities of both chitinase and β-1,3-glucanase were found to be unaffected by the LCO treatment, relative to the control, in the treated first trifoliolate leaf of both nodulating (Figure 4.5A, 4.12A) and non-nodulating soybean plants (Figure 4.5B, 4.12B), at all three sampling times. The activity of both enzymes was measured for the second, untreated, leaf to measure for possible systemic effects, but LCO treatment also failed to affect the levels of these enzymes in this leaf (Table 4.4). However, the basal activities of these two enzymes were much higher in the second trifoliolate leaf than the the first. This is consistent with the levels of SA in the first and second trifoliolate leaves (Figure 4.2A, B): higher levels of leaf PR protein activity are associated with higher levels of leaf SA. The fact that the LCO-driven increase in leaf SA does not lead to an increase in chitinase or β-1,3-glucanase suggests that the plant defense response is able to distinguish between the beneficial signaling molecule of rhizobia and the structural components of a pathogenic fungus.

In this study, it was found that LCO treatment increased the activity of GPOX increased by close to two-fold over the control, in the first trifoliolate leaf of the

nodulating soybean, and by more than 50% over the control, in the first trifoliolate leaf of the non-nodulating soybean, 48 h after the leaf was sprayed with 10^{-7} M LCO. Although the LCO effect on leaf SA was observed only at 24 h and not at 48 h, the SA increase would have had to have precedeeded the increase in PR protein activity.

The observation that GPOX activity in the first trifoliolate increased following LCO treatment, presumably due to the increase in leaf SA, and that chitinase and β-1,3-glucanase activities did not increase, suggest that the plant response to LCO is complicated and specific. Given the roles that GPOX plays in plant lignification, especially in response to pathogenic attack, the results of this study are consistent with the known effects of increased levels of SA and the resulting altered regulation of PR proteins.

The GPOX activity of the untreated, second trifoliolate leaf of each plant of each line was also measured and the 10^{-7} M LCO treatment of the lower, first trifoliolate leaf resulted in more than a 75% increase in GPOX activity in the second leaf, relative to the control for nodulating soybean, and more than a 60% increase in the second leaf for non-nodulating soybean (Table 4.4). As the increase occurred in the leaf above the treated leaf, it seems that the LCO treatment induced a systemic response in soybean. Although the LCO effect on SA level was limited to the applied leaf and was not itself systemic, it has been shown that SA, while critical for SAR, it is not the transduced signal (Vernooij *et al.* 1994; Narusaka *et al.* 1999), thus a local increase in SA is nevertheless able to induce broader systemic effects. Interestingly, while basal levels of GPOX activity are greater in the second trifoliolate leaf, the magnitude of response is greater in the first trifoliolate leaf (>100% vs >75%). For nodulating soybean, this could indicate that either the increase in GPOX activity is greater at the site of LCO treatment than at a distal site, or that because the basal level of GPOX activity is higher in the second trifoliolate leaf that there is less capacity for increase. For non-nodulating soybean the pattern was reversed, with a slight increase in GPOX activity for the second trifoliolate leaf. This discrepancy could be due to the fact that there was a divergence of pattern between the two soybean types in SA levels of older and young leaves. The control plants of nodulating soybean had a higher level of SA in the younger, second trifoliolate, relative to the older, first trifoliolate, whereas in non-nodulating soybean, the reverse was true. As

the nodulating and non-nodulating soybean lines are different in genetic aspects other than just the ability to nodulate, it is difficult to make conclusive comparisons, although this might indicate why the mutant line is unable to nodulate: while the proper metabolic mechanisms for SA biosynthesis exist, hormonal responses to LCO may not function in the same way as in nodulating soybean genotypes. That the LCO effect induces systemic effects on the soybean is to be expected, as Prithiviraj *et al.* (2003) and Olah *et al.* (2005) observed growth increases for the entire plant. The increase in SA and the systemic induction of PR protein activity suggests that the LCO treatment may be inducing SAR within the soybean, and bears further study.

