Regulatory control of the symbiotic enhanced soybean bHLH transcription factor, *GmSAT1*

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Abstract

GmSAT1 is a basic Helix-Loop-Helix (bHLH) DNA binding transcription factor expressed in soybean root nodules. *GmSAT1* is a unique protein, in that it is localised on cellular membranes including the symbiosome membrane, which encircles nitrogen-fixing bacteroids in soybean nodules. Its role in the regulation of gene transcription in nodules or in other plant tissues is poorly understood. In this study, *GmSAT1*'s functional activity was investigated through a series of studies that investigated the link between gene activities to functional phenotypes. This analysis included the influence of symbiotic partnerships with rhizobia and AM fungi and non-symbiotic root tissues. In this context, an evaluation of changes in gene transcription with or without *GmSAT1* expression (RNAi-based silencing of *GmSAT1*) was explored at the individual and global gene levels. The data indicates that *GmSAT1;1* and a close relative *GmSAT1;2*, are both expressed in roots and nodules but *GmSAT1;1* displayed an overall enhancement in the symbiotic root nodule. Expression of both genes was reduced with external nitrogen supply to the nodule and inoculated root. Both genes were up-regulated in root and nodule tissues when plants were supplied low levels of phosphate. Using an improved method for transgenic hairy roots, developed as part of this thesis project, *GmSAT1* was silenced using a RNAi construct. Tissues (roots and nodules) were analysed for changes in global gene expression using microarray analysis, the impact on symbiotic relationships (rhizobia and AM fungi) and genetic and biochemical responses to phosphorus supply. Transcriptome analysis identified networks that *GmSAT1;1* may be associated with, including a suite of putatively active circadian clock regulators operating in nodules, phosphorus responsive genes in roots, cell wall maintenance and or stress defence signaling pathways, nitrogen transport and metabolism and genes linked to auxin and gibberellin regulatory pathways.

The influence of phosphorus and the AM fungal symbiosis was investigated in more detail. Loss of *GmSAT1* activity altered AM colonisation, causing a reduction in root colonisation when grown at reduced external P. At higher P levels, colonisation remained unchanged.

Shoot P content was significantly increased at both low and high external P supply in the *GmSAT1* silenced plants, indicating a potential role of *GmSAT1* in mediating P homeostasis.

The impact of gibberellins (GA_3) on *GmSAT1* expression and activity was also investigated. Using both qPCR and native promoter:GUS fusion constructs in transformed soybean hairy roots and nodules the expression of *GmSAT1;1* in roots and nodules decreased with external supply of GA_3. In parallel experiments, RNAi *SAT1*-silenced plants showed similar responses with GA_3 treated plants, where nodule number and weight decreased while plant height significantly increased. Furthermore, microarray analysis indicated *GmSAT1* negatively interacts with known gibberellin-responsive genes, including *GASA6*, *GAMA-TIP*, *CLE2*, *MTO3*, *GIP1*, *TPS11*, and *GBF1*.

The overall findings of this study have shown that *GmSAT1* is an important TF to soybean with a broad transcriptional imprint which influences both root nodule symbiosis and AM fungal symbioses. Its activity appears to be linked to multiple genetic signaling networks that involve phosphorus and nitrogen metabolism, hormone activity and regulation of the circadian clock.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide.

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Statement of Authorship

1. **Manijeh Mohammadi-Dehcheshmeh, Esmaeil Ebrahimie, Stephen D. Tyerman, Brent N. Kaiser (2013)** A novel method based on combination of *semi-in vitro* and *in vivo* conditions in *Agrobacterium rhizogenes*-mediated hairy root transformation of *Glycine* species. In Vitro Cellular and Developmental Biology – Plant, [Published online: 21 November 2013]. Presented in Chapter 3

MM conducted the research. EE helped in *G. canescens* germination and nodulation as well as statistical analysis. MM and BNK designed the experiment. MM, EE, BNK and SDT wrote the manuscript.

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Presentations

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Abbreviations

ABA	Abscisic acid
AM	Arbuscular mycorrhizal
bHLH	Basic Helix-Loop-Helix
BLAST	Basic local alignment tool
CCaMK	calcium-calmodulin-dependent protein kinase
CRE	cytokinin receptor
DMF	Dimethylformamide
DMI2	Does not Make Infections
EDTA	Ethylenediaminetetraacetic acid
ENOD	Early nodulation gene
ER	Endoplasmic reticulum
ER	Endoplasmic reticulum
GA	Gibberellin
GFP	Green fluorescent protein
GO	Gene Ontology
GUS	β-glucoronidase
H2O2	Hydrogen peroxide
IAA	Indole acetic acid
IPD3	interacting protein of DMI3
kb	Kilobase
kDa	Kilodalton
LB	Luria broth (medium)
LNP	Lectin nucleotide phosphohydrolase
LYK3	LysM receptor kinase 3
µM/M	Macro/ millimolar
MA	Methylammonium (chloride)
MeJA	Methyl jasmonic acid

N	Nitrogen
NFP	Nod factor perception
NFR	Nod factor receptor
NF-YA	Nuclear Factor Y
NIN	Nodule inception
NORK	NODULATION RECEPTOR KINASE
NSP	Nodulation signaling pathway
NUP	nucleoporin
OD	Optical density
P	Phosphorus
PAR	parabolic aluminized reflector
qPCR	quantitative PCR
RNA	Ribonucleic acid
RNAi	RNA interference
RNA-SEQ	RNA Sequencing
RO water	Reverse osmosis water
SYMRK	symbiosis receptor-like kinase
TEM	Transmission electron microscopy
TF	Transcription factor
TMD	Transmembrane domain
TMD	transmembrane domain
wk/d/h	week/ day/ hour
YEM	Yeast extract mannitol (medium)

Chapter 1

1. Literature review

1.1 Introduction

Legumes are agriculturally important crop plants because of their ability to form an endo-symbiosis with soil-borne nitrogen-fixing bacteria of the *Rhizobium* sp. In this symbiosis, the bacteria reduce N_2 to ammonia and exchange this for reduced carbon from the plant. The reduced carbon is used as an energy source for bacterial metabolism, carbon skeletons for ammonia assimilation and the ammonia enables the plant to grow in soils that have little or no available nitrogen (N). Hence the symbiosis between legumes and N_2-fixing rhizobium is both environmentally and economically important.

Establishment of a legume-Rhizobia symbiosis requires an exchange of molecular signals between the two symbionts. Understanding the genetic processes that support the initiation and development of the root nodule, as well as the maintenance of N fixation, has been a topic of long-standing interest to plant biologists. Improved understanding of the symbiosis will help to increase the efficiency of N fixation in legumes and further expand their use in sustainable plant based agricultural production systems. This review will discuss the development and activity of N fixing legume nodule with particular focus on the signaling events, which are believed to regulate legume *Rhizobium* symbiosis and signaling which are similar in both AM and legume *Rhizobium* symbioses .

1.2 Nodulation and signaling pathway

1.2.1. Nod factor perception

The first step of nodule initiation starts with legume root hairs exuding specific flavonoid compounds into the rhizosphere to attract compatible *Rhizobium* sp. These compounds induce expression of nodulation specific genes (nod genes) in compatible bacteria which then proceed to activate the synthesis and secretion of specific lipo-chitooligosaccharides or 'Nod factors' (Lerouge et al., 1990). Nod factors are diffusible signals that when received by the appropriate plant can elicit a number of different symbiotic developmental responses in different layers of the root such as the epidermis, cortex and pericycle that are required for nodulation (Oldroyd and Downie, 2008). All the Nod factors have a basic structure but depending on the bacterial species, the lipooligosaccharides carry a number of different modifications which act as an important determinant of bacterial host specificity (Schultze and Kondorosi, 1998). Nod factors are recognized by Nod factor receptors on legume root hair cells. Two essential Nod factor receptors have been identified in *Lotus japonicas* and *Medicago truncatula*. The receptors are encoded by nodulation-specific receptor-like kinases or *NFR1* (Nod factor receptor 1) /*LYK3* (LysM receptor kinase 3) and *NFR5* (Nod factor receptor 1) /*NFP* (Nod factor perception) (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006). However, previous biochemical studies have suggested alternative candidates for Nod factor perception exist which includes an extracellular apyrase and a lectin nucleotide phosphohydrolase (*LNP*) (Etzler et al., 1999). *LNP* is localized to the epidermal cell surface of young roots and more specifically on the surface of the root hairs (Etzler et al., 1999). In a recent study it has been shown that antisense of *LNP* blocks both nodulation and AM colonization suggesting involvement of this gene in the common symbiosis pathway (Roberts et al., 2013) (Figure 1.1).

1.2.2 Infection and nodule initiation

Nod factors induce a number of different developmental responses in root cells that facilitate both the infection process and subsequent nodule development (Oldroyd and Downie, 2008) (Figure 1-1A). One of these responses is the development of an intracellular infection structure, called the infection thread, which facilitates bacterial penetration into the root. There are several genes known to be involved in the development of the infection thread including, the symbiosis receptor-like kinase (*SYMRK*/ *DMI2* (*NORK*) (Endre et al., 2002; Stracke et al., 2002), two nucleoporin genes (*NUP85* and *NUP133*), and two cation channels *CASTOR*/*POLLUX*/*DMI1* (Imaizumi-Anraku et al., 2005; Charpentier et al., 2008). Disruption in the activity of any of these genes will block early stages of the infection process (reviewed by (Murray, 2011)). *SYMRK* is also involved in nod factor mediated induction of leghemoglobin (Sandal et al., 2002).

Another response to nod factor perception is the generation of calcium oscillations (i.e. calcium spiking) in the nucleus of infected cells. Calcium spiking acts downstream of all the above mentioned genes and is required for the subsequent induction of downstream nodulation events (Oldroyd and Downie, 2004), such as the interaction between *CCaMK*/*DMI3* and *CYCLOPS*/*IPD3*. *CCaMK*/*DMI3* encodes a calcium-calmodulin-dependent protein kinase, which is thought to help decipher the calcium oscillations and mediate downstream signaling events important for nodulation and continued bacterial infection (Mitra et al., 2004; Oldroyd et al., 2011). Gain-of-function mutations in *CCaMK* leads to spontaneous nodule development in rhizobia-free conditions (Tirichine et al., 2006). *CCamK*/*DMI3* interacts with and phosphorylates *CYCLOPS*/*IPD3* (Messinese et al., 2007; Yano et al., 2008; Horváth et al., 2011). This interaction is essential for both rhizobial and mycorrhizal colonization to occur. Loss-of-function mutants for both *CCaMK* and *CYCLOPS*, individually disrupt both root-hair curling and cortical cell division (Tirichine et al., 2006; Yano et al., 2008). Interestingly, nodule formation can be returned in the cyclops mutant with the overexpression of *CCaMK* (Yano et al., 2008).

Downstream of *CCaMK* and *CYCLOPS* are several TFs required for successful nodulation, including *NSP1*, *NSP2*, *NIN1* (NODULE INCEPTION), *HAP2.1*, *ERN1*, and *ERF1* (Grønlund et al., 2003; Kalo et al., 2005; Smit et al., 2005; Combier et al., 2006; Andriankaja et al., 2007; Marsh et al., 2007) (Figure 1-1A).

- *NSP1* and *NSP2* (Nodulation-Signaling Pathway 1&2) encode *GRAS* family transcriptional regulators essential for Nod-factor signaling in both epidermal and cortical cells (Oldroyd and Sharon, 2003; Kalo et al., 2005; Smit et al., 2005; Hirsch et al., 2009). Mutations in *nsp1* and *nsp2* block nod-factor induced ENOD gene activation and prevent the gain of function effect of CCaMK on nodulation (Hayashi et al., 2010). NSP1 and NSP2 are also active in cortical cells, where loss of function (*nsp1* and *nsp2*) disrupts cortical cell division.
- *NIN* is a TF expressed in both epidermal and cortical cells. In *M. truncatula*, *NIN* expression is dependent on the binding of *NSP1* and *NSP2* to its promoter (Hirsch et al., 2009). NIN is also linked to the downstream cortical cell-signaling cascade involving the cytokinin receptor (*LHK1/CRE1*). *NIN* expression responds to cytokinin application (Heckmann et al., 2006) and is dependent on the activity of either *LHK1* or *CRE1* (Murray et al., 2007). These activities suggest *NIN* is a primary regulator of cortical cell division in a developing nodule. The activity of nodule expressed NIN's appears to be specific. Yokota et al (2010) examined functional complementation of *L. japonicus nin* mutants with a NIN homolog from rice, *OsNLP1* (Yokota et al., 2010). The mutant line failed to process infection in a *nin* mutant, whereas *nsp1* or *nsp2* mutants produced normal nodulation after introducion of *NSP1* and *NSP2* homologs from rice (Yokota et al., 2010). Recently, different subunits of the *L. japonicus* Nuclear Factor Y (*NF-YA1* and *NF-YB1*) have been shown to be transcriptional targets of NIN (Soyano et al., 2013).
- *NF-Y* and the *M. truncatula* homolog *HAP2-1*, are CCAAT-binding TFs that have a critical role in nodule development. In *M. truncatula*, RNAi silencing of *HAP2-1* or over

expression of the *HAP2-1* gene specific microRNA169, results in nodule abortion (Combier et al., 2006).

- *ERN1* is an *AP2-like* TF from the *ERF* (Ethylene Response Factor) subfamily. ERN1 is necessary for Nod factor–induced gene expression and for spontaneous nodulation activated by *CCaMK*/*DMI3* (Middleton et al., 2007).
- *NDX* and *EFD* are TFs, which participate in regulation of nodulation and control the number of nodules (Grønlund et al., 2003; Vernié et al., 2008). *NDX* is a homeodomain (HD) protein, belonging to the HD-ZIP TF group. In *L. japonicus*, down-regulation of *Ljndx1* and *Ljndx2* through an antisense expression approach resulted in the production of more nodules but failed to produce well-developed lenticels and vascular tissues (Grønlund et al., 2003). *EFD* belongs to the *ERF* (Ethylene Response Factor) subfamily. *EFD* is a negative regulator of root nodulation suppressing initiation and development of nodules through inhibition of cytokinin signaling (Vernié et al., 2008).

1.2.3 Effects of hormones in nodule organogenesis and development

Regulation of nodule organogenesis involves the participation of plant hormones, TFs and microRNAs (reviewed by (Crespi and Frugier, 2008). Cytokinins have a positive influence on nodulation. When applied exogenously to legume roots, cytokinins activate nodule initiation (including root cortical cell division and induction of early nodulin gene expression) in a similar manner to that induced by external rhizobial Nod factors (Cooper and Long, 1994; Fang and Hirsch, 1998). Both cytokinins and Nod factors activate the expression of critical nodulation genes, including the *GRAS* transcription factors, *NIN* and *NSP2* (Gonzalez-Rizzo et al., 2006; Murray et al., 2007). Recently, the cytokinin receptor (*MtCRE1*) has been linked to the induction of this signaling pathway (Plet et al., 2011). Nodule organogenesis is decreased by activation of a cytokinin-degrading cytokinin oxidase in *L. japonicus* (Lohar et al., 2004). Altogether, initiation of nodule organogenesis appears to depend on the activation of cytokinin signaling pathways, which may involve crosstalk with the Nod factor-signaling cascade.

During nodule organogensis, cytokinin-signaling pathways interact with other hormones including auxin. *MtPINs* (auxin efflux carriers) are induced by the MtCRE1-mediated cytokinin-signaling pathway resulting in local auxin accumulation at the initiation of the nodule primodia (Wasson et al., 2006; Plet et al., 2011). Auxins are also important to nodule organogenesis (Mathesius et al., 1998; Boot et al., 1999; de Billy et al., 2001; Benková et al., 2003; Wasson et al., 2006; Pii et al., 2007). The auxin import carrier (*AUX1*) and the auxin-amido synthetase (*GH3*) are activated during the development of the nodule primordia in both *Medicago truncatula* (de Billy et al., 2001) and in white clover roots (Mathesius, 2008). Furthermore, nodule numbers per root also increase when roots are inoculated by rhizobia harboring an auxin biosynthetic enzyme (Pii et al., 2007). Auxin is also important at later stages of nodule development. Using the auxin reporter GH3 fused to b-glucuronidase (*GUS*) in *L. japonicus* root, Takanashi et al. (2011) revealed strong auxin induction in the central cylinder of the root and vascular tissue before inoculation (Takanashi et al., 2011). However after inoculation, GH3:GUS expression increased in the outer cortex of the root and across the nodule vascular tissue (Takanashi et al., 2011). This would suggest auxins are involved in many cell types and stages of nodule development.

1.2.4 Control of nodulation and Auto-regulation

Although nodule formation is critical for N fixation it appears the plant controls the number of symbiotic N-fixing nodules. In addition to the previously mentioned TFs (*NDX* and *EFD*) there are a number of external and internal factors that act as negative regulators of nodulation. Stress inducible hormones including, ethylene, abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) negatively regulate nodulation (reviewed by (Ryu et al., 2012). Both JA and ethylene reduce Nod factor-induced ENOD11 expression and root-hair calcium spiking/sensitivity to Nod factor (Penmetsa and Cook, 1997; Oldroyd et al., 2001; Sun et al., 2006). Mutations to ethylene response genes, such as *EIN2*, result in increased nodule number (Penmetsa and Cook, 1997; Penmetsa et al., 2008). Application of ABA also blocks root-hair response to nod factor and reduces the number of nodules. However it

appears that the effect of ABA is at the stage between root hair swelling and curling, suggesting that ABA may ultimately control the number of nodules formed on roots (Suzuki et al., 2004). Salicylic acid (SA) also inhibits Rhizobium growth in a dose-dependent manner (Stacey et al., 2006). Taken together these stress-associated hormones appear to be negative controllers of nodulation, a trait possibly linked to their native role in plant defense to selected biotic stimuli sensitivity.

The other pathway that plants use to regulate nodulation is called auto-regulation of nodulation (AON) (reviewed by (Ferguson, 2013). AON involves a root- derived signal (presumably a CLE peptide) (Lim et al., 2011) which is transferred from the root to the shoot where the signal is received by a receptor kinase (*HAR1*/ *NARK*) activating a shoot-derived inhibitor (*SDI*) that is transferred to the roots to inhibit nodulation (Reid et al., 2012). Mutation of the NARK / HAR1 receptor kinases, results in a super-nodulation phenotype in soybean and *L. japonicus*, respectively (Krusell et al., 2002; Searle et al., 2003). Interestingly *HAR1*/ *NARK* plays a similar role in regulation of arbuscular mycorrhizal colonization (Schaarschmidt et al., 2013).

1.3 Common symbiosis pathway

Legumes can form symbiotic relationships with arbuscular mycorrhizal fungi (AM). The AM fungal symbiosis improves the surface area of the root allowing for an improvement in the absorption of minerals, such as phosphorus, ammonium and zinc from the soil solution. To manage both a rhizobia and AM fungal symbiosis, legumes have developed a common symbiosis pathway involving shared molecular strategies (highlighted in Figure 1 and Table 1-1) by which the host and symbionts (rhizobia or AM fungi) can successfully recognize each other in order to proceed with the symbiosis (Bonfante and Genre, 2010; Oldroyd, 2013). At present, the common sym genes are mainly involved in the early infection process and include co-utilised genes including *DMI2/SYMRK*, *NUP133*, *NUP85*, *CASTOR/POLLUX/DMI1*. *CCaMK/DMI3* and *CYCLOPS/DMI3* (Oldroyd, 2013). Considering the much earlier evolution of the AM symbiosis to that of the eudicots (~ 400 million years) it is suggested the

rhizobia symbiosis has utilized pre-existing signaling cascades evolved from AM activities (Parniske, 2008).

1.4 Similar TF's in the AM and Root nodule symbiosis

NSP2 is the only known TF known to be involved in the AM and root nodule symbiosis. Initially it was thought *NSP2* was specific to rhizobial nodulation (Kalo et al., 2005; Smit et al., 2005). However, recently it has been linked to the AM symbiosis as well (Gobbato et al., 2012). The activity of *NSP2* in both symbioses may involve selected interactions with two GRAS proteins, *NSP1* and *RAM1*. A complex of NSP2::NSP1 promoting nodulation responses while a complex of NSP2::RAM1 promotes the mycorrhizal-specific response (Gobbato et al., 2012).

Recently it has been reported that two members of CCAAT-binding family TF, *GmNF-YA1a* and *GmNF-YA1b* are positive regulators of AM colonization in soybean and that its activity is inhibited by a *NARK*-mediated signal (Schaarschmidt et al., 2013). Before this study, most of known CCAAT-binding family TF had been reported to be involved in nodulation (Marsh et al., 2007; Soyano et al., 2013). Interestingly, involvement of the microRNA169 family, which also regulates the stability of *GmNF-YA1a* and *GmNF-YA1b* TFs have been shown in tomato to be down-regulated in leaves under Pi deficiency, but up-regulated in either AM or high-phosphate-treated plants (Gu et al., 2010). Other members of this family such as microRNA169d*/e.2*/l*/m* and microRNA169d, l, are also up-regulated in AM colonised roots of *M. truncatula* with high levels of expression in the phloem and around fungal hyphae (Devers et al., 2011). These results indicate a different family member of microRNA169 and its target, *NF-YA* TF participate in AM or rhizobial symbiosis which reinforces the hypothesis that the rhizobia symbiosis has exploited pre-existing signaling cascades evolved from AM activities.

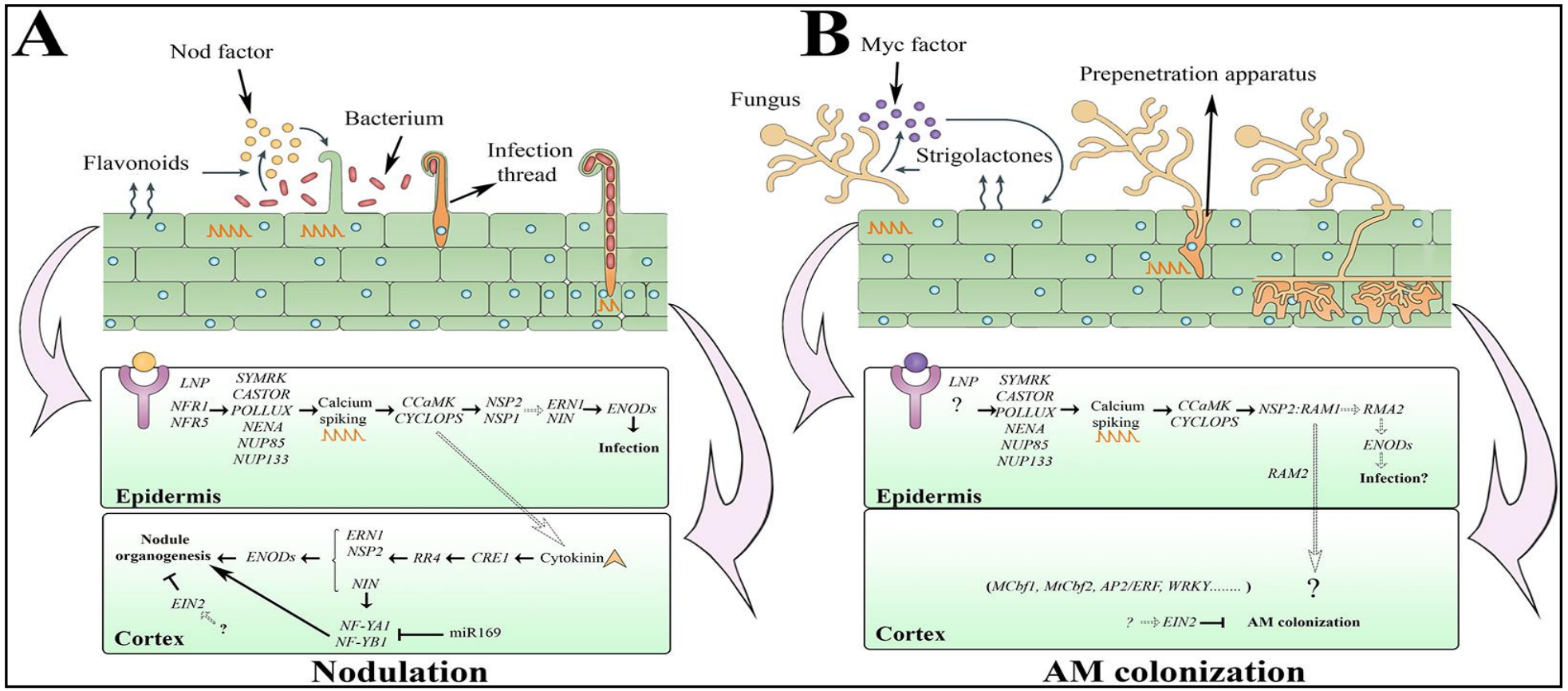


Figure 1.1 Similar signaling pathways between nodulation and AM colonization.

Signaling pathways at early stages are similar in both symbioses. In both symbiosis, plant roots exude compounds to attract symbionts (strigolactones signal AM fungi and flavonoids to signal compatible rhizobia) . and in response both symbionts lipochitooligosaccharides (LCO) which called Myc factor (for AM) and Nod factor (for rhizobia). Perception of Nod/Myc factors activates a series of similar genes in epidermis layer which role of each gene has described in Table (1.1). The difference between both symbiosis is mainly in cortex and most signaling event especially in AM symbiosis is unknown. Black arrows indicate functional links, based on experiments and genetic data; and white arrows indicate hypothetical relationships. (Modified from Oldroyd, 2013).

Table 1.1 Comparison of AM and rhizobial symbioses.

Stage	*Common SYM activities*	*Reference*
Pre-infection	In both symbioses, plant roots exude compounds to attract symbionts. Strigolactones are released to signal AM fungi and flavonoids to signal compatible rhizobia.	Reviewed by (Oldroyd, 2013)
	AM fungi and rhizobia produce lipochitooligosaccharides (LCO) with variable structures called Myc factor (for AM) and Nod factor (for rhizobia).	(Lerouge et al., 1990; Maillet et al., 2011)
Perception	Perception of Nod/Myc factors involves the activation of a LysM receptor kinase. An NFR5-like receptor is predicted to mediate perception of Myc factor. NFR1/LYK3 and NFR5/NFP are involved in perception of nod factor.	(Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006; Op den Camp et al., 2011; Gough and Jacquet, 2013)
	LNP is an apyrase and is considered a Nod factor-binding protein. Loss of LNP blocks both nodulation and AM colonization.	(Roberts et al., 2013)
Signalling pathway	*SYMRK/ DMI2 (NORK)* is a SYM gene, where loss of activity impairs intracellular infection at the epidermal cell layer of the roots in both symbioses.	(Wais et al., 2000; Endre et al., 2002; Stracke et al., 2002)
	CASTOR/POLLUX/DMI1 is a SYM gene. In the AM symbiosis, fungal entry into root epidermal cells of mutants is blocked while in the rhizobial symbiosis, mutants failed to form infection threads.	(Catoira et al., 2000; Harrison et al., 2002; Imaizumi-Anraku et al., 2005; Charpentier et al., 2008)
	NUP85, *NUP133*, *NENA* are SYM genes. Mutations in these genes disrupt the AM symbiosis, preventing the penetration of the cortical cell layer. In the rhizobial symbiosis, loss of activity prevents infection thread release of bacteria.	(Kanamori et al., 2006; Saito et al., 2007; Groth et al., 2010)
	Calcium spiking is required for induction of downstream genes in both symbioses.	Reviewed (Singh and Parniske, 2012)
	CCaMK/DMI3 is a SYM gene. In the AM symbiosis, gain of function mutants enhances the development of the pre-penetration structure. In the rhizobial symbiosis, root-hair curling and cortical cell division is disrupted in loss of function mutants.	(Tirichine et al., 2006; Hayashi et al., 2010; Takeda et al., 2012)
	CYCLOPS/IPD3 is a SYM gene. In the AM symbiosis, loss of activity reduced colonisation levels. In the rhizobial symbiosis, *CYCLOPS/IPD3* is required for infection thread growth and nodule development.	(Yano et al., 2008; Horváth et al., 2011)
	NSP2 is a SYM gene. Mutant exhibited lack of Nod/Myc factor responses but retained calcium spiking.	(Kalo et al., 2005; Maillet et al., 2011)
	RAM1 is AM specific GRAS domain TF, which its interaction with NSP2 is required for AM colonization. Interaction of another nodulation specific GRAS domain TF (*NSP1*) with NSP2 is required for nodulation.	(Hirsch et al., 2009; Gobbato et al., 2012)
	In both symbioses, plants exploit an auto-regulation pathway, which involves *HAR1/NARK*. In the AM symbiosis *HAR1/NARK* are linked to the down-regulation of *GmNF-YA1a/b* to control AM colonization. In the rhizobial symbiosis, *HAR1/NARK* affects hormone balance to stop nodulation.	(Krusell et al., 2002; Searle et al., 2003; Schaarschmidt et al., 2013)
	EIN2 is involved in both symbioses. It regulates early stage of AM symbiosis and reduces root responses to cytokinins and controls ethylene sensitivity and the number of nodules.	(Penmetsa et al., 2008)
Infection structure	Plant cells provide an infection pathway for symbiont entrance. In the AM symbiosis it is called the prepenetration apparatus and in the rhizobial symbiosis, the infection thread.	(Parniske, 2008)
	Symbionts are surrounded by a plant-derived membrane. In the AM symbiosis this is the periarbuscular membrane (PAM), which is continuous with the plasma membrane of the cortical cell. In the rhizobia symbiosis it is the symbiosome membrane (SM) derived from the plasma membrane upon infection thread release.	(Trindade et al., 2010; Montanini et al., 2011)

1.5 *GmSAT1* a novel TF with unknown role in symbiosis

1.5.1 Identification of GmSAT1

GmSAT1 is a soybean TF identified in a functional yeast complementation screen (Kaiser et al., 1998). *GmSAT1* was selected based on its ability to complement a yeast ammonium transport mutant (26972c) on low ammonium concentrations (1 mM), where two of the native yeast ammonium transporters (*mep1* and *mep2*) are inactive and the third *MEP3* transiently inhibited through a direct protein interaction by a dysfunctional mep1-1 (Kaiser et al., 1998). *GmSAT1* was found to be located on the yeast plasma membrane when expressed in 26972c and on the PBM in soybean (Kaiser et al., 1998). Initially, *GmSAT1* was considered as a possible ammonium transport protein, which moves ammonium into yeast cells and also allows the release of symbiotically fixed ammonium across the PBM to the infected cell cytosol (Kaiser et al., 1998). However, questions remained unsolved on its mode of action as it is primarily a hydrophilic protein with a single transmembrane spanning domain. Further yeast studies showed that *GmSAT1* expression does not complement the growth of yeast on low NH_4^+ medium when all three *mep* transporters were deleted (Marini et al., 2000). It was suggested that the role of *GmSAT1* in complementing the growth of 26972c might be associated with up-regulation of *MEP3* (Marini et al., 2000). Further studies demonstrated that *GmSAT1* is likely a bHLH TF localized to the membrane as well as nucleus in yeast (Loughlin, 2007). Also recent microarray experiments have shown that a *MEP3* as well as a large collection of P responsive genes is up-regulated with overexpression of *GmSAT1* in yeast (Mazurkiewicz, 2008).

GmSAT1 is critical for nodulation and loss of its activity resulted in small nodules and chlorotic plants (Loughlin, 2007). However, the exact role of this TF in nodulation and whether it is involved in the AM symbiosis is not clear. *GmSAT1* homologs have been identified in other plants such as *Arabidopsis, Medicago*, rice, grapevines and maize. All of them contain a basic Helix-loop-Helix DNA biding domain and C-terminal hydrophobic

domain and are all structurally similar in size between 35-40 kDa. A few homologs have been partially characterized in other plants. In Arabidopsis, four putative *GmSAT1* homologs exist including At2g22750, At2g22760 (*AtNai1*), and At2g22770. One of the best characterized *GmSAT1* homologs is *NAI1* which appears to regulate the expression of genes related to ER bodies and to plays a key role in the formation of ER bodies (Matsushima et al., 2004).

This thesis documents the characterization of *GmSAT1* in soybean. The research examines its expression pattern during the rhizobial symbiosis and the putative transcriptional networks it participates in across both roots and nodules. As in previous experiments, expression of *GmSAT1* in yeast was associated with up-regulation of N and P transporters (Kaiser et al., 1998; Mazurkiewicz, 2008), in this research I examined expression of *GmSAT1* in response to different level of N and P in soybean. Also this research tries to answer this question whether *GmSAT1*, similar to *NF-YA* and *NSP2* TFs can play a discriminate role in both AM and rhizobial symbiosises. To address this question, an examination of *GmSAT1* RNAi silenced plant in the AM symbiosis is explored indicating a unique link to phosphorus status and AM fungi colonization. Transcriptional networks have been also developed which indicate a role of *GmSAT1* across multiple signaling cascades that includes nitrogen, phosphorus, circadian rhythms and hormone signaling.

Chapter 2

2. Identifying the expression pattern and tissue-specificity of *GmSAT1;1* and its homolog *GmSAT1;2* in soybean

2.1 Abstract

Nodulation is the process by which legumes become competent to host rhizobia in a non-pathogenic relationship. To date, the role of the majority of transcription factors (TFs) expressed in the legume symbiosis has yet to be studied in detail. In this study we have identified that soybean contains 4 homologs of the *GmSAT1* transcription factor, each located on different chromosomes. These include: *Glyma15g06680* (*GmSAT1;1*), *Glyma13g32650* (*GmSAT1;2*), *Glyma05g23530* (*GmSAT2;1*), and *Glyma17g16720* (*GmSAT2;2*). *GmSAT1;1* and *GmSAT1;2* share close sequence similarity and show tissue-dependent expression patterns between root and nodule tissues. Using qPCR, the expression profile of *GmSAT1;1* and *GmSAT1;2* over the course of early nodule and root development were obtained. This data indicated that both N supply and *Rhizobium* inoculation altered the expression of *GmSAT1;1*. Whole tissue expression was complemented with tissue-specific expression of *GmSAT1;1* and *GmSAT1;2* in roots and nodules though promoter::*GUS*-reporter expression analysis in transformed hairy roots. Expression analysis and promoter cloning documented that *GmSAT1;1* is a nodule-enhanced gene while *GmSAT1;2* is expressed similarly in both nodule and root tissues. The highest level of *GmSAT1;1* expression was observed under N deficiency and in the presence of rhizobia during nodule development (19-25 days after rhizobia inoculation). Altogether, the results suggest that *GmSAT1;1* is a nodule-enhanced transcription factor with a high level of expression during the period when nodule development allows for the initiation of nitrogen fixation activity.

2.2 Introduction

Plants require nitrogen (N) for growth where it is an important constituent of key biological processes including, amino acids, proteins, nucleic acids, chlorophyll and numerous organic molecules. Unfortunately, N in the soil is often depleted, particularly in situations involving intensive monoculture crop production. Many plants within the Fabaceae family (i.e. legumes) have developed an alternative strategy that is not dependent on soil N for growth. Many legumes can form a symbiotic relationship with soil based N_2-fixing bacteria, of the *Rhizobium sp*. The symbiosis involves bacterial invasion of the root and the coordinated development of the root nodule, where rhizobia (bacteroids) reside and fix atmospheric N_2 that is exchanged for nutrients delivered by the plant including, sugars, micronutrients and water.

Nodule development and activity ultimately involves multiple plant-based signaling cascades which are required to support these processes. The molecular identification and functional characterization of many of these genes and the signaling networks they are involved in will ultimately lead to a better understanding of the legume symbiosis and its role in nitrogen delivery to the host plant. To date, the identification and functional role of nodule expressed transcription factors (TFs) involved in gene activation in legume nodules is poorly understood. Only a few have been biologically examined which include, *NSP1, NSP2, ERN, EFD, NIN (NODULE INCEPTION), NDX* and *HAP2*-1. This is probably due to their low and generally transient expression, that in many cases are involved in the expression of many genes which participate across multi-faceted signaling cascades that are active during the course of the symbiosis.

GmSAT1;1 is a nodule expressed basic helix loop helix (*bHLH*) TF identified from soybean (Kaiser et al., 1998). *GmSAT1;1* (Glyma15g06680) was initially characterised as an ammonium transporter based on its selection in a yeast complementation assay where it selectively rescued the growth of an ammonium transport mutant 26972c (Kaiser et al., 1998). Since this original study, the role of *GmSAT1* has been better defined where it is now

considered a bona fide membrane bound bHLH transcription factor, when expressed in yeast regulates ammonium transport proteins in yeast, including *Mep3* and a recently identified low-affinity ammonium transporter *AMF1*(Chiasson, 2012; Mazurkiewicz, 2013). In soybean, loss of *GmSAT1;1* activity using RNAi silencing, alters nodule development and nitrogen fixation. The nodules that develop are small and lack the ability to effectively fix and deliver nitrogen to support plant growth (Loughlin, 2007).

The regulation of *GmSAT1;1* expression is poorly understood. Previous studies have shown that expression of *GmSAT1;1* is enhanced in nodules relative to roots (Kaiser et al., 1998) but there is no report on expression of *GmSAT1;2* and little is known about *GmSAT1;1* expression pattern over time and its relationship to external nutrients such as N, a known repressor of nodule development and N_2-fixation activity. To begin answering these questions, the mRNA expression patterns of *GmSAT1;1* and its duplicated homolog *GmSAT1;2*, were evaluated using a combination of online *in silico* expression datasets and plant-based experiments involving soybean root and nodule tissues. *GmSAT1;1* and *GmSAT1;2* expression patterns were also evaluated using promoter::gus constructs that were expressed in transgenic soybean roots and nodules generated using the *Agrobacterium rhizogenes*-mediated hairy root transformation system (see Chapter 3).

2.3 Materials and Methods

2.3.1 In silico based genome identification and expression analysis of GmSAT1 homologs

Phytozome (http://www.phytozome.net/) and blastn were used to identify *GmSAT1;1* homologs present within the sequenced and annotated soybean genome and that of other plants. CLC Genomics Workbench (http://www.clcbio.com/) was then used for sequence alignment and clustering of *GmSAT1;1* homologs based on amino acid sequences (Figure 2.2 and 2.3).

PLEXdb (gene expression resources for plants and plant pathogens, http://www.plexdb.org/) (Dash et al., 2012) and BAR (The Bio-Analytic Resource for Plant Biology, http://bar.utoronto.ca/welcome.htm,) were used to monitor the expression of the *GmSAT1* homologs across different tissues based on publically available soybean transcriptomic datasets and submitted microarray and RNA-SEQ experiments (Figure 2.5).

2.3.2 Plant material and qPCR analysis of GmSAT1;1 and GmSAT1;2 in root and nodule tissues of soybean

Soybeans (*Glycine max* L. cv. Djakal) were grown in Waikerie sand in a growth chamber under a light intensity of 400-600 PAR using mercury halide lamps with a day/night temperature cycle of 28/25°C and a 14/10 hr day/night regime. Based on a designed experimental plan (listed in Table 2.1), pots were watered with the either nitrogen free (-N) or plus nitrogen (+N: 2.5 mM NH_4NO_3) nutrient solution listed in Table (3.1) at three day intervals and watered with distilled water when it was required. Plants were inoculated with *Bradyrhizobium japonicum* USDA110 (inoculum density of 10^5 –10^6cells / ml (Jitacksorn and Sadowsky, 2008) at planting and then again on the following day. In most situations each treatment contained 15 pots with 3 plants per pot. Root and nodule samples were harvested between (11:00 am - 1:00 pm) for RNA extraction and expression analysis across a range of developmental stages. After each harvest, the remaining plants were watered with the

appropriate nutrient solution. For nodules, only the top regions of the main root (crown nodule) were harvested while for the roots, only the main tap-root was selected. All experiments were conducted using a completely randomized design in a single temperature controlled walk-in growth chamber.

Total RNA was extracted from nodules and roots with a Spectrum Plant Total RNA kit (Sigma-Aldrich). DNAeasy from the TURBO DNA-free kit of Invitrogen Company were used to remove any traces of genomic DNA from the extracted RNA. 1 µg of total RNA was used for cDNA synthesis with Superscript III (Invitrogen) and transcript abundance of *GmSAT1;1* and *GmSAT1;2* in each cDNA pool was determined using a Light Cycler (Bio-Rad) qPCR machine using IQ SYBR Green (Bio-Rad, USA) and primers listed in Table(2.2). Relative expression was measured as the mean CT values of three biological replicates. ΔCT values were calculated using Glyma12g05510 (*cons6*) as a reference gene (Libault et al., 2008). The relative expression was then calculated based on the $2^{-\Delta CT}$ formula (Schmittgen and Livak, 2008). For each treatment, tissue (nodule or root) or developmental stage, three technical replications were used and each experiment was repeated twice. Data was subject to an analysis of variance (ANOVA) and a subsequent Paired T-test using MINITAB16 software to compare the expression differences between *GmSAT1;1* and *GmSAT2;2*. Mean separation was performed using Tukey test at 0.05 probability level.

2.3.3 Promoter:GUS analysis of GmSAT1;1 and GmSAT1;2

The regulatory region of *GmSAT1;1*, 1863 bp upstream from its start codon, was PCR amplified from soybean genomic DNA using *GmSAT1;1* PROF/R primers (Table 2.2) and Platinum Taq High-Fidelity (Life Technologies). Using the same approach, the *GmSAT1;2* promoter consisting of 1819 bp upstream of its predicted start codon was amplified with *GmSAT1;2* PROF/R primers (Table 2.2). Primers were designed using FastPCR software (Kalendar et al., 2011). After PCR fragment amplification, the DNA fragment was ligated into the intermediate vector pCR8-TOPO (Life Technologies) and then sequenced. The insert was then gateway recombined into the destination vector pKGWFS7 (Figure 2.1) (Karimi et

al., 2002). 100 ng of plasmid DNA was then transformed to *Agrobacterium rhizogenes* (strain K599) by electroporation.

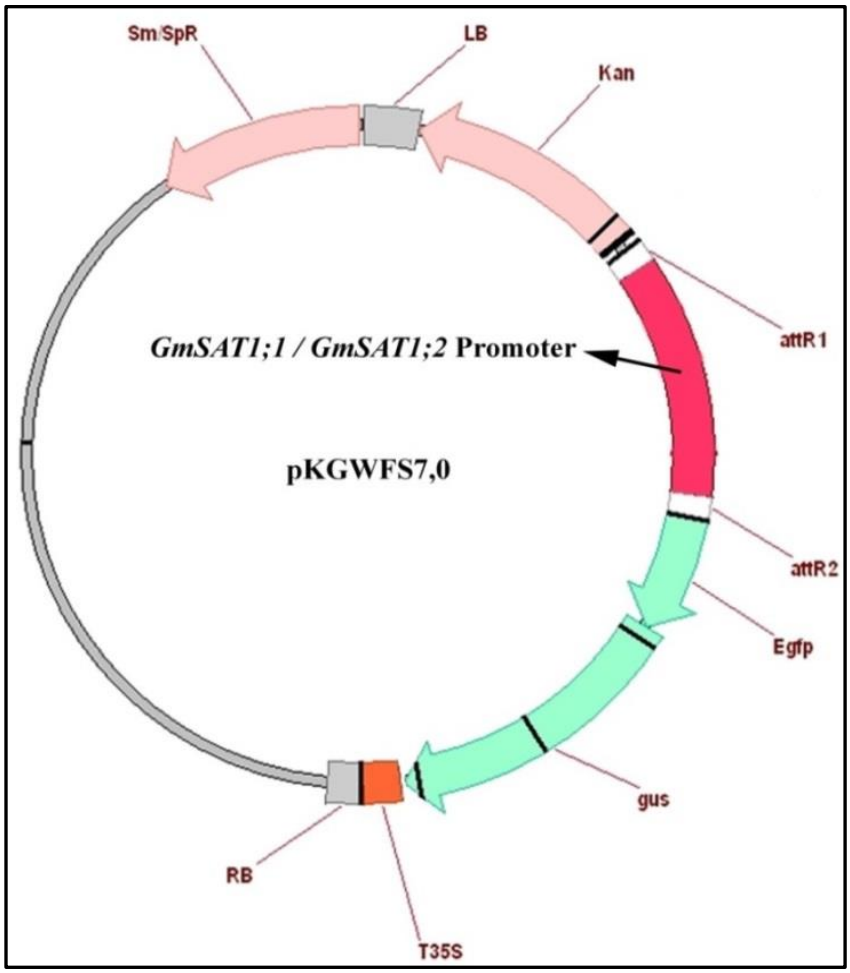


Figure 2.1 Binary construct used for promoter analysis.

The promoter of *GmSAT1;1* and *GmSAT1;2* was inserted into this vector in order to drive GUS/GFP expression (Karimi et al., 2002).

Soybean seedlings were transformed using an optimized *A. rhizogenes*-based genetic transformation protocol (see Chapter 3). Plants with transgenic hairy roots were transferred to larger individual 1 L pots containing sand to develop roots and or nodules. For GUS staining, one entire hairy root, with nodules, excised from the soybean plant was placed in a 50 ml falcon tube and filled with ice cold 90% acetone. Samples were then rinsed twice in sodium phosphate buffer at room temperature for 5 minutes. Samples were covered in GUS staining

buffer (0.1 M sodium phosphate buffer (pH 7), 3% sucrose, 50 mM sodium, 0.5 mM EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.05% (w/v) X-Gluc (5-bromo-4-chloro-3-indolyl-β-d-glucoronic acid) dissolved in 0.5 ml Dimethylformamide (DMF) and infiltrated under vacuum for 30 min before an extended incubation at 37° C for 5 hours. Samples were washed three times with MQ water and used for analysis.

2.4 Results

2.4.1 GmSAT1 is a member of a multi-gene family.

Using Phytozome (http://www.phytozome.net/) and blastn, a sequence based search for *GmSAT1;1* (Glyma15g06680) homologs was conducted. The results showed that *GmSAT1;1* (Glyma15g06680) has three other homologs in the soybean genome including, Glyma13g32650 (*GmSAT1;2*), Glyma05g23530 (*GmSAT2;1*) and Glyma17g16720 (*GmSAT2;2*). I also identified *GmSAT1*-like orthologs in *Arabidopsis*, *Medicago*, *Phaseolus vulgaris* and other plant species. Amino acid sequence alignment of *GmSAT1;1* showed all *SAT1* homologs are highly conserved within the predicted bHLH DNA binding domain, which is involved in the direct binding of DNA, and the transmembrane domain (TMD), while the N-terminal region is more variable (Figure 2.2).

Soybean has previously undergone two genome duplication events approximately 59 and 13 million years ago (Schmutz et al., 2010). The genome consists of many duplicated regions and it is predicted that greater than 75% of the genes within the genome are represented by multiple copies. Since the duplication events the genome has undergone significant gene diversification and loss and it is now considered a diploid genome with significant genetic duplication. Chromsomal sytneny analysis suggest *GmSAT1;1* and *GmSAT1;2* are clearly duplicated genes (Figure 2.4). Phylogenetic analysis showed that *GmSAT1;1* and *GmSAT1;2* cluster together with 89% sequence similarity at the amino acid level. Interestingly, *GmSAT2;1* and *GmSAT2;2* also have the same level of similarity with each other (Figure 2.3 A and B).

Examination of online mRNA expression datasets from soybean (http://www.plexdb.org/ and http://bar.utoronto.ca/welcome.htm,) showed that *GmSAT1;1* and *GmSAT1;2* are expressed mainly in root and nodule tissues, while *GmSAT2;1* and *GmSAT2;2* are expressed in both roots, nodule and shoots (Figure 2.5 A-D). Based on this analysis and previous evidence of *GmSAT1* expression patterns (Kaiser et al., 1998), *GmSAT1;1* and *GmSAT1;2* were evaluated further in both root and nodule tissues using quantitative PCR (qPCR).

2.4.2 Promoter analysis of GmSAT1;1 and GmSAT1;2

Twenty five day old nodules displayed strong GUS activity for *GmSAT1;1* and *GmSAT1;2*. A GUS signal was also detected in root tissues with the *GmSAT1;2* promoter (Figure 2.6 A and B). In a parallel study, both *GmSAT1;1* and *GmSAT1;2* were found expressed in both infected and uninfected cells of root nodules as well as the parenchyma and vascular cells that envelop the infected region of the nodule (Chiasson, 2012).

2.4.3: Expression of GmSAT1;1 and GmSAT1;2 during root and nodule development in the presence of Rhizobia

Rhizobium inoculated soybean seedlings (3 days after planting) were grown for a period of 13 and 40 d in the presence or absence of 2.5 mM NH_4NO_3 (Figure 2.7 A and B). Using qPCR, the expression of *GmSAT1;1* and *GmSAT1;2* in root and nodule tissues was evaluated and is presented in Figure 2.7. In the absence of N, *GmSAT1;1* expression was significantly higher in nodules compared to the roots (Figure 2.7 C). Interestingly, the highest expression for *GmSAT1;1* occurred between days 19-25, which corresponds to a period of nodule development. Furthermore according to previous studies this is the period where N_2-fixation commences (Schubert, 1981; Marcker et al., 1984; Kohl et al., 1990; Sato et al., 2001). *GmSAT1;2* displayed a different expression pattern to that of *GmSAT1;1*, where there was no significant difference in expression of *GmSAT1;2* between nodule and root tissues across the growth period (Figure 2.7 D).

In the presence of 2.5 mM NH_4NO_3, *GmSAT1;1* expression was significantly down-regulated in both root and nodules (Figure 2.7 E). This decrease was most dramatic after 19 d. This response to N is consistent with physiological responses in nodulated roots where N negatively impacts both nodule development and N_2-fixation rates (Imsande, 1986; Kirsch et al., 2001; Fujikake et al., 2011; Naudin et al., 2011). With N supply, expression of *GmSAT1;2* was reduced similar to that of *GmSAT1;1* across both tissues. Again like *GmSAT1;1*, *GmSAT1;2* expression was maintained till about 19 days after planting and then decreased quickly thereafter in both tissues (Figure 2.7 F). Interestingly in contrast with the minus N treatment, expression of *GmSAT1;2* in root was significantly ($P \leq 0.05$) higher than in the nodule.

2.4.4: Expression pattern of GmSAT1;1 and GmSAT1;2 during root development in non -inoculated roots

In this experiment, the expression of *GmSAT1;1* and *GmSAT1;2* in non-inoculated roots of soybeans grown in the presence or absence of 2.5 mM NH_4NO_3 was investigated. Non-inoculated roots were harvested at 4-day intervals until 32 d after germination (Figure 2.8 A and B). RNA was extracted and qPCR performed using *GmSAT1;1* and *GmSAT1;2* primers (Table 2.2). In the presence of N and in the absence of rhizobia, the expression of *GmSAT1;1* was found to be significantly lower than *GmSAT1;2* (Figure 2.8 C and D). Under N deficient conditions *GmSAT1;1* and *GmSAT1;2* expression was less than the + N grown plants however down-regulation of *GmSAT1;1* expression was not significant. *GmSAT1;2* expression did increase as plants grew older (from 12-24 d) then declined prior to the final harvest. *GmSAT1;1* expression remained unchanged during the growth period. There was no N starvation response for either gene (Figure 2.8 C and D).

2.4.5: The influence of N supply on GmSAT1;1 and GmSAT1;2 expression

The aim of this experiment was to define whether *GmSAT1;1* and *GmSAT1;2* expression responds to a short-term (24 and 72 hours) change in N availability (supply or removal of N) in both nodules of inoculated plants and root of non-inoculated plant (Figure 2.9).

In nodules, the supply of N to minus N grown plants quickly reduced *GmSAT1;1* and *GmSAT1;2* expression. This was followed by a recovery of expression of both genes two days later (Figure 2.9 A). However in roots, this reduction in expression was not significant (Figure 2.9 B). The removal of N from plus N grown plants were less dramatic. In roots, *GmSAT1;1* expression increased after 24 h of treatment, then returned to a basal level of expression by 72 h (Figure 2.9 D). In contrast, *GmSAT1;2* expression in roots remained unchanged over the three days. In nodules, there was a significant ($P \leq 0.05$) increase in *GmSAT1;1* and *GmSAT1;2* expression 3 d after removal of N (Figure 2.9 C).

2.5 Discussion

2.5.1 GmSAT1;1 and GmSAT1;2 are active during nodule development but only GmSAT1;1 behaves as a nodule enhanced gene

The functional role of *GmSAT1;1* is still to be defined. In this chapter, I completed a series of experiments to examine *GmSAT1;1* and *GmSAT1;2* expression across the development period that covers the initiation of N_2-fixation in nodules and secondly how both genes respond to N when supplied to plants grown with or without the presence of rhizobia. Previous studies that looked at *GmSAT1* expression primarily focused on the tissue specific expression patterns which indicated *GmSAT1;1* was a nodule enhanced gene (Kaiser et al., 1998; Loughlin, 2007). However there were no report on expression of *GmSAT1;2* and effect of N on expression of *GmSAT1;1* and *GmSAT1;2*. In this study, I have confirmed that *GmSAT1;1* is preferentially expressed in nodules compared to roots particularly when grown in the absence of external N. An interesting aspect of this, is that *GmSAT1;1* expression in nodules coincided with the period where nodule N_2-fixation begins (19-25 d after inoculation) (Figure 2.7 C). Similar trends were not observed with *GmSAT1;2* (Figure 2.7 D), where root and nodule expression were similar and with no obvious relationship with the onset of N_2-fixation. These results reinforce the critical role of *GmSAT1;1* in nodule development and the N_2-fixation process. In support, the promoters of both *GmSAT1;1* and *GmSAT1;2* demonstrated that both

genes are expressed in nodules but only *GmSAT1;1* appears to be nodule enhanced, while *GmSAT1;2* also showed GUS activity in root tissues (Figure 2.6).