The systemic increase in GPOX activity as a result of treatment of the lower, first trifoliolate with 10^{-7} M LCO was also observed in the second trifoliolate of the non-nodulating soybean. The increase was more than 50%, relative to the control. This is substantially less than the increase observed in the second trifoliolate of the nodulating plant (>75%). However, that non-nodulating soybean responds with a systemic increase in PR protein activity that is generally similar to the nodulating soybean, suggests that both soybean types are very similar in their biochemical responses to the LCO treatment. This is unlikely to be the key difference between the two that makes the non-nodulating type unable to enter into symbiosis with *B. japonicum.*

6. Conclusions

In this study, I have shown that the foliar application of LCO to the first trifoliolate leaf of young soybean plants causes a transient increase in SA, occurring at 24 h after treatment. While SA was found to accumulate in control plants, and eventually reached the level of SA in the treated plants, LCO treatment resulted in an earlier accumulation of SA. This increase could account for the mitogenic abilities of LCO, given that SA has been shown to induce MAP kinases which have mitogenic properties. The LCO-driven increase in SA is limited to treated leaves, and does not result in a systemic increase in SA elsewhere in the plant, including the younger, second trifoliolate leaf or in the roots. This may, in part, be due to the fact that, for nodulating soybean, the second trifoliolate leaf has SA levels twice as high as that of the first trifoliolate leaf; similar studies involving cucumber found that not only did the younger leaves have naturally higher levels of SA, but that those levels were unaffected by an elicitor that caused the accumulation of SA in the older, treated leaves.

Given that the transient increase in SA accumulation was not accompanied by a systemic increase, and that expression of both the *PAL1* and *IFS2* genes was unaffected by LCO treatment, it is possible that the SA accumulation observed in this study was the result of biosynthesis from the isochorimsate pathway, with isochorismate as the precursor, and not via the phenylalanine pathway. However, given that the microarray experiment showed the gene encoding cinnamic acid 4-hydroxylase is upregulated after LCO treatment, the increase in SA could still be synthesized from the phenylalanine pathway, but as a result of an increase in cinnamic acid 4-hydroxylase and not PAL.

While direct comparisons cannot really be made between the nodulating OAC Bayfield cultivar and the mutant non-nodulating Evans-derived line, the magnitude of increase in SA accumulation as a result of LCO treatment was much greater in the non-nodulating line, relative to the control, than was the increase in the nodulating cultivar, even though the nodulating cultivar was found to have substantially higher levels of SA overall. Furthermore, whereas in the nodulating soybean the second trifoliolate leaf had much higher levels of SA than did the first trifoliolate leaf, this pattern was reversed in

the non-nodulating plant. These differences between the two types of soybean may be related to the inability of the mutant to nodulate.

The SA increase following LCO treatment resulted in the local, differential expression of 25 stress-related genes in nodulating soybean. That the majority of those genes were upregulated in the LCO-treated plants suggests that the effects of LCO might be even more pronounced under stressed conditions, so that LCO-treated plants might fare better under stress than untreated controls. A local and systemic increase in GPOX activity was observed in both the nodulating and non-nodulating soybean types. These increases could help to explain the effects of LCO on early plant growth, given the role GPOX is thought to play in plant growth and development. Chitinase and β-1,3-glucanase were unaffected by LCO treatment in both types of soybean, suggesting that soybean does not perceive the chitin or the glucose components of the LCO molecule to be the cell wall of a pathogenic fungus: alternatively, the plant could be responding as though the chitin components were those of a symbiotic mychorrhizal fungus. Furthermore, the activity of all three PR proteins was found to be considerably higher in the second, younger trifoliolate leaf, than in the first trifoliolate leaf. Further study is needed to elucidate the nature of the LCO effect on legumes, but these current findings show a strong effect that connects soybean hormone level, gene expression, and PR protein activity, and as a result has advanced the understanding of rhizobial-plant signaling and of the plant defense response. This work suggests that LCO treatment could be used to help crop plants deal with various stresses, for instance, those associated with climate change or the rigours of the northern Canadian growing season.

7. Acceptance or Rejection of Hypotheses

Hypothesis 1:

Foliar application of LCO changes SA levels.

Results related to hypothesis 1: In this study, it was found that when sprayed with LCO, the amount of SA of the treated leaf increased significantly at 24 h relative to the control. Thus, we **accept hypothesis 1.**

Hypothesis 2:

Foliar application of LCO will change *IFS2* and *PAL1* gene expression.

Results related to hypothesis 3: The LCO treatment did not result in a significant change in either *IFS2* or *PAL1* gene expression at any time-point. Thus, we **reject hypothesis 2.**

Hypothesis 3:

Foliar application of LCO results in the differential expression of stress-related soybean genes.

Results related to hypothesis 3: Twenty-five different stress-related genes of the first trifoliolate leaf were found to be differentially-expressed 48 h following treatment with LCO. Thus, we **accept hypothesis 3.**

Hypothesis 4:

Foliar application of LCO results in a change in local and systemic PR protein levels.

Results related to hypothesis 4: The activity of one of the PR proteins, GPOX, increased significantly both locally, and distal to, the site of treatment. Thus, we **accept hypothesis 4.**

8. Suggestions for Future Research

To expand on the work reported here, and to elucidate the role of the lipo-chitooligosaccharide on the plant defense response and metabolism, the following research remains to be done:

1. Expand the sampling time between 0 and 24 h to observe the rate of increase of SA, and to expand the sampling time between 24 h and 48 h to observe the rate of increase of GPOX activity.

This study examined the soybean response at three different timepoints, covering 48 hours. Expanding the sampling time to include more timepoints would enable us to ascertain when precisely the effect on SA and PR protein activity is first observable.

2. Determine the relative gene expression profile of soybean isochorismate synthase (ICS), in response to the LCO treatment.

Despite our best efforts, we were unable to quantify the relative gene expression of ICS2 via QRT-PCR without simultaneously amplifying another gene. Furthermore, an HPLC-quality isochorismate standard is currently unavailable for purchase, so we were unable to monitor the consumption of isochorismate as SA is produced via HPLC. Solving either of these two problems would facilitate the study of the ICS gene and SA biosynthesis via the isochorismate pathway.

3. Repeating these experiments for the two parents of the non-nodulating line, to determine which of the elements of the response to LCO may be due to genotype, and which of the elements are due to the line's inability to nodulate.

The two types of soybean studied in this experiment were good targets for examining different soybean genotypes, however; to draw conclusions based on the difference in response of the non-nodulating line and attribute these differences to its' inability to nodulate, the two parent lines, Evans and L66-2470, must be obtained and tested.

4. Determine the effect of the LCO treatment on the activity of other proteins.

The effect of LCO on three distinct PR proteins was studied in this work. Given that an increase in PR protein activity generally follows SA accumulation in plants, it is therefore likely that activity of other PR proteins, such as ascorbate peroxidase or cellulase, and/or

other antioxidant proteins such as superoxide dismutase, may be induced as a result of the LCO treatment. In addition, determining the effect of LCO, or lack thereof, on activity of PAL and IFS would confirm the results reported here, that the LCO treatment does not affect either of those two enzymes.

5. Use real-time PCR to confirm the effect of LCO on the cinnamic acid 4-hydroxylase gene.

As a result of the LCO treatment, an increase was observed in the transcript abundance of the enzyme cinnamic acid 4-hydroxylase, critical to SA biosynthesis via the phenylalanine pathway. Performing QRT-PCR on this soybean gene would confirm that it is upregulated as a result of the LCO treatment.

6. Use microarrays to observe changes in soybean gene expression at 0 h and 24 h following LCO application.

This study examined soybean gene expression in response to the foliar application of LCO, at 48 h. Repeating this experiment at the 0 h and 24 h time-points would enable us to observe changes in gene expression over time, and determine when the effects of LCO on gene expression can first be seen.

7. Apply a chronic exposure of LCO to the soybean leaves via daily treatments, and perhaps mimic a pathogenic attack situation.

This work was focused on the study of the transient effects of a one-time application of LCO to the first trifoliolate leaf of soybean. A chronic, daily exposure to the signaling molecule may engender a response of greater magnitude, or induce different effects than the single application of LCO, and must therefore be studied.