2.5.2 Short-term responses GmSAT1 expression to N supply and removal

Previous experiments demonstrated that the expression of *GmSAT1;1* and *GmSAT1;2* was down regulated with extended supply of N in both nodule and root tissues. However in the absence of rhizobia, N stimulated expression of both genes, which increased as plants aged. There is a possibility that this increase may be solely due to N-starved plants (-N treatment) growing poorly and the +N treatments showed better expression as a result. The short-term N supply and starvation experiment (Figure 2.9) indicated N supply results in a quick reduction in *GmSAT1;1* and *GmSAT1;2* expression in nodules but then recovers over the next few days ($P \leq 0.05$). When N was removed over a three-day period there was a significant increase in expression of both genes in nodule (Figure 2.9 C). However in roots there was only a significant increase ($P \leq 0.05$) in expression of *GmSAT1;1* after 24 hours (Figure 2.9 D). This supports the notion that the expression response is N linked but may require secondary signaling involving the symbiosis with rhizobia.

Table 2.1 Experimental plan to monitor *GmSAT1;1* and *GmSAT1;2* expression across developmental and N nutritional states in both nodules and root tissues.

Experiment	*Treatment*	*Rhizobia inoculation*	*Nitrogen (2.5 mM NH_4NO_3)*	*Studied tissues*	
				Root	Nodule
Expression of GmSAT1;1 & GmSAT1;2 in inoculated plants	Treatment 1	√	√	√	√
	Treatment 2	√	-	√	√
Expression of GmSAT1;1 & GmSAT1;2 in non- inoculated plants	Treatment 1	-	√	√	-
	Treatment 2	-	-	√	-
Effect of N supply on expression of GmSAT1;1 & GmSAT1;2	Treatment 1	+Rhizobia, +Nitrogen → Removal of nitrogen source			√
	Treatment 2	+Rhizobia, –Nitrogen → Addition of nitrogen source			√
	Treatment 3	-Rhizobia, +Nitrogen → Removal of nitrogen source		√	
	Treatment 4	-Rhizobia, –Nitrogen → Addition of nitrogen source		√	

Table 2.2 List of primers used.

Name	*Sequence*
GmSAT1;1qPCR F	TTATTGCTCAGATGGATATGGAA
GmSAT1;1qPCR R	GACGACCGAAAAATGCATAAC
GmSAT1;2qPCR F	TTATTGCTCAGATGGATATGGAA
GmSAT1;2qPCR R	TCGGAATGCATAACGAGTTTC
cons6 F	AGATAGGGAAATGGTGCAGGT
cons6 R	CTAATGGCAATTGCAGCTCTC
GmSAT1;1 PRO F	CAAGGATTAGGATTATCCAC
GmSAT1;1 PRO R	AGAAAGCCACATATACTC
GmSAT1;2 PRO F	CAATAGTCACCAATAGAG
GmSAT1;2 PRO R	GAGAACAATTCTTTGGAACTTGC

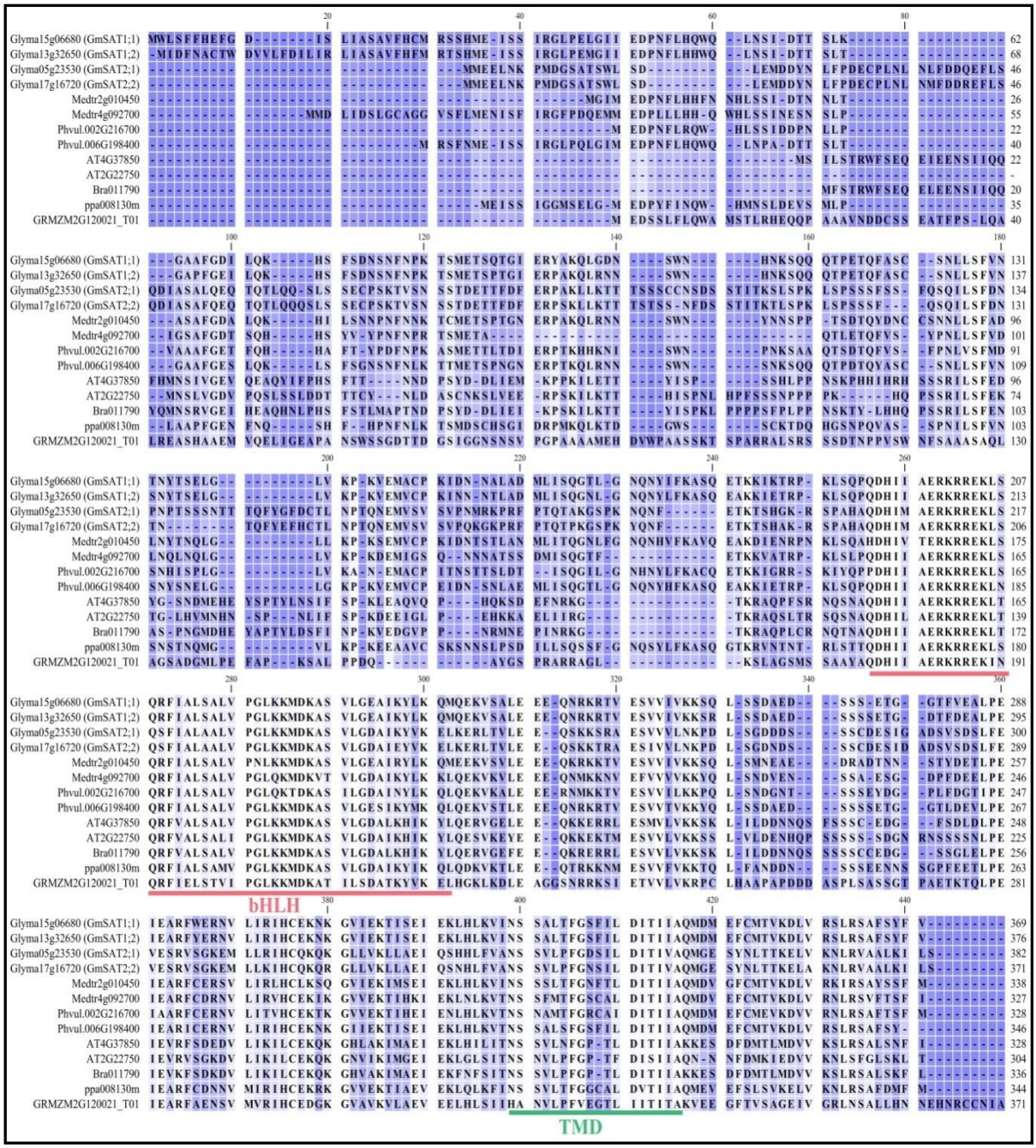


Figure 2.2 Clustal alignment of protein sequences from SAT homologs in soybean and other plants.

Amino acid alignments of SAT proteins were completed using CLCbio Genomics Workbench (http://www.clcbio.com/) in different plants including soybean (Glyma15g06680, Glyma13g32650, Glyma05g23530, and Glyma17g16720), *Medicago* (Medtr2g010450 and Medtr4g092700), *Arabidopsis* (AT4G37850 and AT2g22750), *Phaseolus vulgaris* (Phvul.006G198400 and Phvul.002G216700), *Prunus persica* (ppa008130m.g), *Zea* mays (GRMZM2G120021) and *Brassica rapa* (Bra011790). The putative bHLH region is indicated by a red line and transmembrane domain (TMD) by a green line. Predict protein web site (https://www.predictprotein.org/) used to predict TMD.

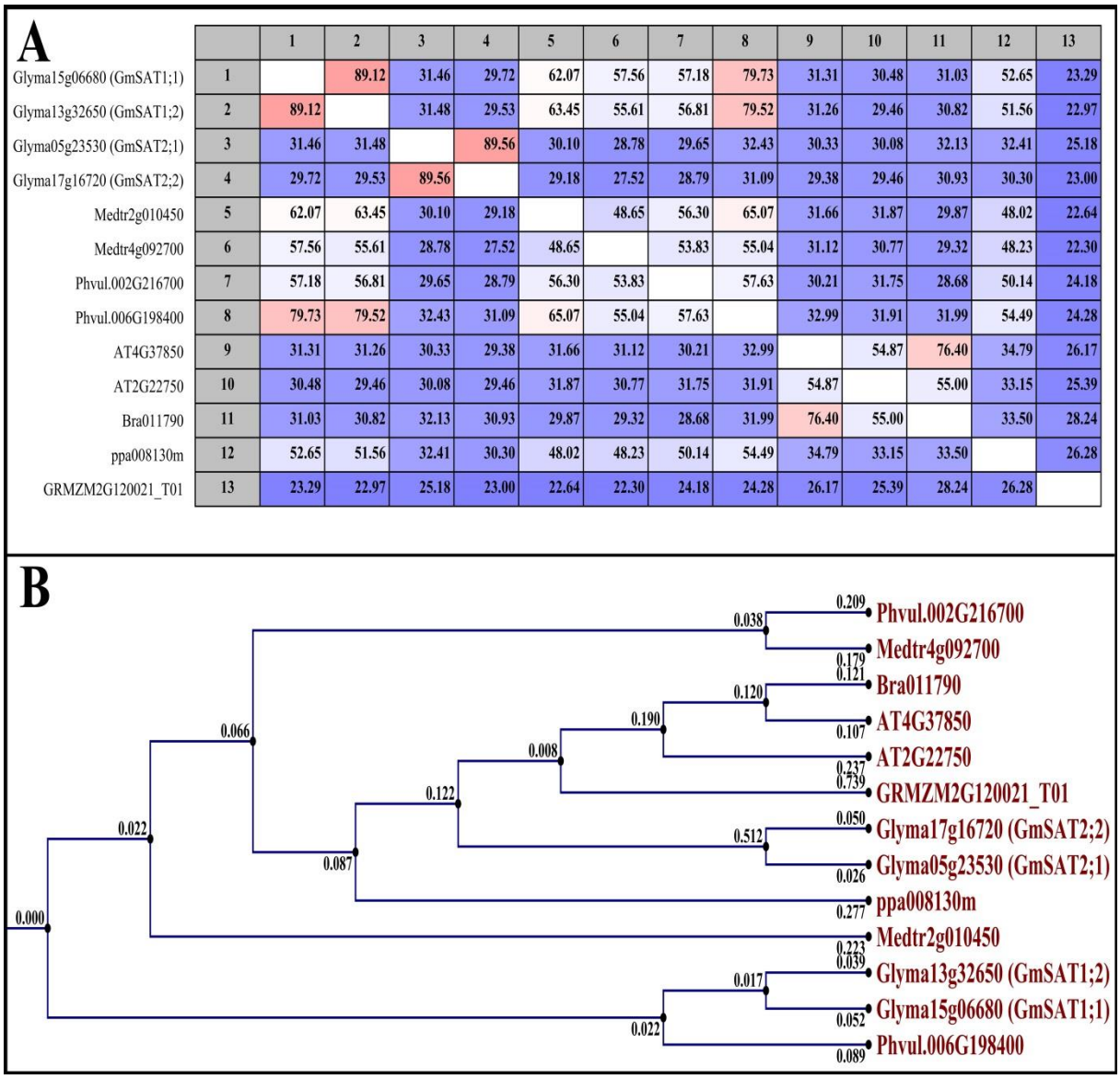


Figure 2.3 Identity table and phylogeny tree of SAT homologues in soybean and other plants.

A) Percentages of identity of amino acids and B) phylogenetic tree were calculated and created using CLCbio Genomics Workbench (http://www.clcbio.com/). *GmSAT1;1* shared around 89% amino acid with *GmSAT1;2* and *GmSAT2;1* shared the same precent of identity with *GmSAT2;2*. The strongest identity to other plants included the legumes *P. vulgaris* (Phvul.006G198400) and *M. truncatula* (Medtr2g010450) with 79 and 62 % identity, respectively.

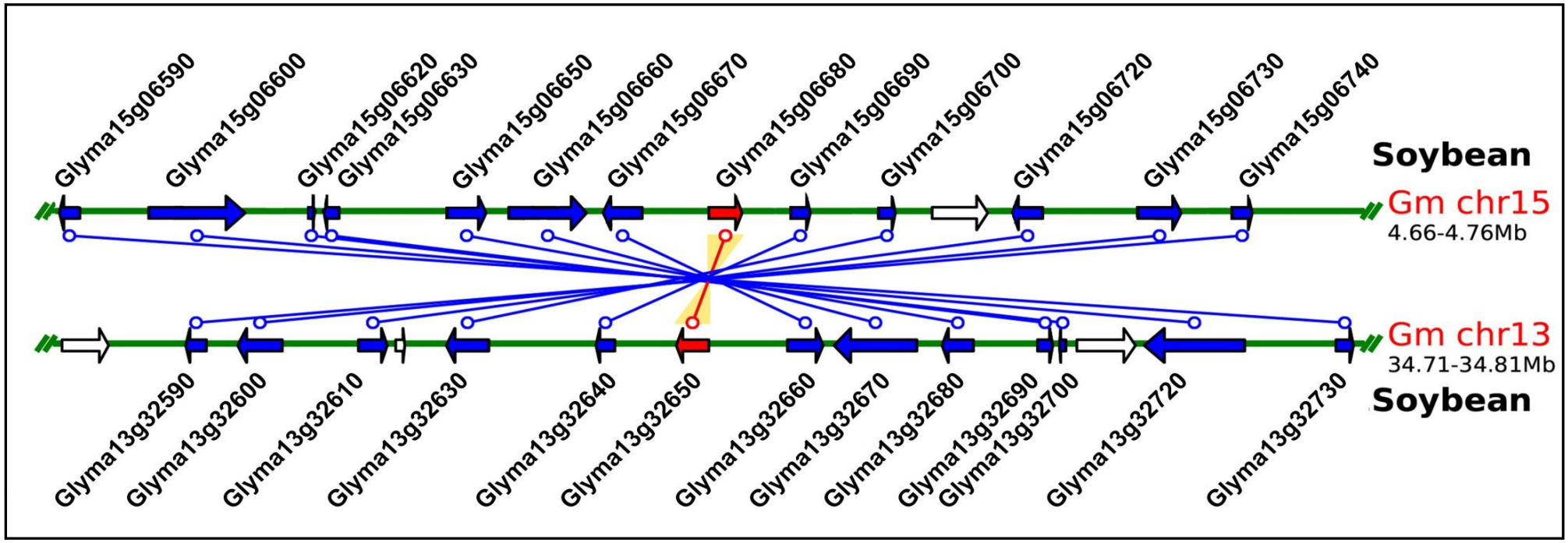


Figure 2.4 Syntenic relationship *GmSAT1;1* and *GmSAT1;2*.

Syntenic relationship of 100 kb of chromosome 15 and chromosome 17 of soybean where *GmSAT1;1* (Glyma15g06680) and *GmSAT1;2* (Glyma13g32650) are located. Chromsomal sytneny analysis was performed using the Genome Duplication Database (http://chibba.agtec.uga.edu/duplication/index/home). *GmSAT1;1* and *GmSAT1;2* are shown in red.

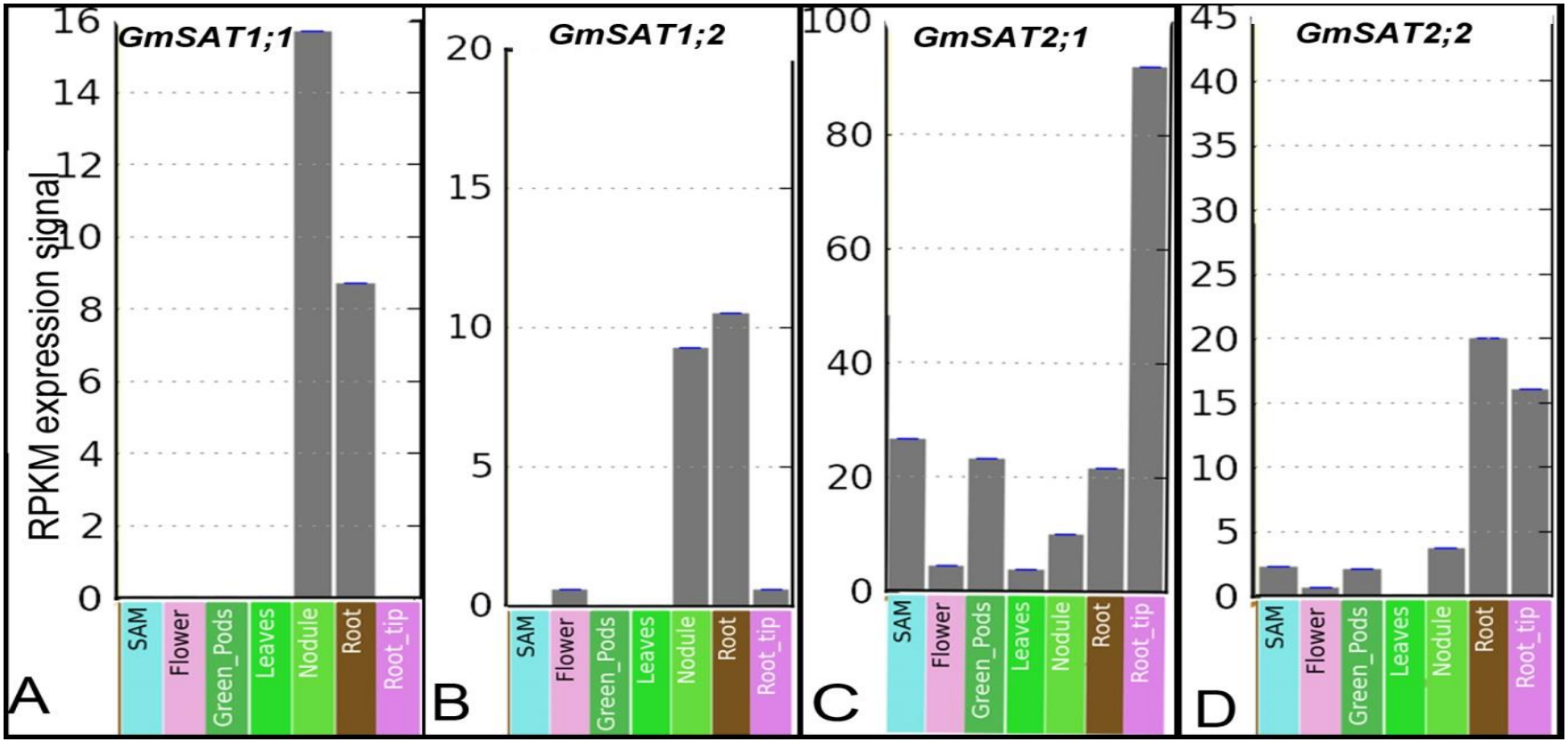


Figure 2.5 *In silico* expression analysis.

In silico expression analysis of *GmSAT1;1* (Glyma15g06680), *GmSAT1;2* (Glyma13g32650), *GmSAT2;1* (Glyma05g23530), and *GmSAT2;2* (Glyma17g16720) using the BAR database (http://bar.utoronto.ca/welcome.htm). Expression is expressed as RPKM (Reads per kilo base of transcript per million total reads). Plant tissues from Minsoy line of soybean (*G. max*) were harvested 3 weeks after germination and inoculated with *B. japonicum* USDA 110 (Langlois-Meurinne et al., 2005).

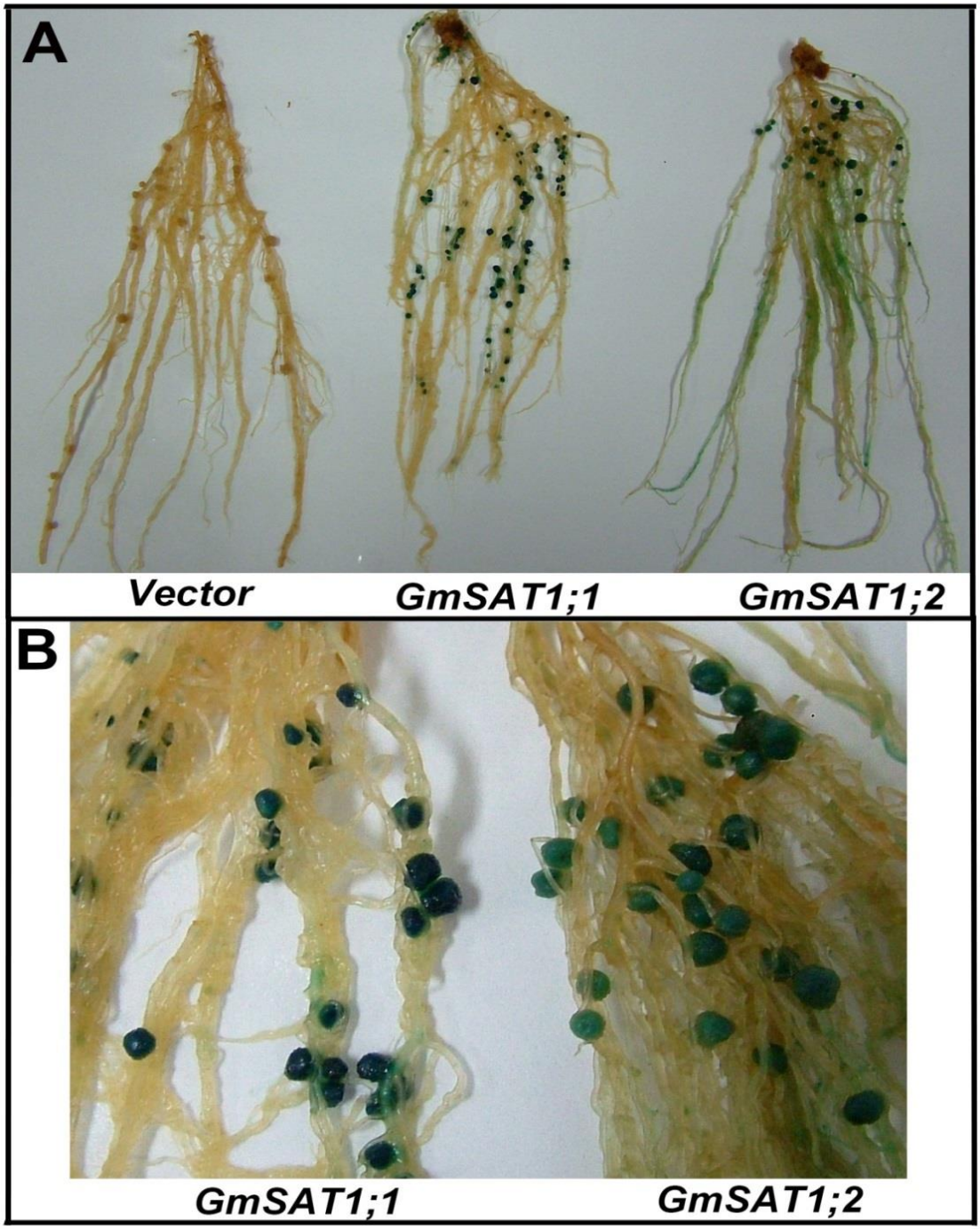


Figure 2.6 *GmSAT1;1* and *GmSAT1;2* promoter activity visualized using a *GUS* reporter in *A. rhizogenes* transformed roots and nodules.

A) *GmSAT1;1* and *GmSAT1;2* promoters are active in nodule tissues. *GmSAT1;2* promoter activity is also observed in root tissues. No *GUS* expression was observed in the empty vector control. B) *GUS* staining of nodules (*GUS* activity indicated by blue stain) based on the expression of either *GmSAT1;1* or *GmSAT1;2* promoters.

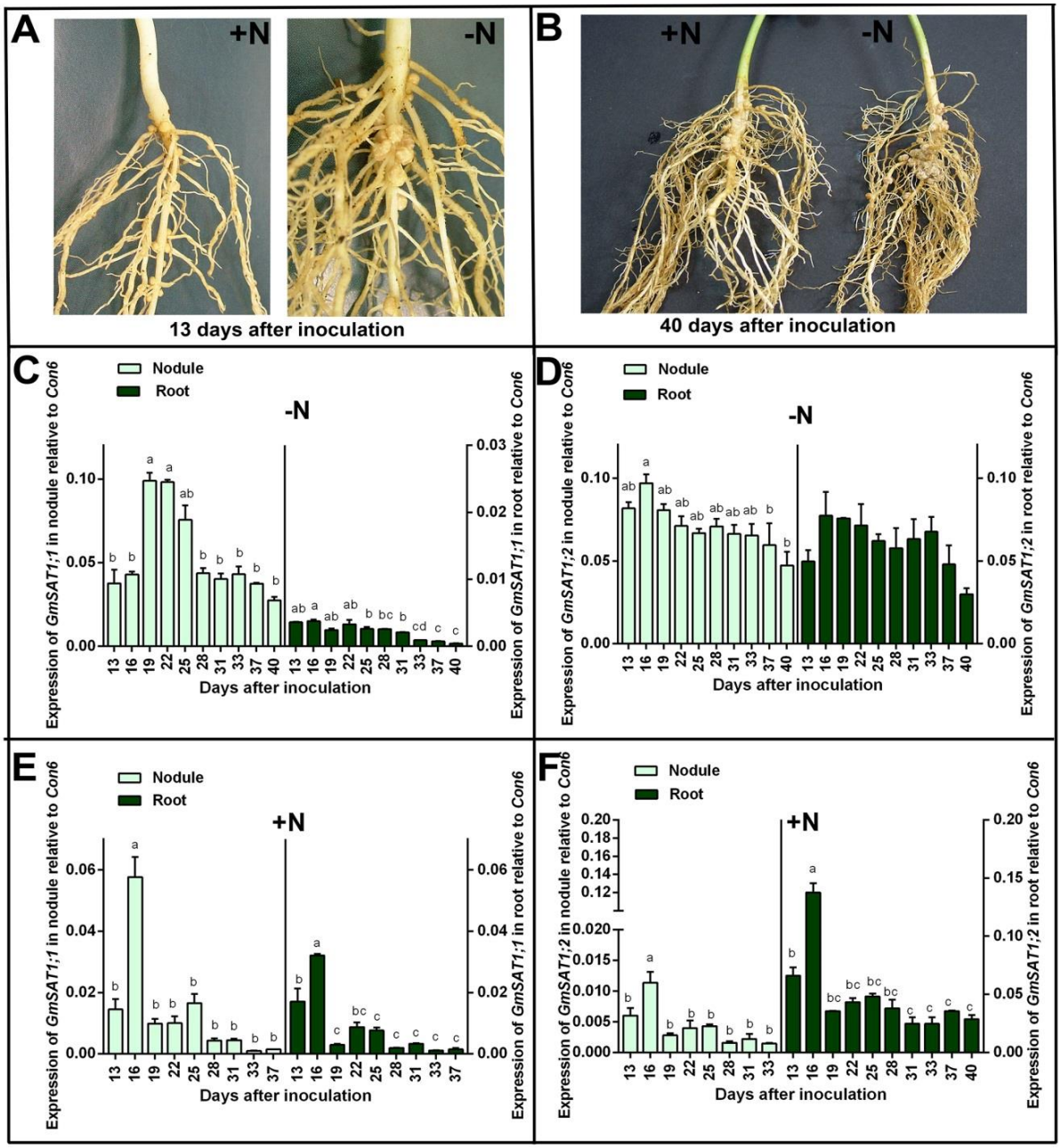


Figure 2.7: Expression pattern of *GmSAT1;1* and *GmSAT1;2* during root and nodule development in the presence (+N) or absence (-N).

(A-B) Pictures of nodulated soybean roots at 13 and 40 d after rhizobia inoculation with and without N. (C, D) Expression of *GmSAT1;1* and *GmSAT1;2* without 2.5mM NH_4NO_3. (E, F) Expression of *GmSAT1;1* and *GmSAT1;2* in nodules and roots grown with 2.5mM NH_4NO_3. Data represents the mean ± SE (n=3 plants).

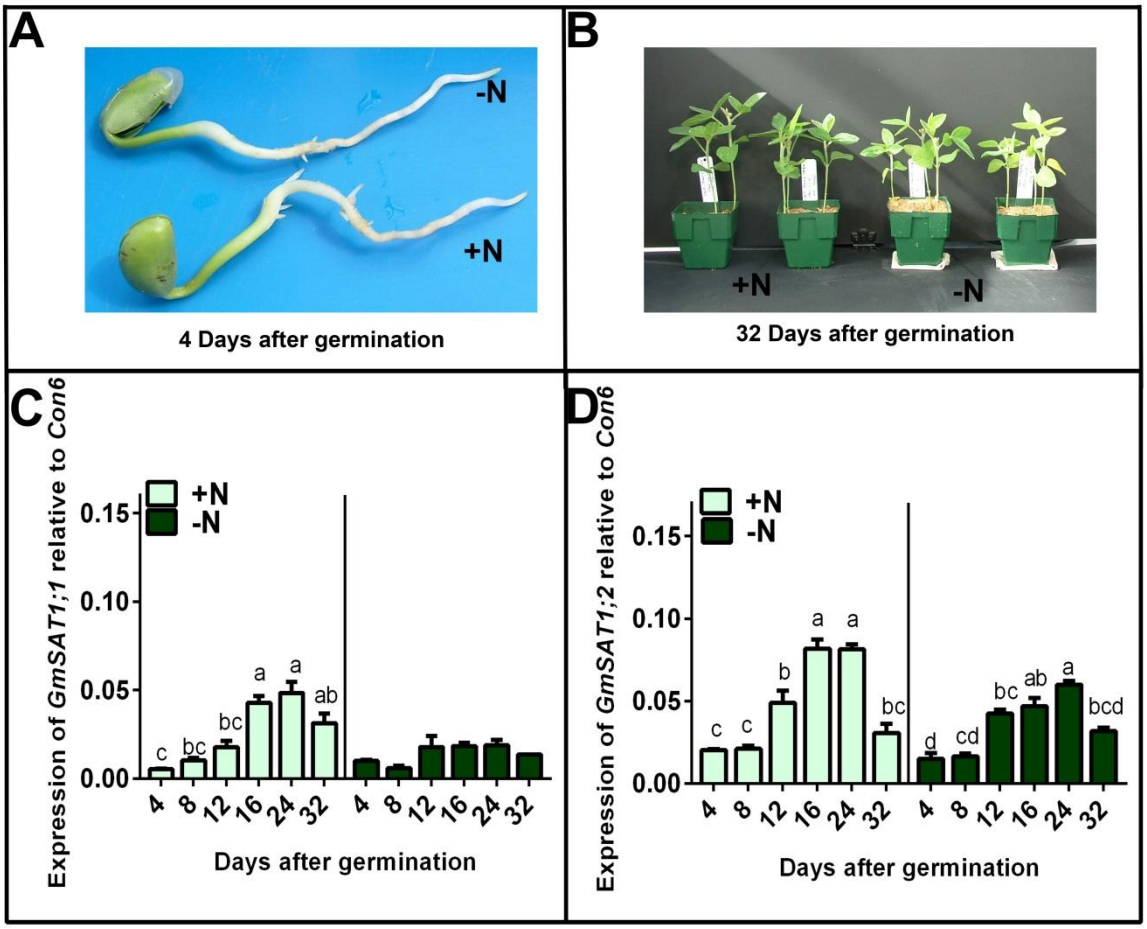
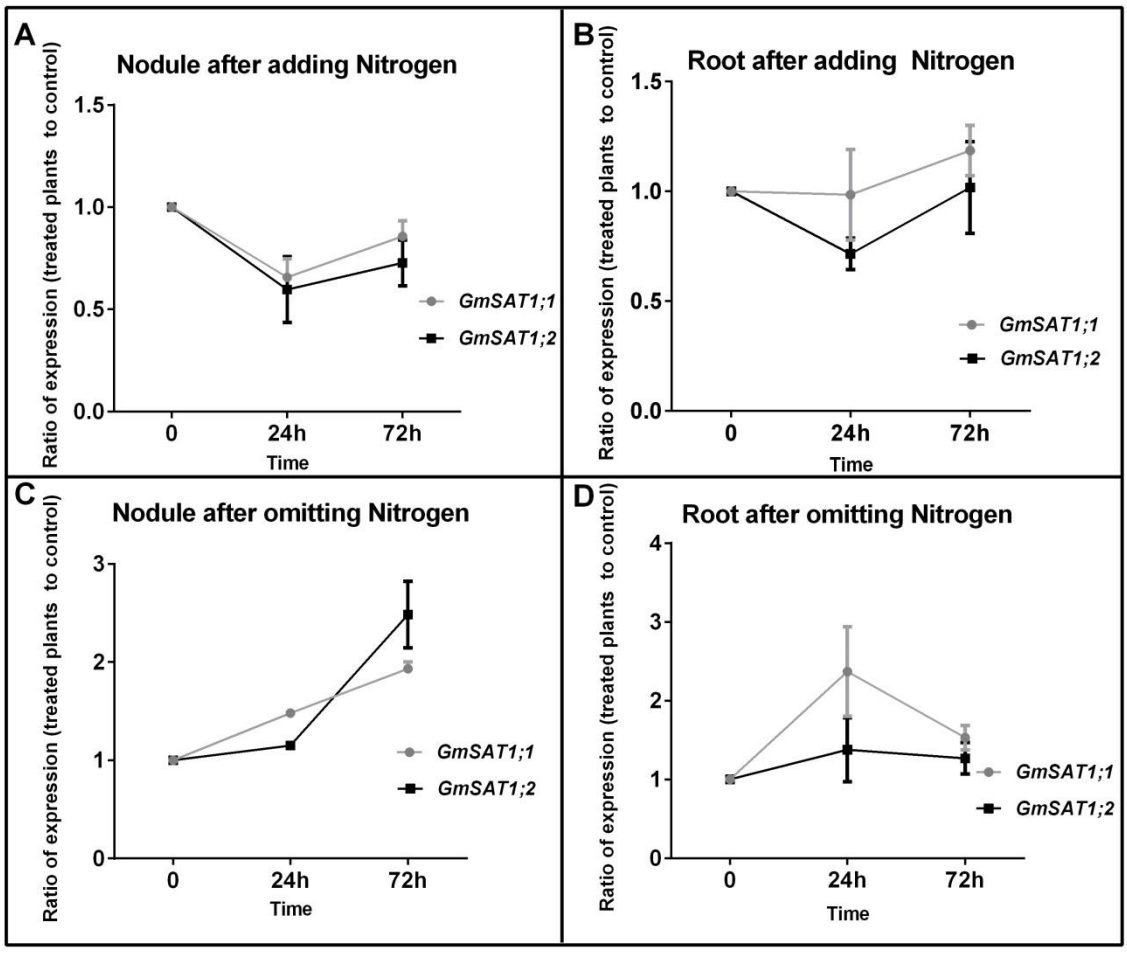


Figure 2.8: Expression of *GmSAT1;1* and *GmSAT1;2* in non-inoculated roots.

Root tissues were collected every 4 d from 4 to 32 d after germination. (A-B) Plant images at 4 (A) and 32 (B) d after germination. (C) Root *GmSAT1;1* expression in the presence and absence of N source in root tissue. (D) Root *GmSAT1;2* expression in the presence and absence of N source in root tissue. Data represents mean ± SE (n=3 plants).

Figure 2.9: The effects of changes in N availability on *GmSAT1;1* and *GmSAT1;2* expression.

(A, C) *GmSAT1;1* and *GmSAT1;2* expression in nodules (A) and roots (C) after removal of nitrogen (-N) from the growth medium. (B, D) *GmSAT1;1* and *GmSAT1;2* expression in nodules (B) and roots (D) after supply of nitrogen (+N) to plants previously grown in the absence of external N (-N). Data represents the mean ± SE (n=3 plants).



Chapter 3

3. A novel method based on combination of *semi-in vitro* and *in vivo* conditions in *Agrobacterium rhizogenes*-mediated hairy root transformation of *Glycine* species

3.1 Abstract

Despite numerous advantages of the many tissue culture-independent hairy-root transformation protocols, the process is often compromised in the initial *in vitro* culture stage where inability to maintain high humidity and the delivery of nourishing culture medium decreases cellular morphogenesis and organ formation efficiency. Ultimately this influences the effective transfer of produced plantlets during transfer from *in vitro* to *in vivo* conditions, where low survival rates occur during the acclimation period. We have developed an intermediate protocol for *Agrobacterium rhizogenes* transformation in *Glycine* species by combining a two-step *in vitro* and *in vivo* process that greatly enhances the efficiency of hairy root formation, and which simplifies the maintenance of the transformed roots. In this protocol, cotyledonary nodes of *Glycine max* and *G. canescens* seedlings were infected by *A. rhizogenes* K599 carrying a reporter gene construct constitutively expressing GFP. Glass containers containing sand and nutrient solution were employed to provide a moist clean microenvironment for the generation of hairy roots from inoculated seedlings. Transgenic roots were then non-invasively identified from non-transgenic roots based on the detection of GFP. Main roots and non-transgenic roots were removed leaving transgenic hairy roots to support seedling development, all within one month of beginning the experiment. Overall, this protocol increased the transformation efficiency by more than 2-fold over traditional methods. Approximately 88% and 100% of infected plants developed hairy roots from *G. max* and *G. canescens,* respectively. On average, each infected plant

produced 10.9 transformed hairy roots in *G. max* and 13–20 in *G. canescens*. Introduction of this simple protocol is a significant advance that eliminates the long and genotype-dependent tissue culture procedure while taking advantage of its optimum *in vitro* qualities to enhance the micropropagation rate. This research will support the increasing use of transient transgenic hairy roots for the study of plant root biology and symbiotic interactions with *Rhizobium* spp.

3.2 Introduction

Soybean (*Glycine max* [L.] Merr.) is one of the world's most important cultivated legume crops due to its high seed oil and protein content. The genus *Glycine* is divided into two subgenera *Soja* and *Glycine* (Pueppke, 1988) . The subgenus *Soja* contains two annual species: *G. max* and *G. soja*. The subgenus *Glycine* also includes 24 perennial wild species indigenous to Australia. The cultivated soybean has a relatively narrow genetic base hindering its further genetic improvement (Hartman et al., 2000; Lu, 2004). In contrast, *G. canescens*, a wild Australian relative of soybean, has a continent-wide distribution and can serve as a source of new genes for disease resistance, nitrogen fixation capacity, and other desirable agronomic traits (Brown et al., 1990; Hartman et al., 2000).

Identifying the mechanisms regulating root-based symbiotic associations between legumes and soil-borne bacteria of the nitrogen-fixing Rhizobiaceae family requires an efficient access to transgenic capabilities (Kereszt et al., 2007). *Agrobacterium rhizogenes*-mediated hairy-root transformation is now a common tool to introduce targeted modification of root and/or nodule expressed genes for forward and reverse genetic approaches. *A. rhizogenes* hairy-root transformation systems were first developed in the model legume, *Lotus corniculatus* (Jensen et al., 1986). Transformed hairy roots provide the opportunity of biotic interaction studies and examining gene function without the need to produce fully transformed plants (Parry et al., 2009). Moreover, transformed hairy roots can be utilized for further cycles of tissue culture to produce fully transformed plants (Vidal et al., 2010).

A. rhizogenes transformation procedures can be performed via one of the following main strategies: *in vitro* (tissue culture-based) and the direct *in planta* (tissue culture-free or *ex vitro* method). Tissue culture-based methods include excising and disinfection of explants, and further culturing of sterilized explants on optimized *in vitro* culture media to accelerate organ proliferation and morphogenesis. For soybean regeneration, segments of hypocotyls, primary-nodes, and cotyledons, have successfully been used as explants for *in vitro* culture (Rech et al., 1988; Olhoft et al., 2007; Kouas et al., 2009). In particular, the use of cotyledonary nodes as explants greatly increases transformation rates and the production of transgenic plants (Olhoft et al., 2003; Pacios-Bras et al., 2003; Somers et al., 2003).

Unfortunately, *in vitro* methods are time consuming, expensive, and often genotype-dependent requiring extensive optimization (Henzi et al., 2000; Somers et al., 2003; Subramanian et al., 2008; Cao et al., 2009; Suzaki et al., 2013). Furthermore, *in vitro* produced plantlets can have a low rate of survival at the time of transferring from *in vitro* to *in vivo* growing conditions (acclimation; (Murray, 2011). In recent years, the use of tissue culture-free (*in planta* or *ex vitro*) root transformation protocols have been widely employed due to their rapidity, low cost, technical simplicity, and ability to overcome genotype specific dependencies (Somers et al., 2003). Development of transgenic nodules directly on the transgenic roots was first demonstrated by Hansen et al. (1989) in *L. corniculatus*, using a protocol used to this day (Hansen et al., 1989). Since then, *A. rhizogenes* transformation has been reported for many legumes including *L. japonicas* (Stiller et al., 1997), *Medicago truncatula* (Boisson-Dernier et al., 2001), *G. max* (Cheon et al., 1993; Kereszt et al., 2007; Suzaki et al., 2013), and *Trifolium repens* (Diaz et al., 1989).

The current *in planta* protocol of soybean employs a rapid tissue-culture-free procedure for induction and growth of transgenic roots by inoculation of *A. rhizogenes* in the cotyledonary node of young seedlings (Kereszt et al., 2007). A pre-requisite for this technique is the requirement to maintain high humidity levels at the infection site for 2–3 weeks for successful transformation (Kereszt et al., 2007). Although possible to achieve, the logistics of maintaining

such conditions outside traditional tissue-culture based-approaches can be problematic often leading to increased incidence of fungal infection and disease, and the loss of successful transformed plants. In addition, the produced transgenic hairy roots cannot be directly used as tissue culture explants and require further disinfection.

Altogether, *in vitro* culture conditions provide an optimum environment for morphogenesis and organ development by maintaining high levels of humidity and adequate nourishment. When omitted within an *in vivo* method, difficulties occur in providing an appropriate micropropagation environment for successful organ development, resulting in lower *in planta* transformation efficiencies.

Here we have combined both *in vitro* and *in vivo* strategies in a hairy-root transformation protocol to exploit the rapidity, ease, and genotype-independency of the *in planta* transformation method, but with the high micropropagation capability of *in vitro* procedures. To do this we have modified the *A. rhizogenes*-mediated transformation protocol previously published by Kereszt et al. (2007) and applied that to *G. max* and *G. canescens* by keeping the infected seedlings in a semi-*in vitro* condition where the efficiency of adventitious hairy root production as well as transformation frequency were found to be greatly improved. Furthermore, we used the pK7GWIWG2D(II) vector for expressing the green fluorescent protein (*GFP*) in both *Glycine* species. Stable *GFP* expression was demonstrated in elongated roots and nodules after infection of transformed hairy roots with different strains of rhizobium.

3.3 Materials and Methods

3.3.1 Plant material

Commercial soybean (*Glycine max* cv. 'Djakal') seed was sourced from New South Wales Department of Primary Industries. Two accessions of *G. canescens* (1270 and 1301) were kindly provided by Dr. Anthony HD Brown CSIRO Plant Industry, Canberra, Australia.

3.3.2 Rhizobium strains

Bradyrhizobium japonicum USDA 110 was provided by Prof. Peter Gresshoff, The University of Queensland and used for inoculation of *G. max*. *Rhizobium* sp. CC1601a was used for inoculum of the two accessions of *G. canescens*. *Rhizobium* sp. (CC1601a) was kindly provided by Dr. J. Brockwell, Division of Plant Industry, CSIRO. CC1601a was first isolated from a *G. canescens* plant grown at Black Mountain, ACT, Australia (Pueppke, 1988).

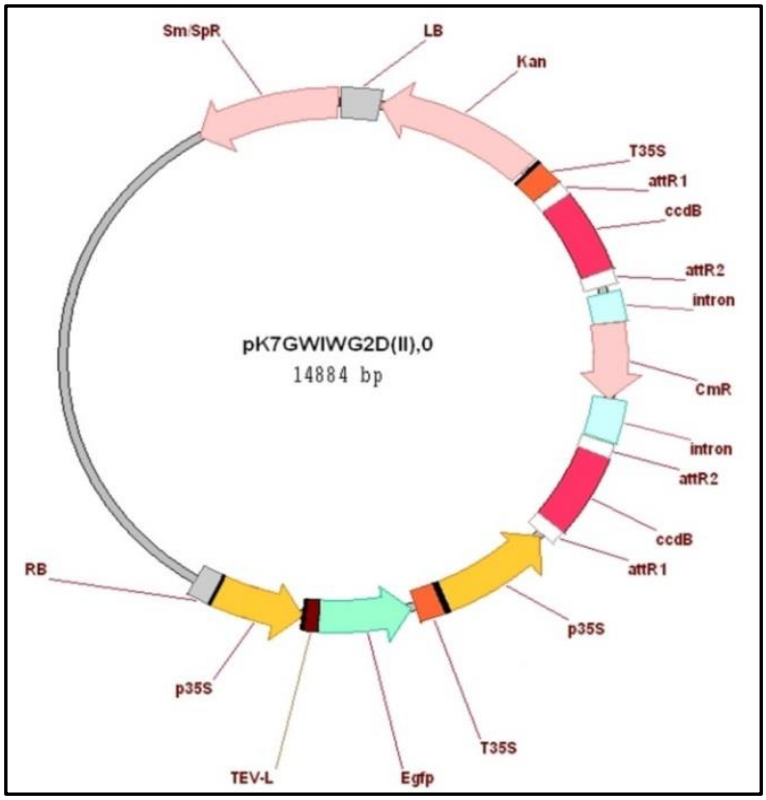


Figure 3.1 Map of pK7GWIWG2D(II) vector used for *A. rhizogenes* K599 hairy root transformation.

The 35S promoter and 35S UTR sequences drive *gfp* and *nptII* (kan) genes (Karami et al. 2002).

3.3.3 Agrobacterium rhizogenes strain and vector plasmid

Agrobacterium rhizogenes (strain K599) was provided by Prof. Peter Gresshoff, University of Queensland. We transformed the T-DNA binary vector pK7GWIWG2D(II) (Karimi et al., 2002) into *A. rhizogenes* which was then used for hairy roots transformation (Figure 3.1). The vector contains the following genes: (1) the selectable marker *nptII* which encodes neomycin phosphotransferase for kanamycin resistance, (2) enhanced green fluorescent protein (*egfp*)

driven by the 35S promoter (*p35S*), and the (3) spectinomycin resistance gene (*Sm/Spr*) for plasmid selection (Figure 3.1).

3.3.4 Seed germination

G. max cv. 'Djackl' and *G. canescens* seeds were surface-sterilized with a hydrogen peroxide/ethanol mix (3% H_2O_2 made up in 70% ethanol). Seeds were surface-sterilized for 2 min by gentle inversion in the hydrogen peroxide/ethanol mix and then rinsed 5–6 times with an excess amount of sterile distilled water (Kereszt et al., 2007).

Seeds were germinated in large (15 cm diameter) sterile Petri dishes containing 2 layers of sterile Whatman™ paper. Under aseptic conditions, ten seeds were placed into each dish followed by the addition of 10–12 mL of sterile distilled water. The Petri dishes were sealed with Parafilm™ and incubated for 5–6 d at 26–29°C in a chamber with minimal lighting (100 PAR, 12-h day, 12-h night). The germinated seeds were allowed to grow to at least 3–5 cm (root tip to expanding cotyledons) and where cotyledons reached either a folded stage in *G. max* (Figure 3.2*A*) or un-folded stage in *G. canescens* (Figure 3.3A).

3.3.5 Preparation of A. rhizogenes K599 for inoculation with additional refreshment

Four days prior to inoculation, *A. rhizogenes* K599 was cultured on LB plates containing 150 mg/L spectinomycin and incubated at 28ºC for 2 d. Two days before inoculation, a single colony was inoculated into 10 mL of LB medium with 150 mg/L spectinomycin and grown overnight at 28ºC with shaking at 200 rpm. One hundred microliters of the overnight grown culture was transferred to 15 mL LB with 150 mg/L spectinomycin and grown to an OD_{600} = 1.2–1.5. Bacteria were collected by centrifugation for 5 min at 5000 rpm. The pellet was resuspended in 10 mL of sterile MilliQ water, mixed and centrifuged for 5 min at 5000 rpm. The supernatant was discarded and the cell pellet re-suspended in 70 µL of sterile MilliQ water and used for inoculation. This suspension was enough for inoculating at least 20 plants.

3.3.6 Preparation of semi-in vitro conditions

South Australian Waikerie sand, 16–20 grades (Sloan's Sands Pty Ltd, Dry Creek, SA) was washed twice with water and then oven-dried for 12 h at 200°C. Glass jars (15 cm height x 6 cm width and 10.5 cm height x 4.5 cm width) were used for *G. max* and *G. canescens*, respectively. The jars were filled with oven-dried sand (3–4 cm). Nutrient medium (Table 3.1) was added (40–60 mL) to each jar, and the tops of the jars were covered with aluminum foil. The jars containing sand and medium were autoclaved at 121°C for 20 min prior to use.

3.3.7 Inoculation of Agrobacterium

G. max and *G. canescens* seedlings (3–5 cm length, approximately 5–6 d after seed culture) were used for transformation experiments (Figure 2 and 3). Using sterile conditions and in a laminar flow hood, 2–3 µL of *A. rhizogenes* K599 suspension was injected using a 1-mL, 16-G syringe needle into the cotyledonary node and/or proximal part of hypocotyl next to the cotyledon (upper hypocotyl) as shown in Figure 2*A* and Figure 3*A*. To inject ~2–3 µL of *A. rhizogenes* with a 1-mL syringe, the 16-G needle was placed into the prepared *A. rhizogenes* liquid stock with the syringe plunger pushed inward. Pressure was then released from the syringe plunger resulting in ~2–3 µL of *A. rhizogenes* culture being transferred into the needle tip. The needle and culture was then injected into the center of the tissue.

3.3.8 Transferring the infected seedling to semi-in vitro conditions

To enable a higher morphogenesis capacity, the infected plants were transferred to a semi-*in vitro* culturing facility (i.e., the previously described filled jars with sand and nutrient medium). The injected area of the plant was not exposed to the growth medium. This was enabled by carefully transferring the infected seedlings into a 1.5 or 2 mL microfuge tube where the tip had been cut-off (Figure 3.2C; Figure 3.3C). Typically, each jar had 3–5 microfuge containing plantlets.

The jars were placed in a temperature (26°C day, 25°C night) controlled growth cabinet (12-h day, 12-h night, 200 PAR provided by a 1000 W mercury halide lamp) for two weeks. High light

density and temperature should be avoided in this stage, since they sharply reduce the transformation efficiency. After two weeks, hairy roots emerged and elongated from the inoculation site (Figure 3.2C; Figure 3.3C).

Once hairy roots reached the length of 1–2 cm (2 weeks after infection), the main root, unnecessary shoots, and leaves were cut and removed. In most situations the lateral shoots were kept rather than terminal shoots as hardening of lateral shoot was found to be more successful. Seedlings with hairy roots were transferred to germination trays (48-cell, cell size: 4.5 × 4 × 4.5 cm) or small pots (4.5 × 12 × 4.5 cm) filled with sand and transparent lids (Figure 3.2D). Then, the plants were transferred to a higher temperature (28°C) and light intensity (400–600 PAR, mercury halide lamps) growth chamber for growth. The plants were kept in trays or small pots for 1 wk, covered for the first 2 d to retain humidity. During the first week, small pots were watered (10 mL per pot) every second day with nutrient solution, which included a nitrogen source (see Table 3.1).

After the first week of transplantation, transgenic hairy roots were determined by *GFP* screening, and non-transformed roots were cut and removed (Figure 3.2E, F; Figure 3.3D–F). A noticeable feature of the semi-*in vitro* cultivation procedure was that hairy root organogenesis was highly active with good root proliferation. In all experiments, *GFP* expression was detected with Fluorescence Stereo Microscope (Leica FLIII) attached to a Leica camera. *GFP* excitation was obtained by a 470-nm excitation filter. For *GFP* visualization, plants within the microfuge tube were carefully removed from the trays/pots and visualized on the stereomicroscope. Plants with only transgenic roots were transferred to larger individual 1-L pots containing sand to develop roots and or nodules (Figure 3.2G; Figure 3.3G). The number of produced hairy roots per infected plant and the number of hairy roots with *GFP* expression per infected plant were recorded to evaluate *A. rhizogenes*-mediated transformation.

3.3.9 Inoculation of transformed roots of G. max and G. canescens with rhizobium strains

Two days after GFP selection of transgenic roots and transfer to 0.9-L pots, both *G. max* and *G. canescens* plants were inoculated with *Rhizobium* strains to begin the symbiosis and nodule production. Soybean was inoculated with a commercial *Rhizobium* sp. strain USDA 110 (*B. japonicum* USDA 110). In contrast, a local *Rhizobium* sp. strain CC 1601a was used for inoculation *G. canescens*. To grow the rhizobium strains for inoculation, USDA 110 and CC 1601a were streaked from a glycerol stock onto the surface of YEM plates (containing 10 g/L mannitol, 0.4 g/L Difco™ yeast extract, 0.8 mM $MgSO_4 \cdot 7H_2O$, 1.7 mM NaCl, and 3 mM $K_2HPO_4 \cdot 3H_2O$ solidified by 15 g/L agar [bacteriological grade]), and incubated at 29ºC for 3–4 d. A colony of USDA 110 or CC 1601a was placed in 50 mL liquid YEM medium and grown for 3 d at 29ºC with shaking at 200 rpm. Cells were then diluted with water to an OD of 0.05 (approximately 10-20× dilution) and added directly to individual pots (25 mL per 1-L pot). Two days after the first inoculation, a second identical inoculation was performed.

Soybean pots were watered every 2 d with 50 mL of minus nitrogen containing nutrient solution (500 µM $MgSO_4 \cdot 7H_2O$, 200 µM KH_2PO_4, 50 µM K_2HPO_4, 250 µM KCl, 25 µM Fe-Na-EDTA, 250 µM $CaCl_2$, 11.5 µM H_3BO_3, 2 µM $MnSO_4 \cdot H_2O$, 2 µM $ZnSO_4 \cdot 7H_2O$, 0.5 µM $CuSO_4 \cdot 5H_2O$, 0.5 µM $Na_2MoO_4 \cdot 2H_2O$) to encourage nodulation and nitrogen fixation.