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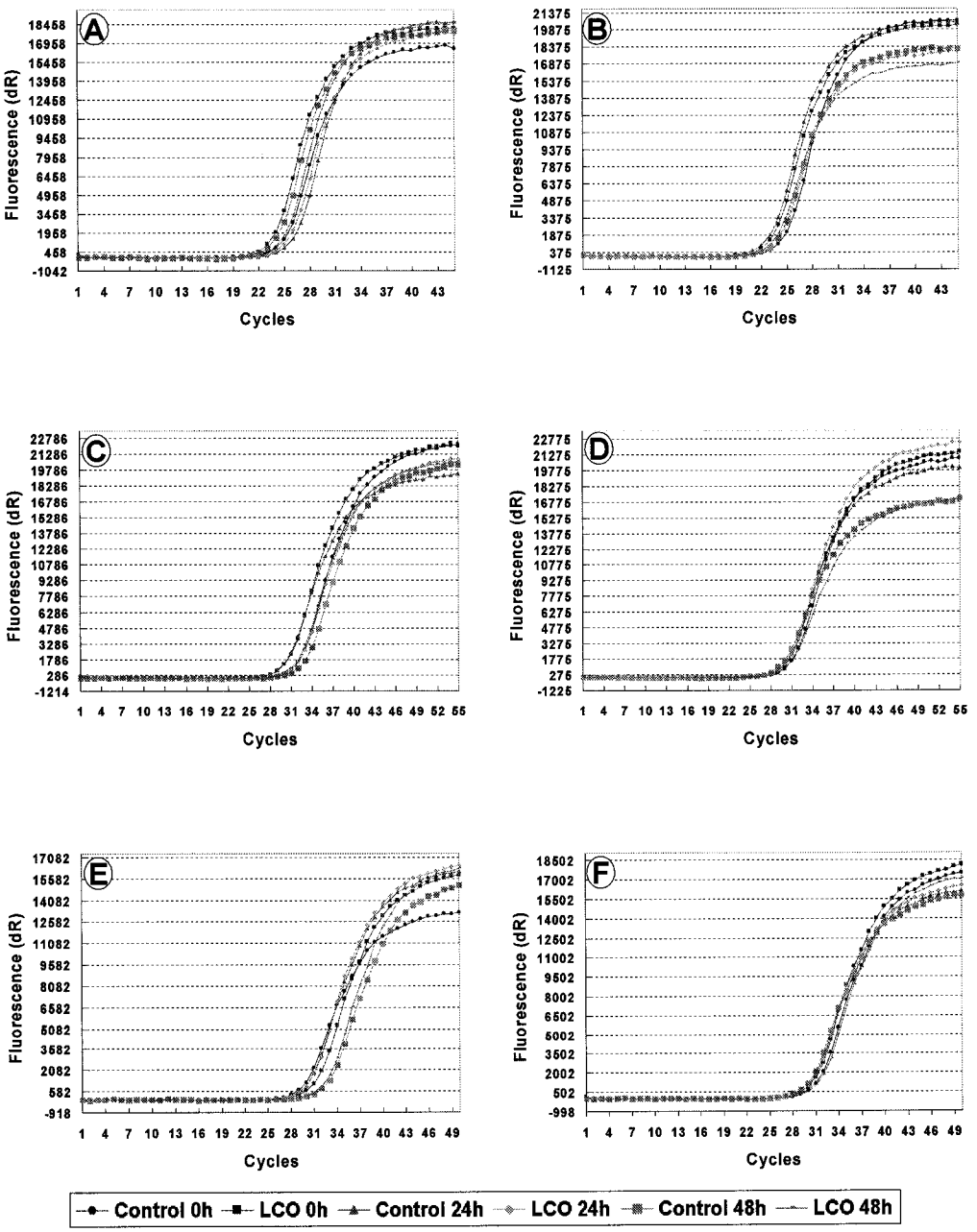
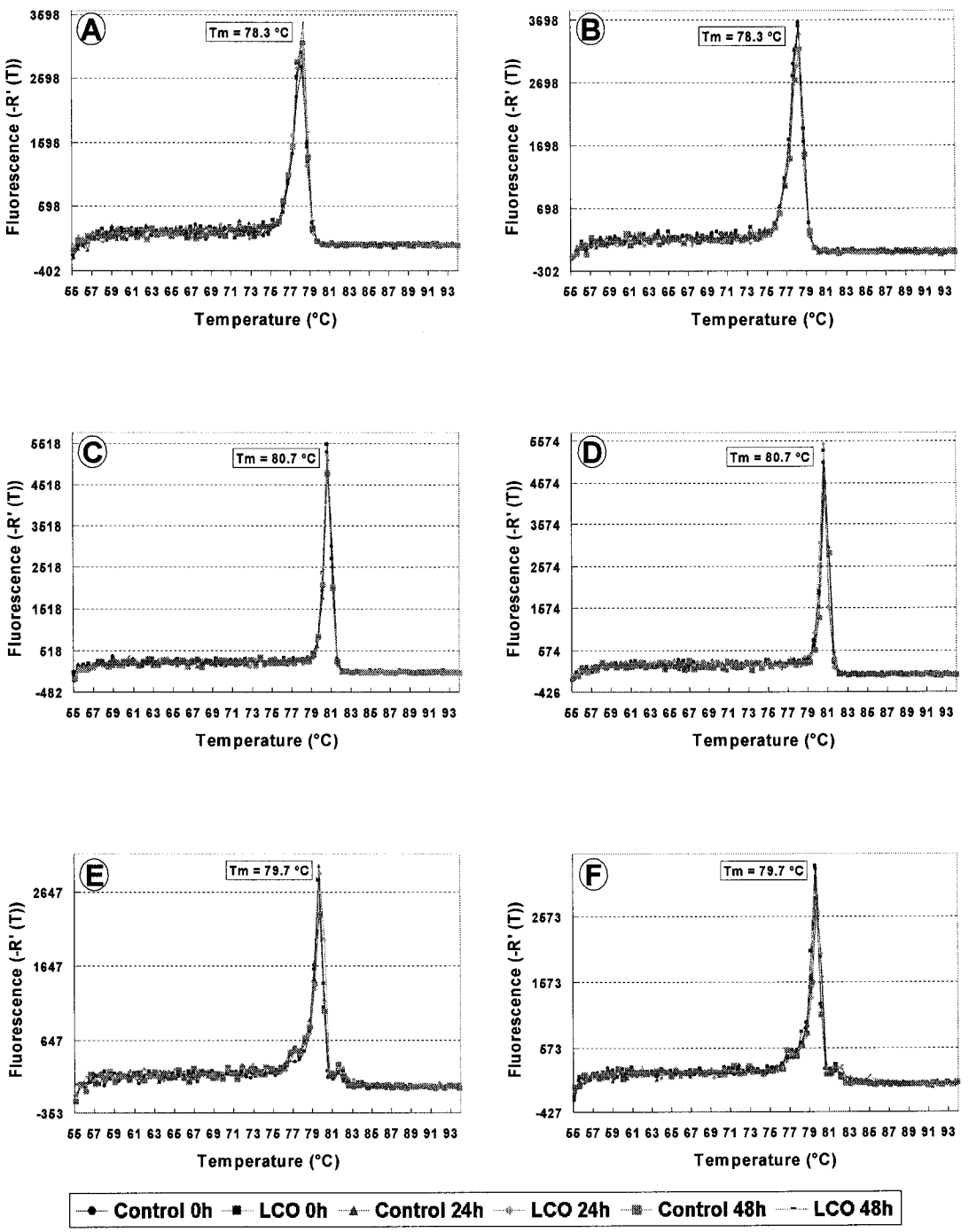
10. Appendices