In soybean, 4 wk after inoculation, the elongated roots and nodules were examined for stable *GFP* expression. With *G. canescens*, *GFP* expression was detected 8 wk after inoculation due to its slow-growth.

3.3.10 Comparing the efficiency of the semi-in vitro method (this study) with previous hairy root transformation protocols

The major differences between the hairy-root transformation method we propose here and those previously utilized and described (Kereszt et al., 2007; Cao et al., 2009) are the inclusion of a semi-*in vitro* culturing step and repeated refreshment of the bacterial cells before inoculation. In a separate experiment, we compared the efficiency of our new protocol to the widely used *A.*

rhizogenes protocol (Kereszt et al., 2007; Cao et al., 2009). Thirty plants were inoculated for each transformation protocol, and subsequently, the number of plants with hairy roots, the number of generated hairy roots per plant, and the number of transformed hairy roots (GFP-expressing roots) per plant were recorded and statistically compared with 2-sample *t* test using Minitab 16 software (www.minitab.com). We repeated this experiment 3 times, where similar results were obtained each time (data not shown).

3.3.11 Testing genotype-independency of the semi-in vitro method in Glycine spp.

To investigate genotype-independency of the presented semi-*in vitro* method in *Glycine* spp., the hairy root transformation efficiencies were compared between two different accessions of *G. canescens* (1270 and 1301) and *G. max* L. cv. 'Djakal'. For each *G. canescens* accession, 30 seedlings were used and for *G. max*, ~200 seedlings were infected by *A. rhizogenes* K599. The number of plants with hairy roots, generated hairy roots per plant, and the transformation frequency (GFP-expressing roots) were recorded and compared using Minitab 16 software (www.minitab.com).

3.4 Results

3.4.1 Semi-in vitro hairy root formation, transformation, and nodulation process.

After injection with *A. rhizogenes*, a small callus formed at the site of infection in both *Glycine* spp. (Figure 3.4 A, B). From the developing callus site, hairy root primordia and developing roots could be observed within 2–3 weeks after infection. The incorporation of a semi-*in vitro* procedure enabled the infected regions of the plants to be maintained at high humidity to encourage callus and root development. It also ensured the separation between the infected region of the plant from the sand within the jar and the nutrient solution provided during growth. This humid environment helped to generate a prolific mass of hairy roots without much root death (Figure 3.2C; Figure 3.3C, D). In the transgenic hairy roots, we found high levels of stable expression of GFP in both *G. max* and *G. canescens* (Figure 3.2F; Figure 3.3E, F). Upon inoculation of rhizobia, we found GFP expression in both nodules and roots (Figure 3.2H).

When compared to the commonly used *in vivo* protocol (Kereszt et al., 2007; Cao et al., 2009), the semi *in-vitro* protocol produced significantly more total and transformed hairy roots ($P < .05$; Figure 3.5). The semi-*in vitro* protocol therefore improved the transformation efficiency by at least 3-fold ($P < .05$).

3.4.2 Efficiency of the semi-in vitro hairy root transformation protocol with G. max and G. canescens

In *G. max*, the semi-*in vitro* protocol resulted in ~88% of *A. rhizogenes*-infected plants successfully developing hairy roots; with on average there were ~11 hairy roots per plant (Table 3.2). Upon screening for GFP expression we found there were on average 7–8 transgenic roots per plant. In both accessions of *G. canescens*, all inoculated plants developed hairy roots, with between 13 and 19 hairy roots per plant (Table 3.2). High transformation efficiency was also evident in *G. canescens*, where all of infected plants displayed strong GFP expression. In contrast, in cultivated soybean, the frequency was less at 71.5%. We found no differences in response to plant infection, hairy root formation, and transformation between the two *G. canescens* accessions ($P > .05$, 2-sample *t*-test). To the best of our knowledge, this is the first report on *A. rhizogenes* transformation of *G. canescens* using an *in vivo*/semi-*in vitro* transformation protocol. There appears to be no genotype dependency of transformation success within the two *G. canescens* accessions tested; this is in contrast to that previously reported (Rech et al., 1988; Kouas et al., 2009) using an *in vitro*-based transformation method. Transformation efficiency was thus very high and allowed many plants to be developed containing a transgenic root system (Table 3.2).

3.5 Discussion

Accelerated methods for obtaining transgenic roots have been developed using *A. rhizogenes*. The transformation procedure is completed by either a tissue-culture-based (*in vitro*) or tissue-culture-free (*in planta*) method.

Tissue culture (*in vitro*) conditions promote highly efficient organ proliferation and morphogenesis (Ebrahimie et al., 2007), but these conditions often induce various abnormalities such as vitreous leaves and non-functional vascular tissues and stomata (Murray, 2011). As a result, the transfer of *in vivo* tissue culture plantlets to soil and normal atmospheric conditions can delay the hardening process and result in low survival, mainly due to water loss and desiccation (Murray, 2011). In addition, genotype-dependency that facilitates somatic organogenesis is one of the major limitations of the *in vitro* culture method (Suzaki et al., 2012). In contrast, the rapidity of tissue-culture-free transformation protocols (*in planta* or *in vivo* procedures) must deal with the loss of optimum *in vitro* culture conditions (high humidity and a nourishing culture medium) that enhance callus and root primordia formation/growth. This trade-off results in a decrease of tissue morphogenesis and organ formation efficiency.

To address the problem of poor survival of tissue culture raised plantlets, Ziv (2011) proposed the use of *in vitro*-acclimation. This procedure emphasized that *in vitro*-produced tissues required hardening and or acclimation much earlier in the tissue culture process instead of being postponed until the end at final transplanting step. A key aspect of this approach takes advantage of the high morphogenesis and micropropagation capacity of *in vitro* culture (Murray, 2011) that encourages good root development.

In this study, we introduced an intermediate '*in vitro*/*in vivo'* method referred to as semi-*in vitro*. After the *in vivo* infection of plants with *A. rhizogenes,* transformed tissues are transferred to a semi-*in vitro* condition that encourages root proliferation without the development of tissue culture-based abnormalities such as vitrification and phenotypic abnormalities. Our approach can be considered as an alternative approach for *in vitro* acclimation suggested by Ziv (2011). The combination of this approach with the high totipotency of cotyledonary nodes for soybean organogenesis (Cheng et al., 1980; Meurer et al., 1998; Donaldson and Simmonds, 2000) may make it suitable for *A. tumefaciens* based protocols as well.

We believe there are multiple factors which have led to the success of the combined (semi) *in vitro*/*in vivo* protocol. First of all, the ability to maintain high humidity levels during *A. rhizogenes* inoculation within a sterile environment is extremely important. This is a major shortcoming of the traditional *in vivo* protocols where humidity control is often not managed or plants are subjected to deleterious infection. Furthermore, we believe the use of highly totipotent cotyledonary nodes for inoculation enhanced the transformation efficiency. Lastly, the ability to culture plants in a nutrient rich media in sand further enhanced plant growth and health of the developing callus and hairy roots.

This protocol has other advantages including the ability to produce sterile transgenic roots that can be used for other tissue culture purposes such as *in vitro* secondary material production, callus culture, regeneration of whole transgenic plants from transgenic roots, micropropagation, and organogenesis. The rapidity and high transformation efficiency of this protocol creates a useful approach to examine root based biology. For example, the protocol could be used in the rapid transfer of resistance genes into roots where systematic acquired resistance (SAR) is desirable. The method presented in this study provides a fast, non-expensive, and efficient approach for studying function of genes in plant roots and nitrogen fixation.

3.6 Conclusion

To investigate the function of genes in plant roots, reverse and forward genetic approaches (over-expression, RNAi silencing, enhancer traps, etc.) are often required to validate gene activity. For those plants where whole plant regeneration is slow or not feasible; quick and reliable transient based transformation methods provide an important option. The improvements to the traditional hairy-root transformation procedure outlined in this study represents a significant advance in the culturing of hairy and/or transgenic roots eliminating the long and genotype-dependent tissue culture procedure while taking advantage of improved micropropagation via the combination of both *in vitro* and *in vivo* methods.

Table 3.1 Nutrient solution for culturing infected seedlings using the semi-in vitro protocol.

Medium pH was adjusted 6.2–6.4

Chemical	**Concentration**
Macro Elements	
$MgSO_4 \cdot 7H_2O$	500 µM
KH_2PO_4	200 µM
K_2HPO_4	50 µM
KCl	250 µM
Fe-Na-EDTA	100 µM
$CaCl_2$	250 µM
Trace Elements	
H_3BO_3	46 µM
$MnSO_4 \cdot H_2O$	8 µM
$ZnSO_4 \cdot 7H_2O$	8 µM
$CuSO_4 \cdot 5H_2O$	2 µM
$Na_2MoO_4 \cdot 2H_2O$	2 µM
Nitrogen source	
NH_4NO_3	1 mM

Table 3.2 Efficiency of semi-*in vitro A. rhizogenes* hairy root transformation method in *G. max* and *G. canescens*.

Five day-old seedlings of *G. max* L. cv. 'Djakal' and *G. canescens* accessions (1270 and 1301) were inoculated with *A. rhizogenes* K599. Two weeks after inoculation, the percentage of infected plants that developed hairy roots were calculated for each genotype. Three weeks after inoculation, the total number of hairy roots generated and positive GFP-expressing transformed hairy roots were counted per *A. rhizogenes*-infected plant. Values for *G. max* represent mean ± SE (n=200 plants), and for *G. canescens* mean ± SE (n=30 plants) per accession.

***Glycine* species**	***Rhizobium* inoculant**	**% of *A. rhizogenes*-infected plant which developed hairy roots (mean ± SE)**	**No. of generated hairy roots / *A. rhizogenes*-infected plant (mean ± SE)**	**No. of transformed hairy root / *A. rhizogenes*-infected plant (mean ± SE)**	**% of transformed plants**
***G. max* cv. 'Djakal'**	*B. japonicum* USDA110	88.2 ± 4.0%	10.96 ± 0.27	7.69 ± 0.19	71.5%
G. canescens					
Accession 1270	CC1601a	100%	13.7 ± 1.8	12.0 ± 1.2	100%
Accession 1301	CC1601a	100%	19.8 ± 2.2	16.1 ± 1.5	100%

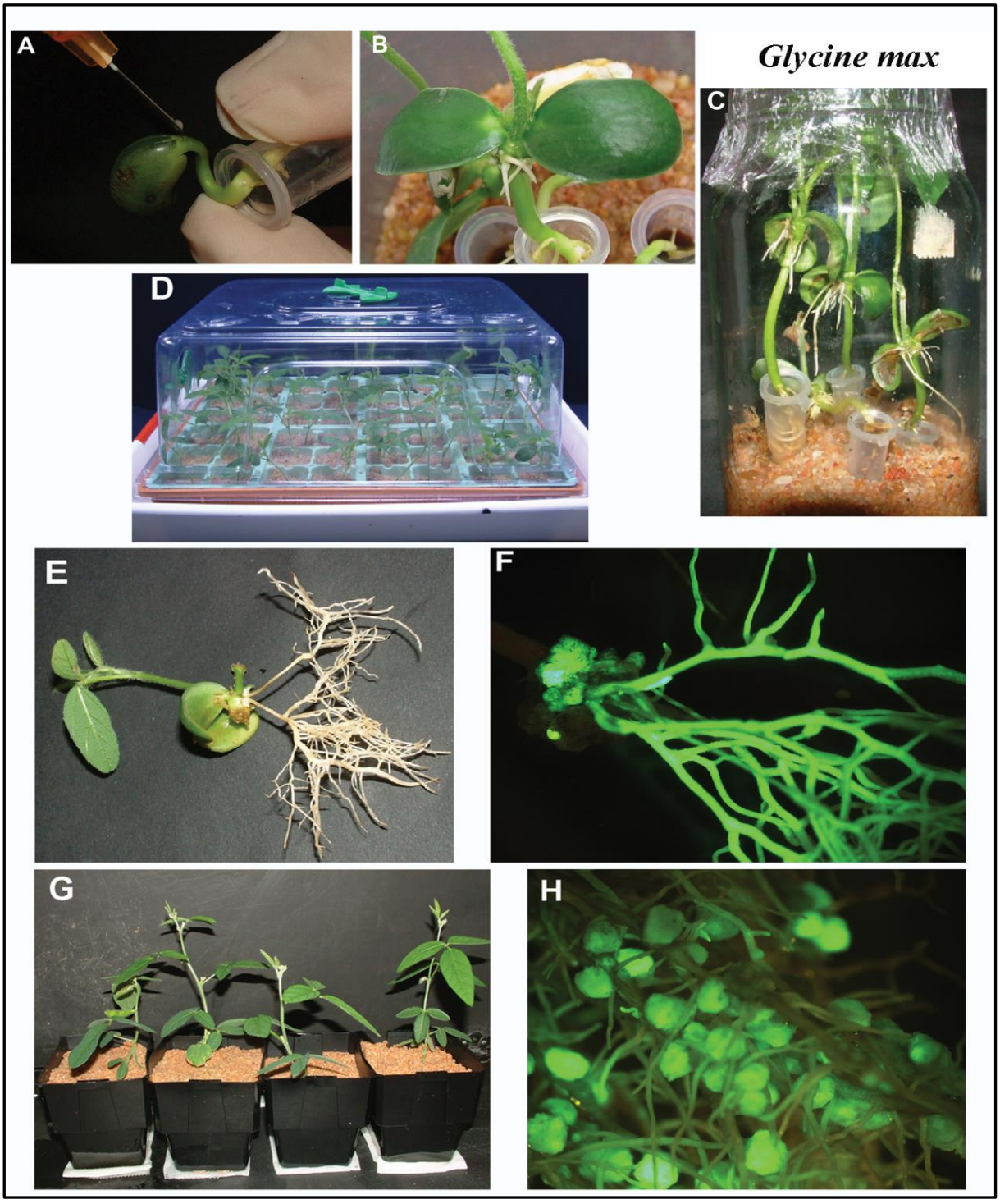


Figure 3.2 *G. max* hairy root transformation with semi-*in vitro* protocol.

A) Infection of 5-day old seedling with folded cotyledons by *A. rhizogenes* K599 around the cotyledonary node area using a 26-G syringe needle. *B*) Initiation of hairy root formation from the infection site. Plants were placed within an open cut Eppendorf tube, which allows for roots to enter the sand media of the glass jar. *C*) Semi-*in vitro* environment produced by sealed glass jars containing sand and nutrient medium. *D*) Germination tray containing plants with adventitious hairy roots (main roots have been removed at the time of transferring to germination tray). *E*) Plant with elongated adventitious hairy roots 1 week after transfer to germination tray. Plants at this stage are ready for GFP detection and transfer to larger size pots. *F*) Selection of transgenic hairy roots by GFP expression and removing of non-transformed hairy roots. *G*) Hairy root transformed-plants in 0.9-L pots after inoculation with *B. japonicum* USDA 110. *H*) Transgenic nodules with high levels of GFP expression.

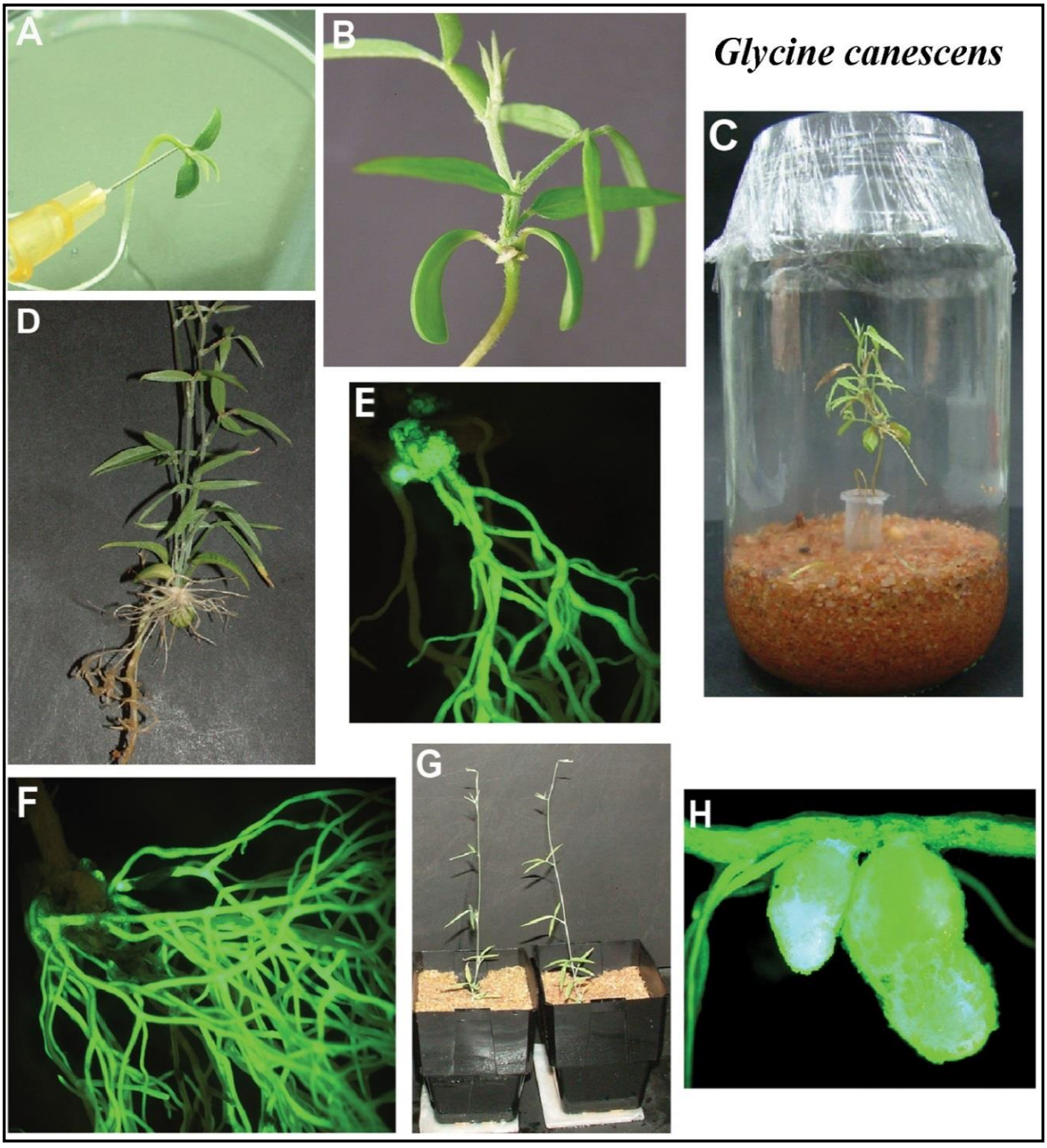


Figure 3.3 *G. canescens* hairy root transformation using the semi-*in vitro* protocol.

A) Infection of 6-day old seedlings with un-folded cotyledons by *A. rhizogenes* around the cotyledonary node area using a syringe needle. *B*) Initiation of hairy root formation from the infection site. *C*) Semi-*in vitro* environment produced by sealed glass jars containing sand and nutrient medium. *D*) Composite plants containing main (primary) and hairy roots 14 d after inoculation. *E*) Plant with elongated adventitious hairy roots 1 wk after transfer to small pots; plants at this stage are ready for GFP detection and transfer to larger pots. *F*) Selection of transgenic hairy roots by GFP expression and removing of non-transformed hairy roots. *G*) Hairy root transformed-plants in 0.9 L pots after inoculation with *Rhizobium* sp. strain CC 1601a. *H*) Transgenic nodules with high levels of GFP expression.

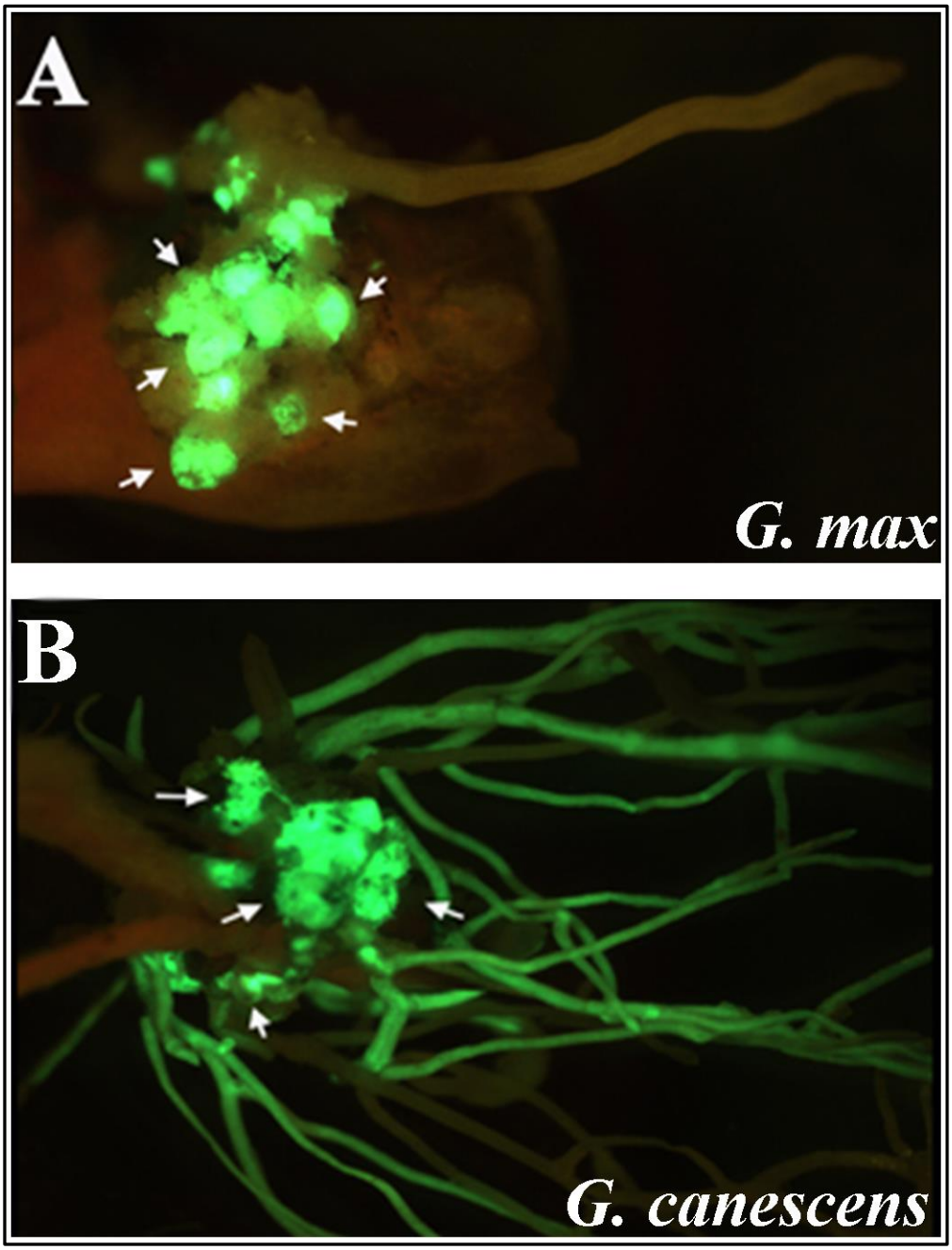


Figure 3.4 GFP-expressing totipotent calli and transgenic root primordia.

GFP expression within *A*) *G. max*. and *B*) *G. canescens* calli and transgenic root primordia. *Arrows* show root primordia.

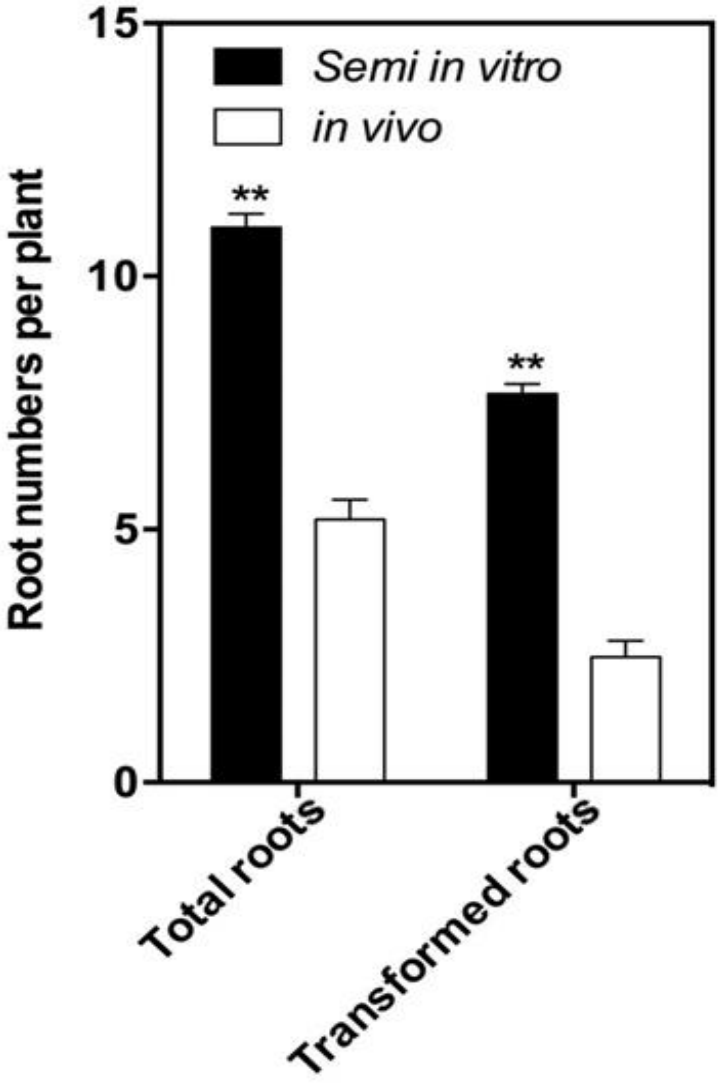


Figure 3.5 Enhanced transformation efficiency of semi-*in vitro* method versus previously published *in vivo* hairy root transformation method in *G. max*.

The semi-*in vitro* protocol produced significantly ($P \leq .05$) more hairy roots and transformed (GFP-expressing) roots per *A. rhizogenes*-infected plant than the previous *in vivo* method developed by Kereszt et al. (2007). Data represents mean ± SE (n=30 *A. rhizogenes*-infected plants per protocol).

Chapter 4

4. Functional and genetic analysis of *GmSAT1* activity in soybean

4.1 Abstract

The functional activity of *GmSAT1* was examined using a reverse genetics approach involving combined RNAi silencing of both *GmSAT1;1* and *GmSAT1;2* (*GmSAT1*) , in *Agrobacterium rhizogenes* mediated roots and nodules. Analysis of silenced tissues (*sat1*) revealed that the loss of *GmSAT1* affects nodule formation and development resulting in predominantly ineffective nodules and reduced nodule number. Loss of *GmSAT1* was less evident in the form of a visual phenotype in *sat1* root comparison to controls. However light microscopy of root cross-sections indicated a disruption in the development of the vascular tissue within the stele. Using RNAi-silenced tissues a microarray-based experiment was performed on both nodules and roots to investigate the genetic effects of *GmSAT1* silencing. Loss of *GmSAT1* expression had a relatively small impact on gene expression in both nodules and roots but did identify a number of genes and gene networks changing in line with the loss of *GmSAT1* activity. Using the changes in gene expression, a number of putative gene expression networks were developed through combining genetic ontology analysis and published literature datasets, which together provided a hypothetical overview of *GmSAT1* activity in both root and nodule tissues. The results of this study indicated that *GmSAT1* silencing is accompanied by a significant down-regulation of genes involved in light responsiveness/circadian rhythms, hormone regulation, biotic stress and nitrogen and phosphorus linked metabolic activities. These experiments suggest *GmSAT1* is more than a

symbiosis specific transcription factor *per se* but rather a TF with multiple targets and processes involved in a range of different signaling pathways.

4.2 Introduction

Using a reverse genetics approach, involving the RNAi silencing of *GmSAT1* in hairy roots, Loughlin (2007) found that disruption of *GmSAT1* activity reduced the general fitness of developing nodules. The nodules were predominantly small and ineffective as a nitrogen-fixing organ. When plants were grown solely on the nodulated transgenic roots without external nitrogen supply, shoot growth was compromised typified by chlorotic leaves and stems (Loughlin, 2007). This preliminary analysis suggested *GmSAT1* plays a key role in the *Rhizobium* symbiosis and its target appeared to be linked to nodule development and or activity. Unfortunately, the soybean genome sequence was not available at the time of these experiments, limiting the development and access to whole genome expression analytical tools (e.g. microarrays, RNA-SEQ) that could be used to help pinpoint the mechanism by which *GmSAT1* operates.

The processes of nodulation and nodule development are complex genetic phenomenon involving the cooperation of a large number of plant genes, many specifically expressed only upon initial infection or during root nodule development and activity. Since *GmSAT1* has now been confirmed as a DNA binding transcription factor (Chiasson, 2012) it is logical to expect that loss of *GmSAT1* activity may alter the expression of many genes directly or indirectly and that potentially could have a broad impact on both root and nodule gene expression. As *GmSAT1* is also expressed in non-symbiotic tissues and in non-legumes, there is a strong possibility that *GmSAT1* may also influence other signaling pathways not related to the *Rhizobium* symbiosis. It is important that the role of *GmSAT1* as a regulatory mechanism is defined to help understand it potential role in both symbiotic and non-symbiotic systems.

To address this, the impact of *GmSAT1* expression on nodule and non-inoculated root development was re-evaluated using RNAi silencing. Microarray analysis was then employed

to explore whole genome expression patterns that could highlight underlying regulatory mechanisms. Changes in gene expression were also used to construct potential *GmSAT1* mediated molecular networks by combining *Gene Ontology* (GO) analysis (Ashburner et al., 2000; Consortium, 2008) with relationship algorithms (Pathway Studio) that cross referenced published literature datasets.

4.3 Materials & Methods

4.3.1 Construct preparation for RNAi silencing for soybean

Harpin binary RNA expression vector of PK7GWIWG2D(II) was used for silencing of *GmSAT1* and evaluating the role of *GmSAT1* in nodule and root activity. A 359 bp portion of the *GmSAT1* 3'UTR was cloned with the primers *GmSAT1*-RNAi-F (TCCCCGAAAGGTACATGAAG) and *GmSAT1*-RNAi-R (CAACAAAGGCCTGTCTGTCA), and inserted into pCR8-TOPO (Life Technologies). The fragment was recombined into pK7GWIWG2D(II) (Karimi et al., 2002). This vector contains GFP construct with 35S promoter which facilitates selection of transformed cells based on GFP expression (Chapter 3, Figure 3.1). The 35S promoter had been demonstrated to be active in the infected cells of nodule in soybean and other plants such as *Sesbania spp.*, *Arachis hypogea* and *Phaseolus spp*. (Van de Velde et al., 2003; Estrada-Navarrete et al., 2006; Govindarajulu et al., 2009; Sinharoy et al., 2009), but in some other plants such as, *Vicia hirsuta*, *M. sativa*, and *M. truncatula* the 35S promoter was only expressed in un-infected cells (Quandt et al., 1993; Samac et al., 2004; Auriac and Timmers, 2007) Either the constructed plasmid or empty vector was introduced into *A. rhizogenes* K599 using the electroporation method (Mattanovich et al., 1989).

4.3.2 Plant material and experiment design

Preparation of plants for morphological and physiological experiment

Soybeans were grown in two different ways. In the first method seeds were germinated and then transformed with either empty vector or *GmSAT1* RNAi construct according to the method described in Chapter 3. The only difference with this approach was that the main root was retained as an internal control for each replication. So each plant was contained one transformed hairy root and one untransformed main root. Two days after transplanting to a larger pot to establish both root systems, plants were inoculated with *B. japonicum* USDA 110. Twenty eight days after inoculation, nodules of transformed roots were harvested for analysis. This analysis was carried out on 15 RNAi silenced, and 10 empty vector control plants. In the second method, plants were transformed according to the method described in Chapter 3 where only transformed roots were retained. Fifteen plants for each construct were grown on a single transformed root and planted individually in a 1 L pot. Twenty-eight days after inoculation, roots and nodules were harvested for analysis. Root sections for light microscopy were taken from ~10 cm above the root tip of empty vector or RNAi silenced roots and fixed for microscopy analysis (see section 4.2.3).

Preparation of plants for microarray experiment

Glycine max cv. Djakal was transformed using *GmSAT1* RNAi construct and empty vector (control) according to the methods described in Chapter 3. Two sets of plants were prepared for this experiment containing 22 *GmSAT1 RNAi* plants and 10 empty vector controls.

Plants with just one transgenic root were transferred to individual pots containing river sand and watered with nitrogen-free Herridge's media (Chapter 3 Table 3.1). To generate nodules, plants were inoculated with *B. japonicum* USDA110 as described in Chapter 2 at planting. After 26 days, nodules from individual transgenic roots were picked for RNA extraction, frozen in liquid N_2 and stored at -80°C. In a second set of plants, non-inoculated hairy roots were watered with media containing 2.5mM NH_4NO_3. After 26 days non-inoculated roots

were harvested and used for RNA extraction. To identify transgenic hairy roots the roots were screened for GFP expression using a Fluorescence Stereo Microscope (Leica FLIII) attached to a Leica camera. For both nodules and roots, individual sample reps were selected based on the level of RNAi silencing of *GmSAT1;1* across the population set using qPCR analysis. Four samples from the control and the RNAi tissue pools were selected for microarray analysis.

4.3.3 Light and electron microscopy

For light microscopy, nodules and root specimens were fixed at 4°C for 24 h in fixative (0.25% v/v glutaraldehyde and 4% v/v paraformaldehyde and 4% w/v sucrose made up in PBS (130 mM NaCl, 7mM Na_2HPO_4 and 3mM NaH_2PO_4, (pH7.3)). Samples were washed twice for 10 min in PBS plus 4% (w/v) sucrose. All samples were dehydrated in an ethanol/water series (70%, 90% and 100% ethanol) for 3 x 20 min each. Dehydrated samples were then embedded in either paraffin for light microscopy or in LR White resin for electron microscopy.

For paraffin embedding, ethanol was gradually replaced by xylene using a xylene/ethanol series (25%, 50%, 75% and 100% xylene) for an hour each. The samples were then kept in 100% xylene overnight. Xylene was replaced by molten paraffin the following day where paraffin was changed twice a day for 3 days until the xylene was completely removed. The infiltrated samples were embedded in paraffin and stored at 4°C before sectioning. Sections (1 µM) were stained with toluidine blue and examined by a light microscope.

For LR White resin embedding, specimens were incubated overnight in a 1:1 mix of ethanol: LR White resin (London Resin Company Ltd). Specimens were then transferred to LR White resin and incubated for 48 h, with three changes of resin. Specimens were transferred to small gelatine capsules filled with LR White resin and embedded at 50ºC for 1-3 d. Sections ~ 90 nm were stained for 5 min with 3% (v/v) uranyl acetate in 70% (v/v) ethanol and then washed. The sections were stained with lead citrate (Venable and Coggeshall, 1965) and examined by a Philips CM 100 transmission electron microscope (Adelaide Microscopy).

4.3.4 Performing microarray experiment

Extracted total RNAs (see procedures in Chiasson D. 2012) were sent to Ramiciotti Centre (The University of New South Wales, Sydney) who performed the microarray experiment. 100 ng of total RNA from four replicates were labeled and hybridized to the most recently available Soybean 1.0 ST array, according to the manufacturer's instructions (Affymetrix).

4.3.5 Analysis of microarray data

Data was received in Cell file format consisting of 16 Cell files from the Ramiciotti Centre. The associated library file (CDF file) and annotation file (CSV file) for the Soybean 1.0 ST array was downloaded directly from the Affymetrix website (http://www.affymetrix.com/). The microarray data was normalized for quality control (QC) analysis (Gentleman, 2005) using Expression Consol software, downloaded from the Affymetrix website. The quality of the root and nodule array experiments is presented as both box plot and raw density histograms (Figure 4.2 A-D). Normalization efficiency was then carried out using the RMA algorithm (Lim et al., 2007). After normalization, the means of the relative log of signal expression across all samples had shifted to the same level (Figure 4.2 C&D). Based on this analysis, one of the nodule empty vector Cell files which displayed significant variability and noise across the array was removed from the data set.

FlexArray software (genomequebec.mcgill.ca/FlexArray/, McGill University, Canada) was used for statistical analysis of microarray data. In both nodule and root data sets, RNAi silenced samples were compared with the empty vector controls using the Bayesian T-test. As there were only 3-4 replications in the microarray experiments per tissue, I chose to use the Bayesian and/or Empirical Bayes methods to generate more accurate statistical calculations, compared to commonly used methods, including two-sample t-test, F-test (Fisher test) and PCA (principle component analysis (Fox and Dimmic, 2006; Baseri et al., 2011). Genes with $p<0.05$ and at least 2-fold over-expression or down-expression were tagged as significantly differentially expressed compared to the controls (either up or down regulated). To recheck the quality of array data sets, a heat map of expression signals of the top 50 down-regulated

and up-regulated genes in both the nodule and root arrays were analysed by the FlexArray software (Figure 4.3 A-B). Based on the heat maps, one of the root RNAi Cell files which displayed significant variability in gene expression patterns amongst the replications (Figure 4.3 B) was removed and the data reanalyzed.

4.3.6 Functional genomics (Gene Ontolog) of modulated genes in root and nodule tissues and Chromosome localization

Gene Ontology (GO identifiers) of differentially expressed genes in both nodules and roots were identified from the Soybean Functional Genomics Database (http://bioinformatics.cau.edu.cn/SFGD/) (Appendixes 1, 2, 3, 4). The MAPMAN program (http://mapman.mpimp-golm.mpg.de/pageman/) was then used to classify genes into distinct gene ontology groups (Figure 4.9, 4.10). To identify statistically different functional groupings MAPMAN classifications were analysed using IDEG6 algorithms (http://telethon.bio.unipd.it/bioinfo/IDEG6_form) (Pacios-Bras et al., 2003) (Figure 4.9, 4.10 and Tables).

4.3.7 Construction of GmSAT1-based gene networks

The development *GmSAT1*-based gene networks were completed using Pathway Studio software 9 (http://www.elsevier.com/online-tools/pathway-studio/biological-database) which combines published literature with known protein-protein interaction networks. The software is equipped with several layout algorithms to draw links and visualization of the network such as the shortest path, near neighbors, union selected sub-networks, regulatory detection algorithms (based on inhibitory effects of microRNAs, promoter binding etc.) (Nikitin et al., 2003). The shortest path length is the path with the smallest number of links between a pair of nodes (Managbanag et al., 2008). To construct networks, first the shortest path algorithm was employed using different sets of gene lists including down and or up-regulated nodule and root genes in various combinations. Union selected sub-network algorithm, was then used to identify and join small networks from complex constructed networks from the previous algorithm. Significant sub-networks were detected based on a Fisher exact test within the

Pathway package (Subramanian et al., 2005). Constructed sub-networks were enriched by adding specific entities or relationships (such as regulatory detection algorithms and predicted gene networks *GmSAT1* may be involved with.

4.3.8 Prediction of microRNAs targeting GmSAT1;1

Most plant miRNAs bind to their targets with perfect or near-perfect complementarity (Jones-Rhoades and Bartel, 2004). The psRNATarget server (http://plantgrn.noble.org/psRNATarget) (Dai and Zhao, 2011) was used to predict potential microRNA interactions with *GmSAT1;1*. Prediction of small RNA target in this server is not only based on reverse complementary matching between small RNA and target transcript using a proven scoring schema, but also based on target-site accessibility evaluation by calculating unpaired energy (UPE) required to 'open' secondary structure around small RNA's target site on mRNA (Dai and Zhao, 2011). To predict microRNAs targeting *GmSAT1;1*, maximum expectation was set at 5 to get higher prediction coverage but target accessibility was kept low at 25 for more precise target recognition. Using this server several microRNA were found which the role of most of them was unknown including miR4362, miR4392, miR4413a, miR4413b, miR5040, miR1520c, miR156p, miR156t, miR156f and miR393b.

4.4 Results

4.4.1 Loss of GmSAT1 activity represses nodule development and activity in soybean

RNAi silencing reduced *GmSAT1* expression (*sat1*) between 70 and 90% in nodules and 50 to 70% in roots across four independent transgenic events (Chiasson, 2012). *sat1* nodule growth (fresh weight) and nodule number per root were found to be significantly reduced ($p \leq 0.05$) (Figure 4.4 A, B, D-F). As previously observed by Loughlin (2007), nodulated *sat1* plants grew poorly when supplied nutrient solution lacking external nitrogen. This resulted in chlorotic shoots relative to the control (empty vector) transformed plants (Figure 4.4 C). Nodules in *sat1* plants also displayed a reduction in leghaemoglobin relative to the empty vector control nodules (Fig 4.4 F). When plants were grown with both transformed hairy roots and non-transformed main roots, nodulation and nodule development was significantly restricted in the *sat1* plants (Figure 4.5) in compare with plants which were grown only on transgenic hairy root (Figure 4.4) which this could be the because of systemic effect of non-transgenic roots on transgenic roots. To avoid this possible systemic effect in next experiments only method two were used. Fresh weight of nodules in the empty vector hairy roots were ~17 times greater than similar aged nodules on *sat1* hairy roots while the number of nodules were ~6 times more than *sat1* hairy roots (Figure 4.5 C, D).

Analysis of the non-inoculated plus nitrogen grown empty vector control roots revealed no immediate impact on root architecture or significant differences in root weights between *sat1* and empty vector controls (Figure 4.4 I, H).

4.4.2 Light and electron microscopy analysis of GmSAT1 RNAi nodule and root

Cross sections of *sat1* nodules showed that the infection region containing uninfected and bacteroid infected cortical cells was small relative to similarly aged empty vector nodules (Figure 4.6 A, B). The infected cells of this region were also small and most of the symbiosomes appeared to contain single bacteroids (Figure 4.6 D, F). This contrasts to what is typical in soybean and seen in the empty vector control nodules, where infected cells are

considerably larger and often contain symbiosomes with multiple bacteroids (Figure 4.6 C, E). A preliminary examination of *sat1* root cell architecture was completed using light microscopy (Figure 4.7). The most notable difference was within the root stele (Figure 4.7). In empty vector (control), the pericycle and vascular poles were well developed while in *sat1* sections, the pericycle was more compact and the maturation of xylem cells into distinct xylem poles was reduced (Figure 4.7). Future work requires more detailed TEM sections of similar tissues to verify if these observations are correct and whether the observed differences are related to cell walls or cell compartments.

4.4.3 Transcriptional profiling using Affymetrix microarrays

RNA extracted from nodule and non-rhizobial root tissues of *sat1* and empty vector control treatments were profiled against Affymetrix SoyGene-1_0-st-v1 arrays. These arrays are designed to contain probe sets for different exons of genes across the annotated soybean genome. Quality control analysis of the hybridisations revealed consistency across the majority of the arrays with the exception of one empty vector nodule array and one RNAi root array, which were subsequently removed from the analysis (Figure 4.2, 4.3). Differentially expressed (up or down regulated) genes in either nodules or roots (*sat1* versus empty vector controls) were identified. All genes which displayed at least a 1.8-fold change in expression across the 3-4 replicates of the RNAi and control tissues were identified as modulated genes. A Bayesian T-test ($p < 0.05$) was applied to identify those showing significance from the empty vector controls. In nodules we identified 108 genes that were significantly down-regulated and 95 that were up-regulated (>2-fold, $p < 0.05$) in *sat1* nodules (Appendixes 1 and 2). In non-inoculated *sat1* roots, 308 genes were significantly down-regulated and 313 genes were found to be significantly up-regulated (>2-fold, $p < 0.05$) (Appendixes 3 and 4). Modified genes were categorized according to their chromosome location. The alignment indicated that all 20 chromosomes were represented with the highest representation on chromosome 17 for nodules (Figure 4.8 A) and chromosome 13 for roots (Figure 4.8 B) where *GmSAT1;1* homologues (*GmSAT1;2* and *GmSAT2;2*) are localized.

4.4.4 Gene ontologies (GO) and identities of down- and up-regulated genes in nodules and roots

Differentially expressed genes in *sat1* tissues were categorized into major GO categories using the MAP-MAN algorithm (Figures 4.9, 4.10). Significant GO categories of down regulated genes in nodules were identified that included: stress, RNA transcription, development as well as subcategories, involving ABC transporters, calcium signaling, auxin metabolism, protein modification, and degradation ($p \leq 0.05$) (Figure 4.9 A and Table). In contrast, over-expressed genes in nodules displayed a tendency to align with categories that included cell wall, cell organisation, and miscellaneous genes (Figure 4.9 B and Table).

In nodules, strongly down regulated genes included an ureide transporter (Glyma20g17440), acyl acid amido synthetase genes *BRU6/GH3.1* (Glyma05g21680, Glyma17g18040, Glyma11g05510) in which are annotated as an auxin amino acid conjugation, light responsive and circadian clock genes including, *Gigantea* (*GI*) (Glyma20g30980, Glyma09g07240), *PRR5* (Glyma04g40640), *PRR7* (Glyma12g07860), *HHP4* (Glyma11g05030), *HPT1* (Glyma10g44170) and the stress responsive genes *ELI3-2* (Glyma18g38670), *TPS04* (Glyma13g25270), *RPS2* (Glyma07g08500), *PDR9* (Glyma17g04360) (Appendix 1). Notable genes up-regulated with the loss of *GmSAT1* were gibberellin responsive genes such as *GASA6* (Glyma19g31480), *GIP1* (Glyma03g12150) and *GAMMA-TIP* (Glyma03g34310) and some transcription factors including, *bZIP42* (Glyma18g51250, Glyma08g28220), *TCP* (Glyma07g08710), bHLH family transcription factor (Glyma20g39220) and *bHLH093* (Glyma02g38240) (Appendix 2).

In roots, down regulated genes fell into functional groups involved in cell wall development, lipid metabolism, stress response and nutrient transport (Figure 4.10A). In contrast, the majority of up-regulated genes were associated with functional groups of RNA synthesis, protein and ethylene metabolism (Figure 4.10B). The genes suggesting a potential role in phosphorus metabolism included, three homologs of the pyridoxal phosphate phosphatase-related protein *HAD1* (Glyma08g20810, Glyma08g20820, and Glyma13g42770), two *SPX*

domain containing genes *SPX2* (Glyma06g07260, Glyma17g12340) and an inorganic phosphate transporter, *PHT1;7* (Glyma03g31950) (Appendix3). Other genes that showed strong down regulation included five uncharacterized major facilitator superfamily (*MFS*) proteins (Glyma03g27840, Glyma03g27830, Glyma04g37320, Glyma13g28450, Glyma11g00710), biotic stress and defence responsive genes such as *DMR6* (Glyma19g04280), *PAL1* (Glyma19g36620), *MYB14* (Glyma06g45550), *SRF6* (Glyma19g45130) *AP4.3A* (Glyma03g25380), *UGT73B5* (Glyma10g42680), *PDR12* (Glyma19g35270) and *UGT73B3* (Glyma02g11640), four nitrate transporters (Glyma02g02680, Glyma11g20090, Glyma13g39850 and Glyma18g20510), and eleven genes encoding putative cytochrome P450 proteins (Glyma08g46520, Glyma02g30010, Glyma10g22070, Glyma10g22000, Glyma10g22080, Glyma10g22060, Glyma10g12700, Glyma10g12710, Glyma03g34760, Glyma08g25950 and Glyma16g24720) which in *Arabidopsis* they have found to be involve in circadian regulation (Pan et al., 2009).

There were a significant number of genes encoding transcription factors, which showed increased expression in *sat1* root tissues. These included the transcription factors: *HAT22* (Glyma0041s00350), *RAP2.4* (Glyma04g11290), *RAP2.6L* (Glyma08g28820), *RAP2.7* (Glyma12g07800), *GBF1* (Glyma11g06960, Glyma16g25600), *WRKY11* (Glyma13g00380), *WRKY27* (Glyma20g30290), *NF-YA3* (Glyma13g16770), *NF-YA8* (Glyma17g05920), *COL9* (Glyma13g11590), *EMB2301* (Glyma16g02200), *MP* (Glyma17g37580) and *BZO2H3* (Glyma19g43420) (Appendix4).

The top two up-regulated genes, Glyma11g34650 and Glyma12g12610 (21 and 13-fold, respectively) had no functional annotation. Domain analysis on both using the pfam database (http://pfam.sanger.ac.uk/) failed to identify a putative protein structure. However, prosite scan analysis using the PBIL website (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_proscan.html) recognized the presence of a protein kinase C phosphorylation site and a Casein kinase II phosphorylation site on both of the proteins, and N-glycosylation and N-myristoylation sites on Glyma11g34650.

4.4.5 Genes changing their expression profiles in nodules and roots

Co-expressed genes

The microarray data showed there were 23 genes, which changed in a similar pattern (either up or down) in both *sat1* root and nodule tissues (Table 4.1). Down regulated genes included: a transcription factor *FRU* (Glyma12g30240), which is involved in the regulation of iron uptake; *SWEET17* (Glyma19g42040), which is a vacuolar fructose transporter; and *PDR12* (Glyma19g35270), which is an ABC transporter (Table 4.1). Genes, which were upregulated in both nodules and roots included the transcription factors *NAC1* (Glyma16g02200), a winged-helix DNA binding protein (Glyma10g30560,) and a multiprotein bridging factor, *MBF1B* (Glyma06g42890). A number of unknown genes were also identified (Glyma06g08100, Glyma13g12190, Glyma13g25520, Glyma17g03850) (Table 4.1).

Alternatively expressed genes

We identified a number of genes in *sat1* tissues, which displayed either upregulation in nodules but down regulation in roots and vice versa (Table 4.2). For example in nodules, loss of *GmSAT1* resulted in a significant number of upregulated genes associated with cell wall modification, which were down regulated in *sat1* roots. This suggests a distinctive transcriptome pattern that is mediated by *GmSAT1* in each tissue and supports a role of *GmSAT1* that extends across many signaling pathways in soybean in the symbiotic or non-symbiotic state.

4.4.6 Building GmSAT1-based gene networks

One of the major goals of this study was to investigate the signaling pathways *GmSAT1* participates in both nodules and roots. We predicted genes, which showed either up or down-regulation in *sat1* tissues would provide a good opportunity to explore putative *GmSAT1*-based molecular networks. To do this, we used a bioinformatics platform called Pathway Studio (Elsevier). The program looks for relationships amongst the expression data we

supply to research findings found in either published journals (~1300 journals) and scientific literature-based datasets (~2.9 million entries). This program boasts one of the most complete databases of protein-protein interaction networks collected through literature mining of different resources such as Pubmed, Google Scholar, and HighWire Press (Managbanag et al., 2008).

Light responsive networks in nodules

Analysis of differentially expressed genes in *sat1* nodules has revealed a potential light responsive network (Figure 4.11 A). The network involves seven classes of genes including the peptide transporter *HPT1* and the uncharacterised heptahelical transmembrane protein *HHP4*, with four other genes including the circadian clock regulators *PRR5, PRR7* and *GI* (Gigantea) and the scare-crow-like *SCL13 GRAS* transcription factor involved in phytochrome signaling (Figure 4.11A). Loss of *GmSAT1* activity has up-regulated *TUB1* (beta tubulin) and down-regulated the other genes (Figure 4.11B, Table 4.3). All down-regulated genes in this network have previously been found to respond positively to light (See Table 4.3 for reference).

Defense response network in nodules

Genes involved in hormonal based plant defense responses were identified in *sat1* nodules. These included the auxin induced genes (*ATL2*), jasmonic acid responsive genes (*PDR9* and *TPS04*), salicylic acid responsive gene (*PDR12*), and *ELI3-2* (an important early defense responsive gene (Figure 4.12A). Expression of all these genes was down-regulated after *GmSAT1* disruption (Figure 4.12B).

Nitrogen linked root signaling pathways

In roots, the genetic impact of a loss of *SAT1* was more robust than nodules where a greater collection of genes shifted in their expression patterns from the empty vector controls. A nitrogen-linked transport and assimilatory pathway was identified that involved selected

members of the nitrate (*NRT2* and *NRT1*), ammonium (*AMT2*), urea/NH_3 (*DUR3*) transporter families and nitrate reductase (*NIA1*) (Figure 4.13A). The network also links in a *WRKY27* transcription factor and a putative glucose MFS transporter *MSS1*. Previously, both *DUR3* and *NRT2.4* have been shown to increase their expression in roots in response to N deficiency (Bi et al., 2007; Kojima et al., 2007). In *sat1* roots, both genes are down-regulated suggesting *GmSAT1* may have a role in nitrogen sensing and or the coordination of the nitrogen deficiency response in roots (Figure 4.13 B Table 4.5). In contrast, genes involved in nitrate uptake and redistribution (*NRT1;1*) and in nitrate assimilation (*NIA1)* were found to be significantly up-regulated with the loss of *GmSAT1* (Figure 4.13 B, Table 4.5). In Arabidopsis, *NRT1;1* and *NIA1* are both known to increase in expression when nitrogen is available (Wang et al., 2009; Konishi and Yanagisawa, 2011). This variation in nitrogen sensing suggests *GmSAT1* may be influencing how roots mediate nitrogen signaling.

Biotic stress, cell wall and lipid metabolism

Network analysis identified a significant number of genes involved in root-based biotic stress responses, cell wall development and lipid metabolism (Figure 4.14 A). This overall defense response network is represented by three potential sub-networks: 1) pathogen responsive genes; 2) cell wall development and maintenance; 3) lipid metabolism. Elements such as JA, lignin, SA, P and auxin are interconnected between the sub-networks. The pathogen responsive sub-network includes the biotic responsive genes such as *PAL1*, *PDR12*, *UGT73B5*, *LOX1*, *AP4.3A*, and *DMR6*. The network shows that the *MYB14* transcription factor could be involved in the network. *MYB14* is known to be up-regulated by JA and pathogen attack (Table 4.6). The cell wall sub-network consists of *CESA2*, *CEV1*, *TUA4*, *TUA2*, *SRF6*, and *SKU5* and the lipid metabolism sub-network contains *DIR1*, *MGD2*, *SOD2*, *PLDP1*, and *NPC4* (Figure 4.14 A). Interestingly, most of the genes in the lipid metabolism sub-network are induced by Pi starvation. Expression of all these genes decreased with the loss of *GmSAT1* (Figure 4.14 B Table 4.6) suggesting potential role of *GmSAT1* in defense responses.