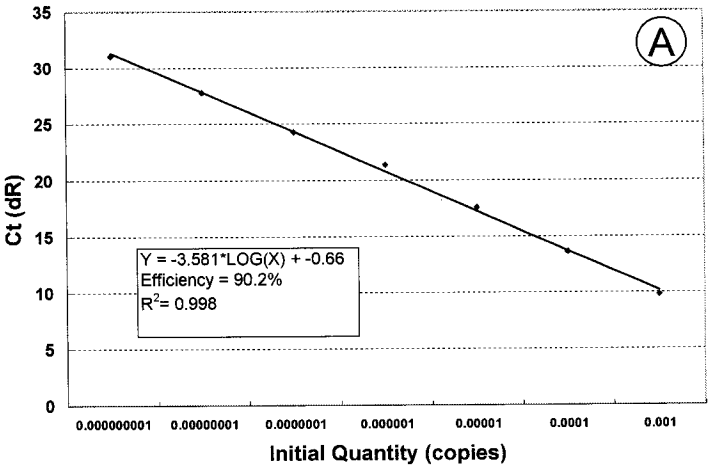
Appendix 1. The QRT-PCR amplification plots of the cv. OAC Bayfield plants for the (A) *actin*, (C) *IFS2*, and (E) *PAL1* gene transcripts and the amplification plots for plants of the non-nodulating line derived from cv. Evans for the (B) *actin*, (D) *IFS2*, and (F) *PAL1* gene transcripts.