Flower development and ethylene responsiveness.

A number of transcription factors involved in flower development were up-regulated in roots with the loss of *GmSAT1* (Figure 4.15 A-B, Table 4.7). This subgroup involved the flower development genes *RAP2.7, ARF6, COL9, ELF3, GBF1, APRR5* and *PAT1*. Interestingly this network was shown linked to ethylene responsive genes through JA and ethylene intermediates (Figure 4.15A).

Phosphorus responsive networks

The microarray expression data showed a strong link between *GmSAT1* and genes involved in phosphorus nutrition including *HAD1, SPX2, PHT1;7, MGD2,* and *RNS2*. Each of these genes has been linked to phosphate availability where activity increases under phosphate deficiency. Network analysis identified a putative interaction between these genes, *GmSAT1* and the phosphate linked microRNAs, miR156 and miR169 (Figure 5.1 Chapter 5). The proposed network also highlighted a potential interaction with signaling cascades that respond to nitrogen starvation. Loss of *GmSAT1*, increases the expression of the transcription factors *NF-YA3* and *NF-YA8* which are regulated by miR169, which itself is known to be repressed by both N and P starvation. The model indicates that there might be a potential link between N and P nutrition possibly mediated through *GmSAT1* cross-talk with micRNA 156.

Hormone networks involving gibberellin and auxin

In general, there were a number of genes involved in gibberellin (GA_3) or auxin metabolism that showed a change in expression in nodules when *GmSAT1* was silenced. For gibberellin-responsive genes, expression was generally enhanced without *GmSAT1* being present. A predicted network of this response is presented in Chapter 6 (Figure 6.1), which indicates gibberellins may influence the expression of *GmSAT1*, possibly in a negative relationship that alleviates *GmSAT1* repression of known GA_3 regulated genes. In contrast, a select number of auxin-responsive genes were found to be down-regulated in the *sat1* nodules and or roots

(Figure 4.16, Table 4.8). Four of these genes are involved in auxin homestasis, including *BRU6* (Glyma05g21680 and Glyma17g18040), *GH3.1* (Glyma11g05510), and *PDR9* (Glyma17g04360) (Li et al., 2007; Růžička et al., 2010; Fu et al., 2011). Expression of *BRU6, PDR9,* and *ATL2* are induced by auxin (Martínez-García et al., 1996; Růžička et al., 2010; Fu et al., 2011). *PDR9* also facilitates indole-3-butyric acid (IBA) efflux and negatively regulate *AUX1* activity which is an auxin influx transporter (Ito and Gray, 2006; Strader et al., 2008). The positive role of auxins in nodule organogenesis is well-documented (Boot et al., 1999; de Billy et al., 2001; Benková et al., 2003; Wasson et al., 2006; Pii et al., 2007; Mathesius, 2008; Ryu et al., 2012).

4.5 Discussion

The major change observed in *sat1* tissues was the impact on nodule development. Loss of *GmSAT1* resulted in small nodules that contained a small infection region and symbiosomes populated with a reduction in bacteroids instead of the multiple bacteroids common in soybean (Figure 4.6 F). Our preliminary results in soybean roots showed that there was a visible change in the endodermal layer of stele and the development of vascular tissue, which contained a high-density of cells which were less developed (Figure 4.7). The mechanism by which *GmSAT1* influences both nodule development and stele of root is unknown.

Using microarray analysis changes in gene expression with or without the presence of *GmSAT1* were evaluated. This analysis across both *sat1* nodules and roots identified a number of potential genetic pathways involving *GmSAT1*. However, it is clear from this analysis that *GmSAT1* most likely interacts across multiple signaling pathways that operate in soybean as it entertains interactions with soil microbes (rhizobia or AM fungi) or as it mediates processes involved in nutrient acquisition, tissue development, plant defense and light regulated processes.

4.5.1 Role of GmSAT1 in biotic defense responses in nodule and root tissues

Biotic stress (pathogen recognition, pathogen defense responses) and ABC transporter were important functional groups found down-regulated (P≤0.05) in *sat1* in both roots and nodules (Figure 4.9 and 4.10). In the predicted networks, several genes belonging to these groups were identified including *PAL1*, *UGT73B5*, *AP4.3A*, *DMR6*, *LOX1*, *MYB14* in root, and *ELI3-2*, *PDR9*, *ATL2*, *TPS04* and *PDR12* in nodules, Figure 4.12 and 4,14). Each of these genes is known to be induced during plant defense responses to pathogens, often through SA or JA mediated signaling cascades (References in Table 4.4 and 4.6). For example, *PDR12*, which was down-regulated in both nodule and root tissues, encodes an ABC transporter. *PDR* genes have been associated with plant biotic stress and are often up-regulated in response to fungal and bacterial pathogens. The mechanism is thought to be governed by salicylic acid, methyl jasmonate, and ethylene signaling cascades, which may bind to cis-regulatory elements in the promoter regions of *PDR* genes (Sasabe et al., 2002; Grec et al., 2003; Stukkens et al., 2005). Unfortunately, the role of these genes in legume root nodules is poorly understood. In soybean and *Medicago*, *PDR* ABC transporters are found to be involved in secretion of genistein, which is a signal flavonoid in rhizobium symbiosis (Sugiyama et al., 2007, 2008; Banasiak et al., 2013). This type of transporter was found to have a function in AM symbiosis as well. In *Petunia axillaris PDR1* ABC transporter regulates the development of AM and axillary branches by functioning as a cellular strigolactone exporter (Kretzschmar et al., 2012).

Pathogen detection and the initiation of pathogen defense responses are often associated with elicitor-based recognition networks (Varbanova et al., 2011). In nodules, loss of *GmSAT1* caused a significant reduction (~-4.34-fold) in the elicitor-based recognition gene, *ELI3-2* (Glyma18g38670). Similarly, this gene was also down-regulated in roots (~1.5-fold). Pathogen recognition at the surface of plant cells is a pivotal step in developing a plants defensive response which is often associated with the production of apoplastic reactive oxygen species (ROS) and the so-called ‘oxidative burst’. ROS production is important in cell

wall strengthening, cell hypersensitivity, defense gene activation and systemic acquired resistance (Torres et al., 2006). Pathogen induced ROS production is often associated with a rapid influx of Calcium (Ca) into the cytosol (Blume et al., 2000; Grant et al., 2000). Interestingly, in GO groupings of modulated genes in nodules, a functional group involved in Ca signaling was found to be significantly down-regulated (Figure 4.9 and Table). It will be exciting to determine what functional link *GmSAT1* has with *ELI3-2* and its potential involvement in microbe interactions within the nodule or root.

Based on the predicted network in root defense responses, there appears to be a link to functional groups of cell walls, and lipid metabolism (Figure 4,14), which were all significantly down-regulated (Figure 4.10 and Table). Lipid biosynthesis is often involved in plant defense and stress responses (Figure 4.14 and Table 4.6). Furthermore, P plays a key function in phospholipid structure. During P starvation stress, plants remodel lipid compounds to cope with P deficiency (Benning and Ohta, 2005; Maoyin et al., 2006; Okazaki et al., 2013). It has been shown that plants growing under phosphorus-limited conditions have a lower concentration of phospholipids and higher concentration of galactolipids and this decrease in phospholipids may allow phosphorus to be used for other critical cell functions (Benning and Ohta, 2005; Maoyin et al., 2006). *PLDz1* is a key enzyme in turning over phospholipids to galactolipids and it has also been shown to be induced by H_2O_2 to decrease the promotion of cell death by H_2O_2, playing a positive role in signaling stress responses (Zhang et al., 2003). P deficiency modifies cell wall compounds by decreasing cell wall polysaccharide contents (Zhu et al., 2012). Cell walls can signal stress responses in plants by induction of JA and ethylene biosynthesis and changes in biogenesis of cell walls can influence stress response signaling (Ellis et al., 2002).

4.5.2 The involvement of GmSAT1 in N transport and metabolism

The altered expression of nitrogen transport and assimilatory genes indicates a role of *GmSAT1* in nitrogen signaling in both roots and nodules. The nitrogen-starvation induced genes (*NRT1;7 AMT2, DUR3, NRT2;4*) were all down-regulated in the *sat1* root tissues

(Figure 4.13 B, Table 4.5). In contrast, we found up-regulation of a nitrate induced transporter (*NRT1.1*) and nitrate reductase (*NIA1*) (Figure 4.13 B, Table 4.5). This contrasting genetic response may indicate roots lacking *GmSAT1* are no longer capable of sensing external or internal nitrogen levels. The link to nitrogen was also found to extend to the nitrogen-responsive transcription factors, *NF-YA.* Three *NF-YA* transcription factors that showed variable expression in both root and nodule tissues were identified (Appendix 3 and 4). The relationship between *GmSAT1* and *NF-YA* may be important as it is a target of miR169 which is expressed in roots upon both P and N starvation (Hsieh et al., 2009; Zhao et al., 2011). There is a possibility that the *GmSAT1* response involving N transport may occur through some form of interaction with this gene.

In nodules, a ureide transporter (Glyma20g17440) was down regulated (2-fold) in *sat1* nodules (Table 4.1). Ureide transport is an important mechanism in soybean nodules which exports bacteroid reduced ammonia in the form of ureides (~80% of translocated N) out of the nodule and into to the plant (McClure and Israel, 1979; Gordon et al., 1985; Collier and Tegeder, 2012). Loss of *GmSAT1* also was associated with down regulation of the nodulin enhanced protein (uricase) which is required for the production of ureides (Collier and Tegeder, 2012). It is still unclear whether *GmSAT1* interact with this protein directly or this expression patterns is simply be the result of a poorly functioning non-nitrogen fixing nodule. Further investigation is required to define this relationship particularly if it is indirect or actually a direct interaction involving a *GmSAT1* mediated signaling cascade.

4.5.3 Possible role of GmSAT1 in crosstalk between ethylene and flowering responsive genes

A number of transcription factors involved in flowering under the regulatory effect of ethylene were identified in *GmSAT1* silenced tissues (Figure 4.15). In agreement with the network outcome, gene ontology analysis shows that genes involved in the ethylene metabolism functional group were significantly altered (Figure 4.10). For instance, *EIN2* is an ethylene response gene, which was up-regulated in *sat1* root. Mutation of this gene has

increased infection in AM symbiosis and nodule number in *rhizobium* symbiosis (Penmetsa and Cook, 1997; Penmetsa et al., 2008).

Altogether, it seems that there is an interaction between *GmSAT1* and ethylene. There was a significant alteration in the GA metabolism functional group as well (Figure 4.10 and Table). This result along with modulation of light responsive genes may suggest a link between light, gibberellin and flowering responsive genes.

4.5.4 Possible cross talk between microRNA and GmSAT1 in hormone and nutrient homeostasis

In a recent study on cell and tissue-specific transcriptome analyses in *Medicago* nodules, it was revealed that a homolog of *GmSAT1*, *MtSAT1* (Medtr2g010450) and several auxin responsive genes are co-expressed at high levels in the meristematic zone of the inderteminate *Medicago* nodule (Limpens et al., 2013). In determinate nodules, auxin-responsive genes are primarily expressed in the vascular tissues of roots and in the parenchyma cells of nodules (Pacios-Bras et al., 2003; Takanashi et al., 2011). Interestingly, *GmSAT1;1* expression is found across the infected region of the nodule but also shows concentrated expression in the peripheral parenchyma cell layer (Chiasson, D. , 2012). Two bHLH transcription factors, *LHW (LONESOME HIGHWAY)* and *MtbHLH1*, have previously been shown to alter vascular pattern development in an auxin-dependent manner in *Arabidopsis* and *Medicago*, respectively (Ohashi-Ito and Bergmann, 2007; Godiard et al., 2011; Ohashi-Ito et al., 2013). *MtbHLH1* (Medtr3g099620) shares 68% similarity with a *GmSAT1* homologue in *Medicago* (Medtr4g098250) (http://www.phytozome.org/). Interestingly, there are similarities in tissue expression patterns between *MtbHLH1* and *GmSAT2;1*, where both are expressed in root tips (http://bar.utoronto.ca/welcome.htm). In nodules both *MtbHLH1* and Medtr4g098250 are expressed in the meristematic zone of nodule and in uninfected cells (Godiard et al., 2011).

The potential relationship between *GmSAT1* and auxin is intriguing as auxin responsive genes were identified as down-regulated in *sat1* nodules including, *BRU6*, *GH3.1*, *ATL2* and *PDR9* (Figure 4.16 B). Induction of genes encoding the auxin import carrier (*AUX1*) or the auxin-

responsive reporter (*GH3*) has resulted in the altered development of nodule primordia in *Medicago truncatula* (de Billy et al., 2001). As auxins display a positive role on nodule development any disruption of auxin signaling could ultimately have an impact on nodule development.

In a recent study, Turner et al. (2013) examined the role of auxin and regulatory miRNAs in the development of determinate nodules. In this study, over-expression of nodule expressed microRNA393 and microRNA160 revealed that hyposensitive plants (microRNA393 over-expressed) nodulated normally whereas hypersensitive plants (microRNA160 over-expressed) had reduced nodule primordium formation. This suggests that minimal or reduced auxin signaling may be required for determinate nodule development. Interestingly, in soybean microRNA393 is induced within 3 hours after inoculation with *B. japonicum.*

Our search to find putative microRNAs revealed a number of microRNAs that may interact with *GmSAT1* mRNA. Interestingly, *GmSAT1* was identified as possible target of a family member of microRNA393, microRNA393b (Figure 4.1). microRNA393b regulates auxin signaling by transcriptional regulation of three F-box auxin receptors, *TIR1*, *AFB2*, and *AFB3* in *Arabidopsis* (Navarro et al., 2006; Parry et al., 2009; Windels and Vazquez, 2011). In Arabidopsis, microRNA393 targets the auxin receptor *AFB3* in a nitrate-dependent manner (Vidal et al., 2010; Vidal et al., 2013). In rice and soybean microRNA393 has also been linked to salinity and alkaline stress and is proposed to function as a negative regulator of plant salt-alkali stress responses (Gao et al., 2011; Li et al., 2011).

Other microRNAs identified include 3 members of microRNA156 (microRNA156 f, p, t) (Figure 4.1). Interestingly, in soybean, different members of microRNA 156 family are expressed in a tissue-dependent manner under P or N starvation (Wang et al., 2013; Xu et al., 2013). For example, in response to long term N starvation, microRNA156f is down-regulated in roots of soybean, while up-regulation of microRNA 156p occurs in shoots (Wang et al., 2013) and in *Arabidopsis* roots subject to P starvation (Hsieh et al., 2009). Although this

study, did not examine the interaction between predicted *in silico*-derived microRNAs and *GmSAT1;1*, the *in silico* analysis suggests a possible relationship may exist.

4.6 Conclusion

Much of the previous work on *GmSAT1* has considered its role in the context of the rhizobia symbiosis and ammonium transport (Kaiser et al., 1998; Marini et al., 2000; Loughlin, 2007). In this study we investigated the genetic role of *GmSAT1* by microarray analysis. The data suggested *GmSAT1* might be involved in plant defense, auxin and GA signaling, nitrogen and phosphorous homeostasis. The altered pattern of vascular tissues in *sat1* root and possible crosstalk between *GmSAT1* and predicted miRNAs in this study is an interesting preliminary result, which needs to be investigated further. In particular, cross sections of roots from different distances from the root tip is required as will be the influence of different hormonal and nutritional treatments to help better understand the cause of the phenotype and its ultimate long-term impact on root growth and development. Taken together, these results suggest that *GmSAT1* is involved in many different networks possibly as a shared element.

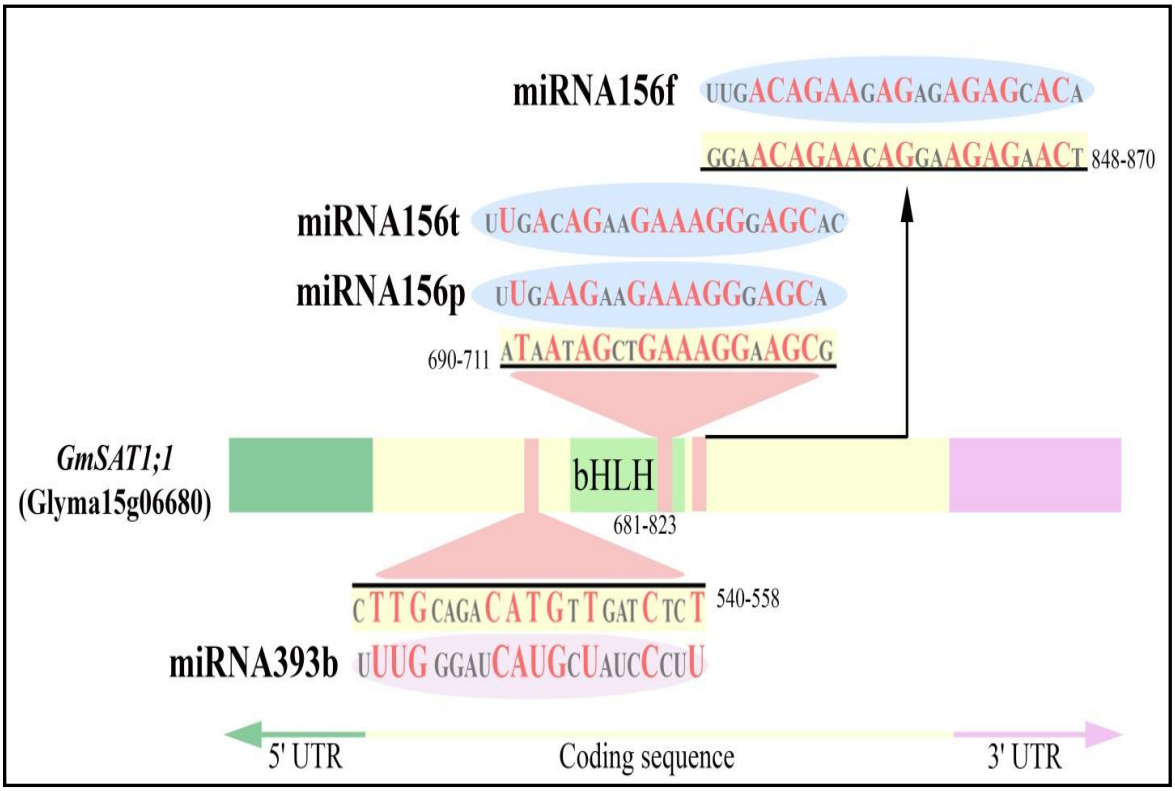


Figure 4.1 Potential microRNAs interactions with *GmSAT1;1*.

Four of potential microRNAs interactions with *GmSAT1;1* predicted by the psRNATarget server (http://plantgrn.noble.org/psRNATarget) based on reverse complementary matching and target-site accessibility evaluation by calculating unpaired energy (UPE) required to 'open' secondary structure around small RNA's target site on mRNA. Two N and P responsive miRNAs, miRNA 156t and p, predicted to interact with the regin of the mRNA encoding bHLH DNA binding domain (light green color) of *GmSAT1*. Sequence of target site on *GmSAT1* shaded with yellow color and sequence of microRNAs shaded

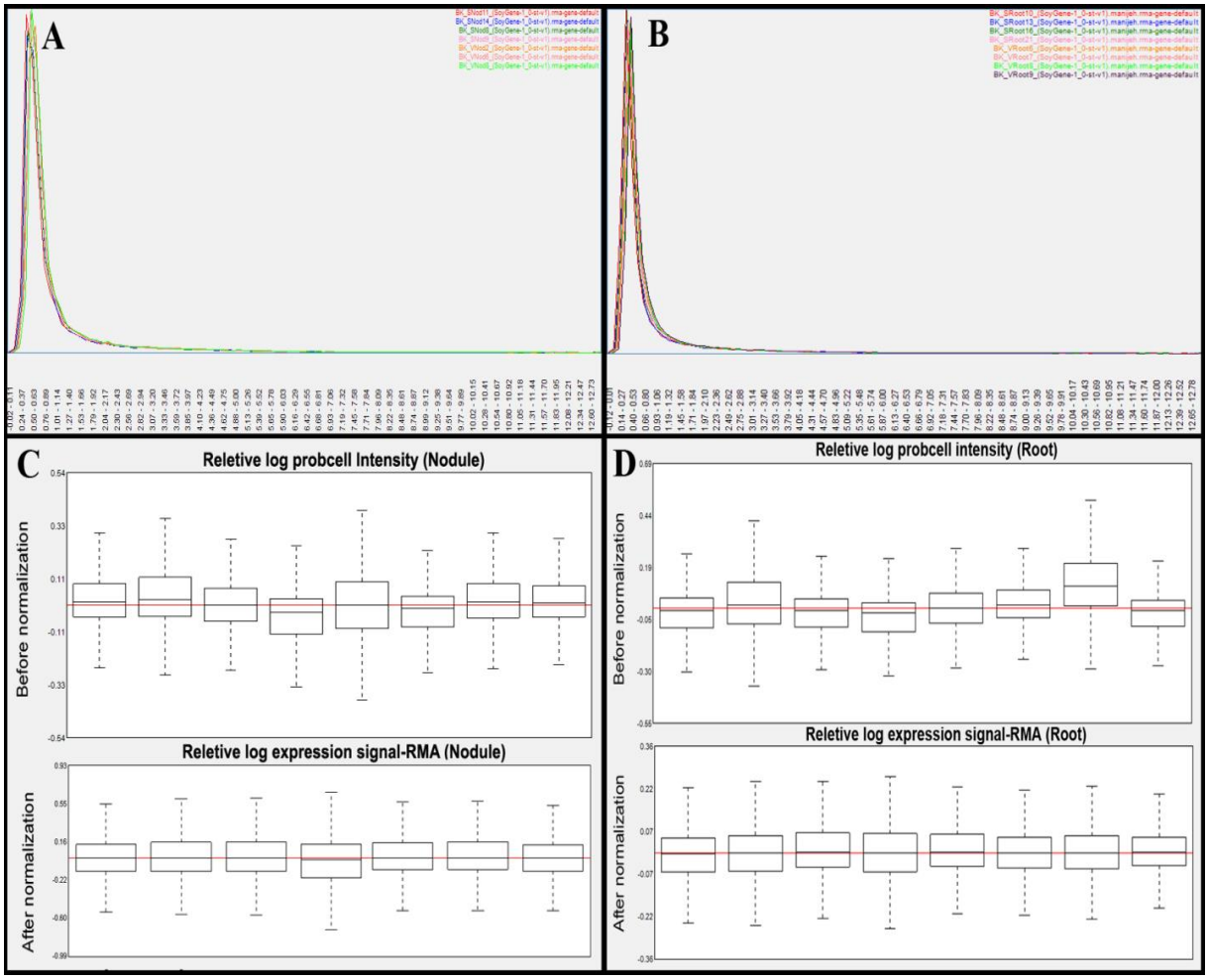


Figure 4.2 Quality control analysis of scanned soybean microarrays.

(A, B) Signal intensity distribution between nodules (A) and root arrays (B). The array signals were normalized using the RMA algorithm. Means of relative log expression signals of all nodule (C) and root (D) samples before normalization (C, D top panel) and after normalization (C, D bottom panel).

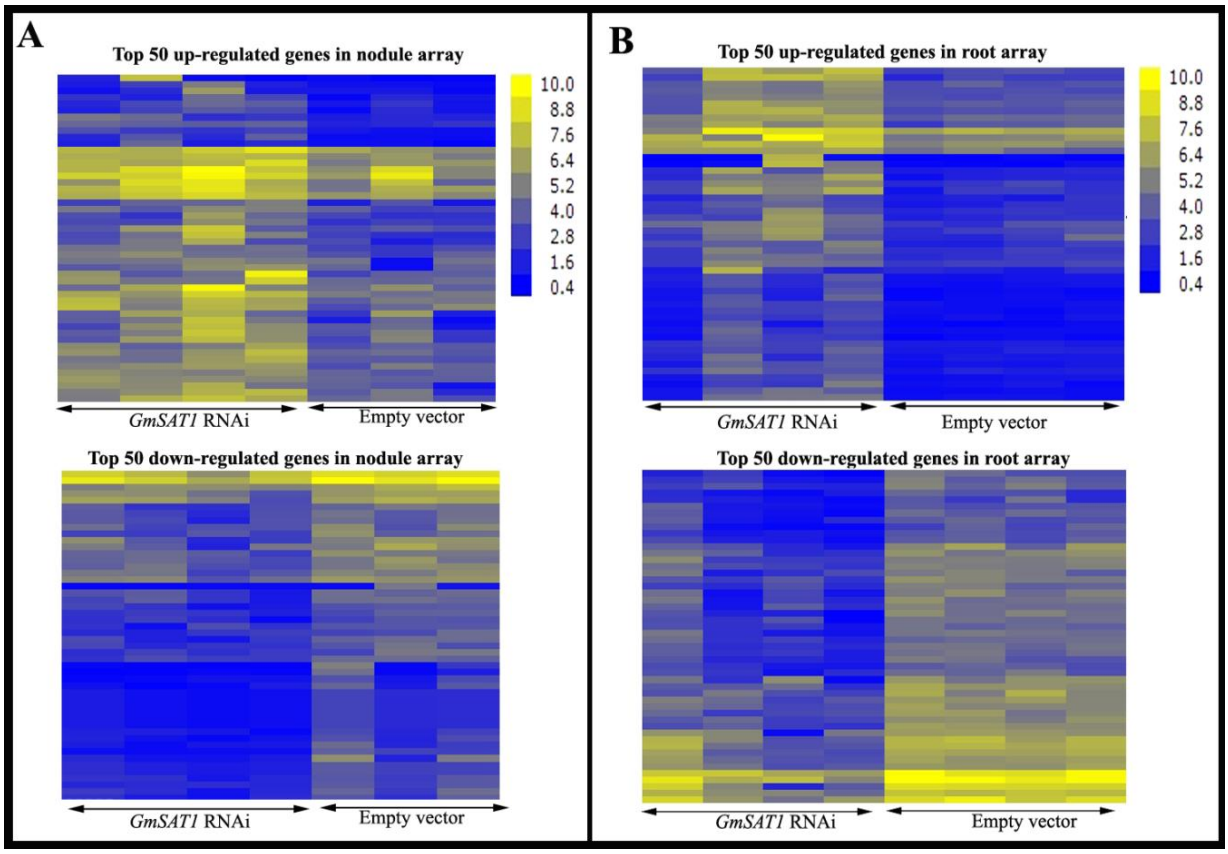


Figure 4.3 Heat map analysis

Heat map analysis of expression signals of the top 50 down-regulated and up-regulated genes in the nodule (A) and root arrays (B) ($p < 0.05$, fold-change > 2). Yellow denotes elevated expression and blue denotes reduced expression across a 10-fold scale. Each column represents a single array where ordered genes in each column are presented as rows across all of the columns.