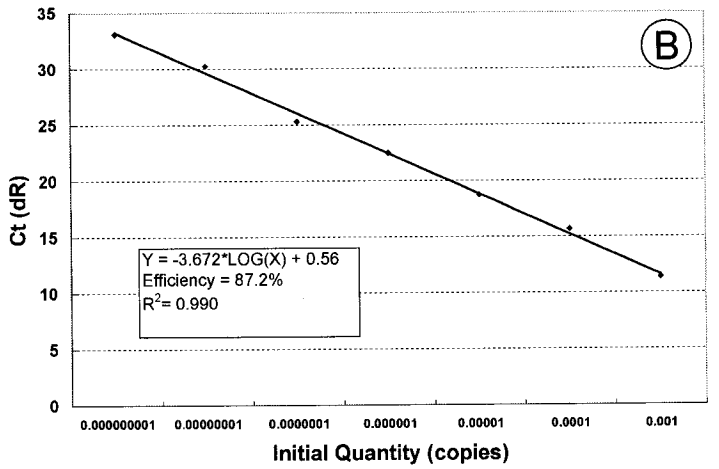


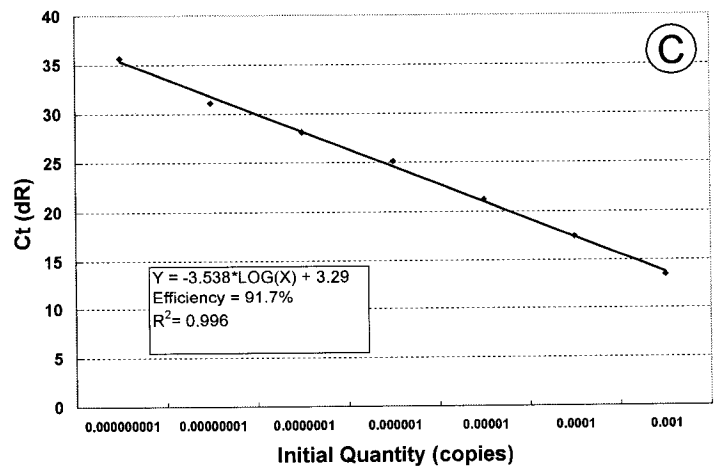
Appendix 2. The QRT-PCR dissociation curves of the cv. OAC Bayfield plants for the (A) *actin*, (C) *IFS2*, and (E) *PAL1* gene transcripts and the dissociation curves for plant of the non-nodulating line derived from cv. Evans for the (B) *actin*, (D) *IFS2*, and (F) *PAL1* gene transcripts.



Appendix 3. The QRT-PCR standard curves for the amplified cDNA of the genes (A) *actin*, (B) *IFS2*, and (C) *PAL1*.







Appendix 4. DNA-agarose gels with amplified cDNA of the genes: (A) *actin* (118bp), (B) *IFS2* (161bp), and (C) *PAL1* (240bp), for the soybean (1) cv. OAC Bayfield and (2) the non-nodulating line derived from cv. Evans. All sample cDNA was synthesized from RNA extracted from the first trifoliolate leaf of soybean plants. The cDNA was amplified, and quantified, using QRT-PCR. The ladder used in these gels is a 100 bp ladder.