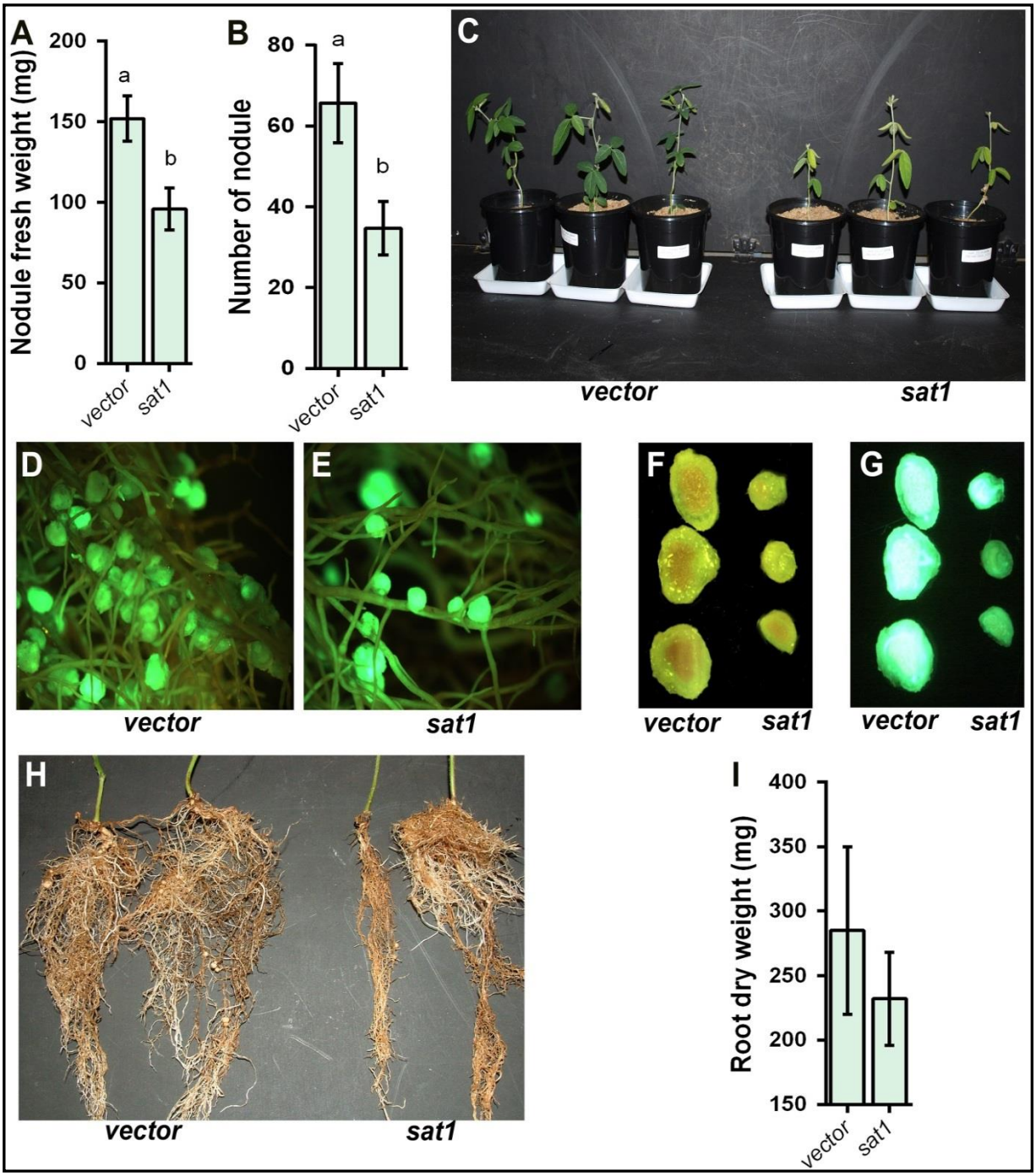


Figure 4.4 Effect of *GmSAT1* silencing on soybeans roots and nodule.

(A) Significant reduction in nodule fresh weight and number in *sat1* plants comparing to vector (n=15, P≤0.05) (C) Comparison of *sat1* with vector, plants grown without external nitrogen on the nodulated transgenic root system. (D-G) Comparison of the number and size of transgenic nodules on hairy roots between *sat1* and vector. (F) Smaller and poorly developed nodules of *sat1* transformed nodules, demonstrating a reduction in the presence of leghemoglobin in the infected region in *sat1* nodules. (H, I) No significant difference in root dry weight between *sat1* and empty vector plant (Data represents mean ± SE).

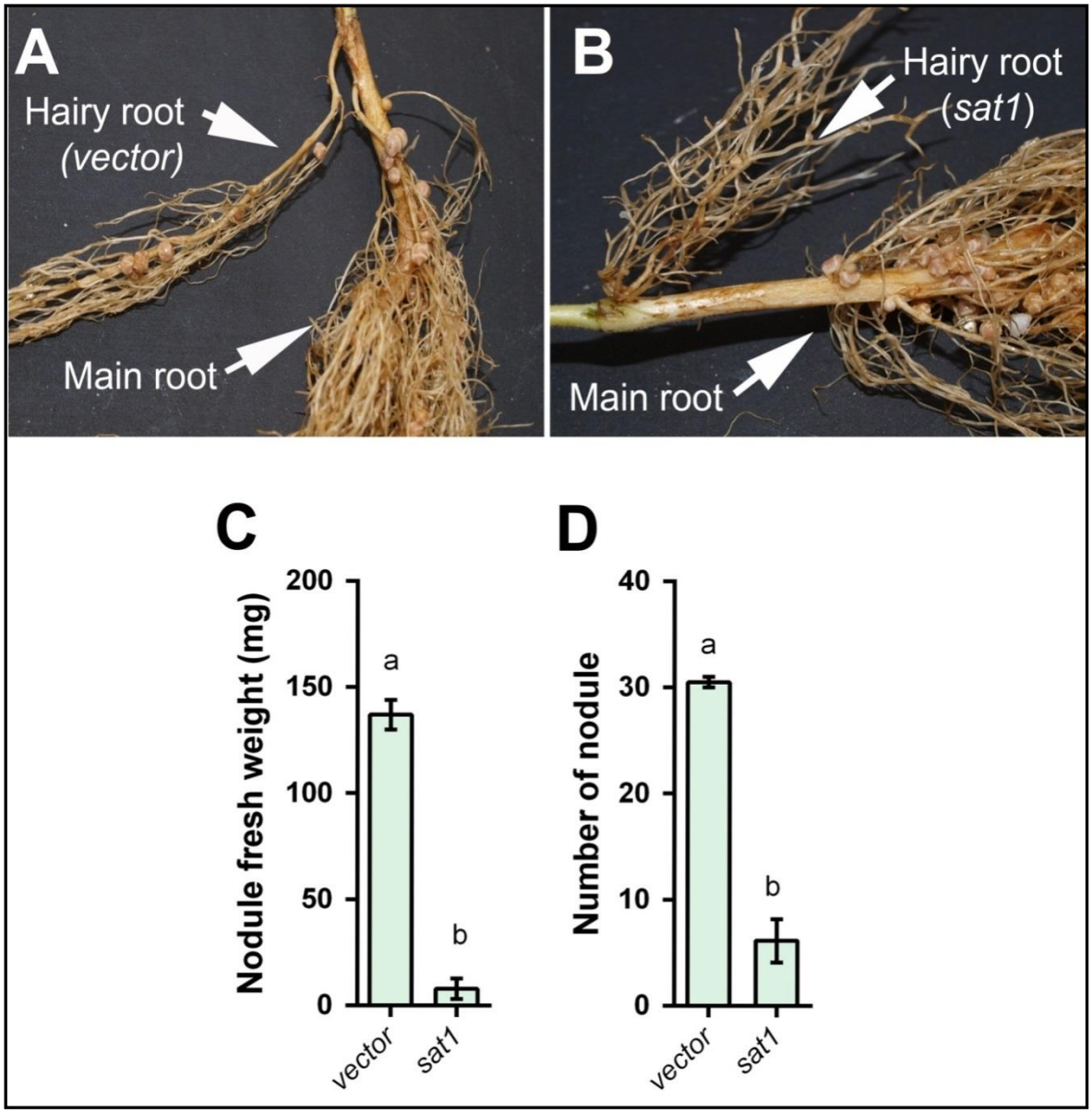


Figure 4.5 Effect of *GmSAT1* silencing on nodulation when transgenic hairy root and non-transgenic main root are nodulated simultaneously.

(A, B) Reduction in number and size of nodule in transgenic hairy roots is more sever when main root is kept attached. (C, D) Significant reduction in nodule fresh weight and nodule number in *sat1* plants comparing to vector (n=10-15, $P \leq 0.05$)

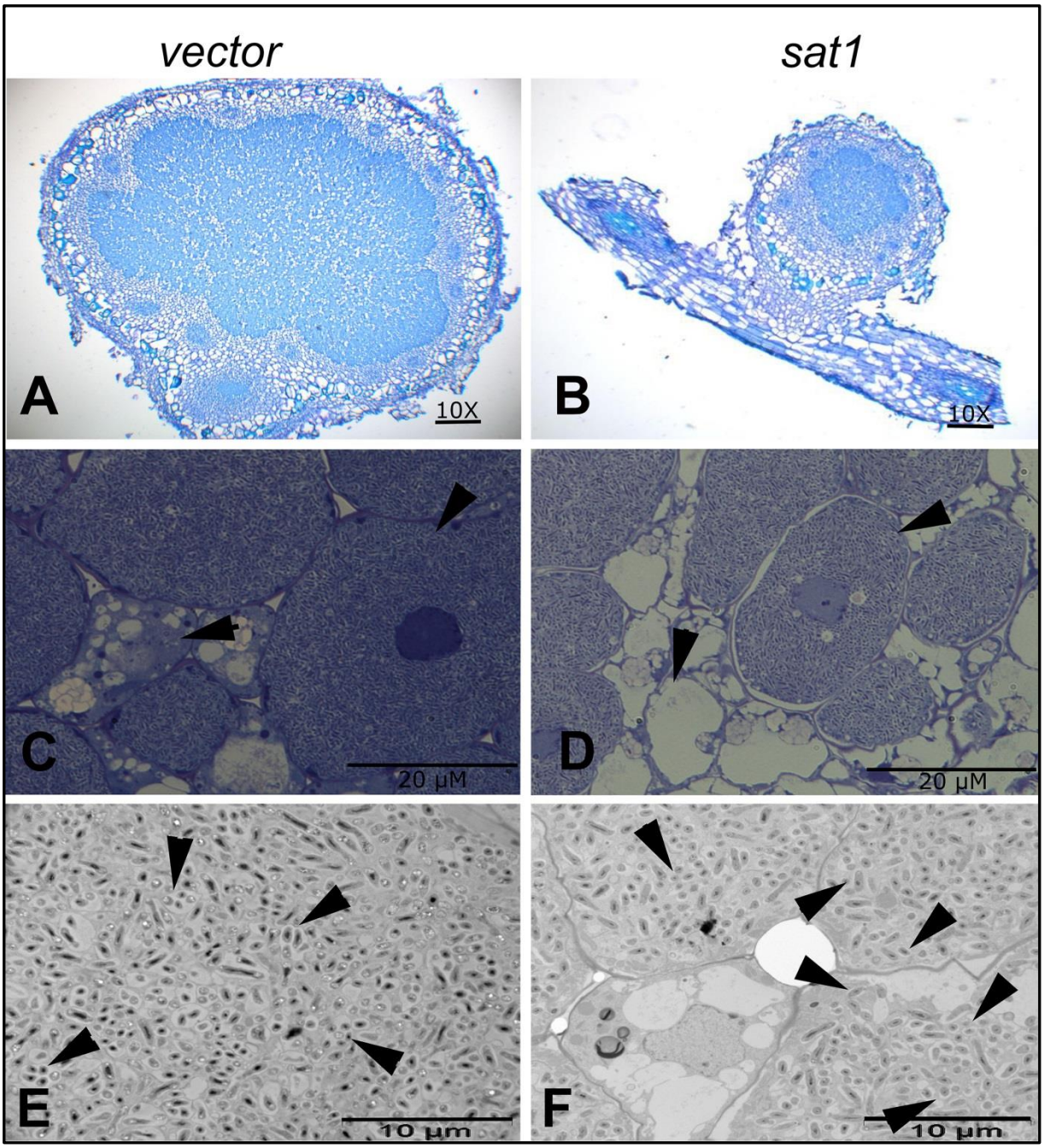


Figure 4.6 Microscopic (light and Transmission electron microscopy) comparisons of nodule structure between *sat1* and vector plants.

(A, B) *sat1* transformed nodules showed smaller infected zone comparing to vector (stained with Toludine blue). TEM cross sections of infected cell in vector (C, E) and *sat1* plants (D, E) (stained with lead citrate). IC: Infected cell, UIC: un-infected cell (bar=20 µm (C and D), bar= 10 µm (E and F)).

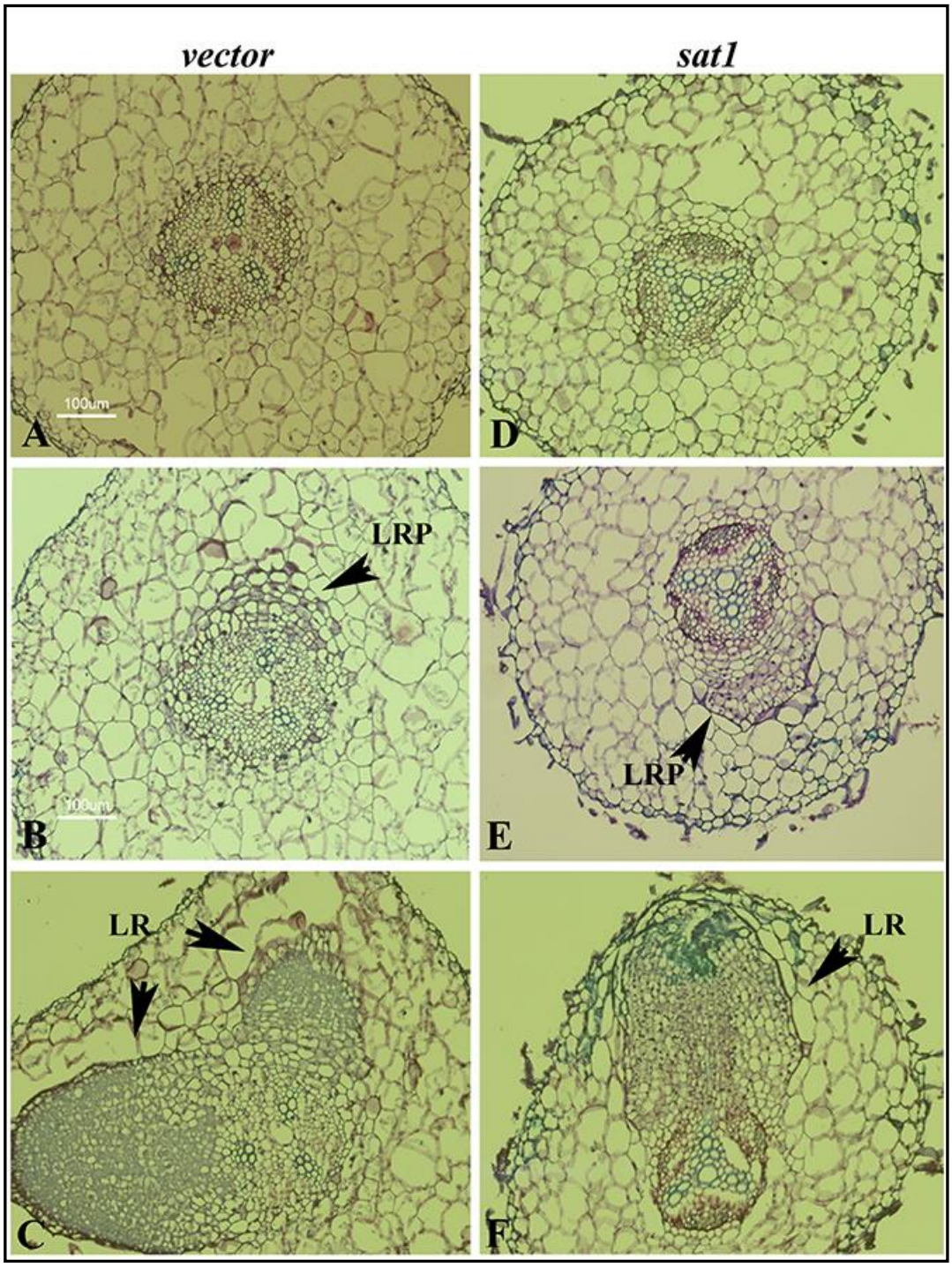


Figure 4.7 Cross section of vector and *sat1* root.

A, B, D, E) Stele tissue in root of vector plants has different pattern in comparison to *sat1* plants and show higher cell density (stained with Toludine blue). Higher cell density in vector plant was observed in lateral root as well (C, F). (LR: lateral root, bar =100 µm).

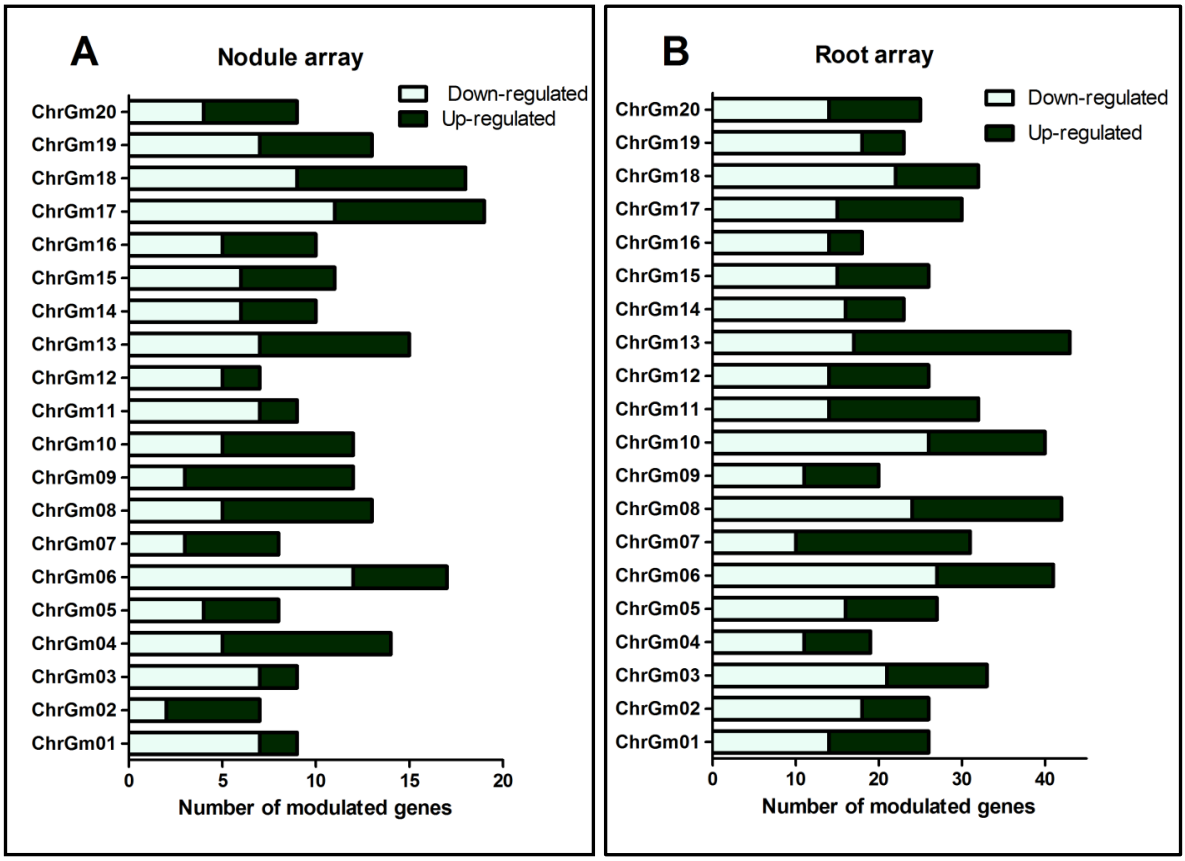
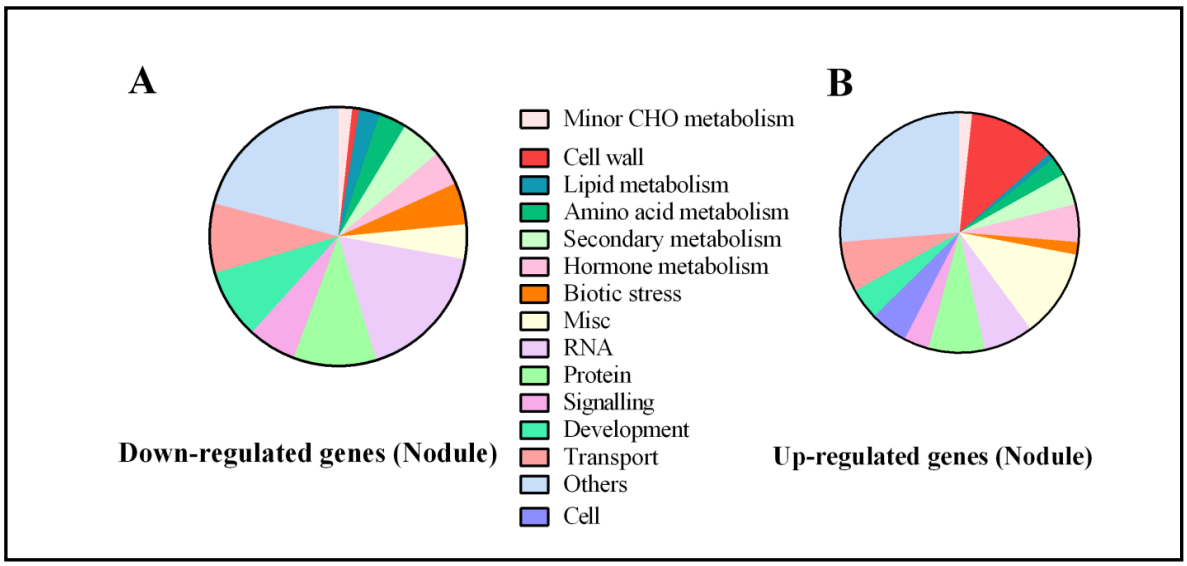


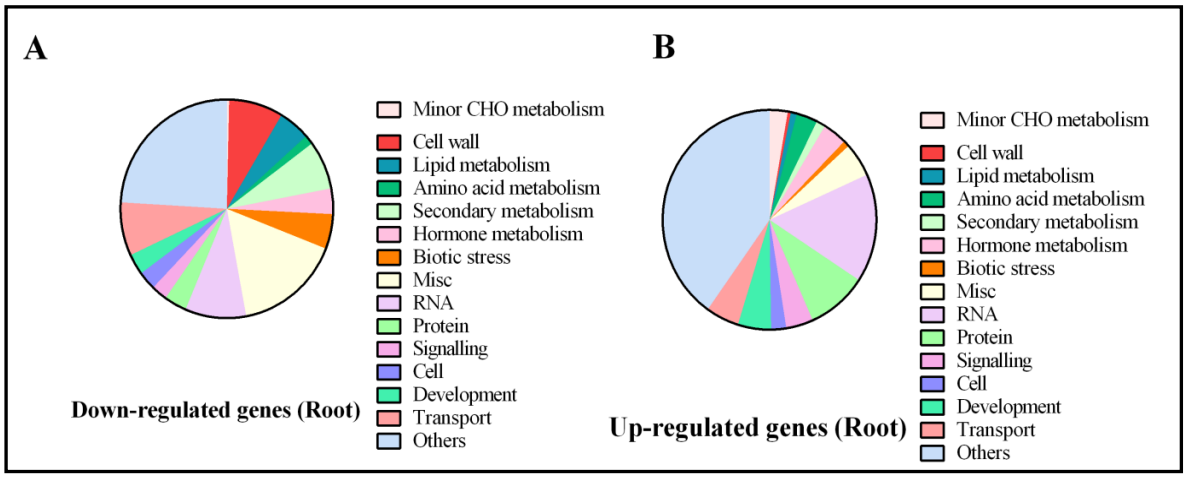
Figure 4.8 Chromosomal location of differentially expressed genes in *sat1* nodule (A) and roots (B).



Functional groups	***No. of down-regulated genes***	***No. of up-regulated genes***	***p-value***
Cell wall	1	14	0.00
Cell wall.modification	1	5	0.04
Hormone metabolism.auxin	3	0	0.05
Stress.biotic	6	2	0.05
misc	5	14	0.01
RNA	20	8	0.00
RNA.regulation of transcription	14	8	0.03
RNA.RNA binding	4	0	0.02
Protein.postranslational modification	3	0	0.05
Protein.degradation.ubiquitin.E3.RING	5	1	0.04
Signaling.calcium	3	0	0.05
Cell	0	6	0.00
Cell.organisation	0	5	0.01
Development	10	5	0.04
Transport.ABC transporters and multidrug resistance	6	0	0.00

Figure 4.9 Gene Ontology (GO) groupings of down-regulated and up-regulated genes in *sat1* nodule tissues.

Presentation of GO groupings of up (A) and down (B) regulated nodule genes. Functional groupings were carried out with the MapMan software and is based on significantly down-regulated and up-regulated genes ($p < 0.05$, fold change > 2). (Table) Functional groupings were statistically compared using IDEG6 webpage and are based on the number of genes within each grouping.



Main functional group	*No. of down-regulated genes*	*No. of up-regulated genes*	*P-value*
Minor CHO metabolism	1	6	0.01
Cell wall	24	1	0.00
Lipid metabolism	14	2	0.00
Amino acid metabolism	4	7	0.05
Secondary metabolism. Flavonoids	11	1	0.00
Hormone metabolism. Ethylene	0	3	0.04
Hormone metabolism. Gibberellin	3	0	0.05
Stress	24	11	0.03
Stress. Biotic	15	2	0.00
Nucleotide metabolism. Synthesis	0	3	0.04
Misc	47	11	0.00
Misc. cytochrome P450	8	2	0.05
Misc. acid and other phosphatases	5	0	0.03
Misc. UDP acid glucosyl and glucoronyl transferases	5	0	0.03
Misc. Seed storage/lipid transfer protein (LTP) family	5	0	0.03
Misc. GDSL-motif lipase	5	0	0.03
Misc. galacto – and mannosidases	4	0	0.06
RNA	27	35	0.00
RNA. Regulation of transcription	20	32	0.00
RNA. RNA binding	4	0	0.05
Protein	10	20	0.00
Protein. Posttranslational modification	2	5	0.05
Protein. Degradation	8	13	0.02
Development	9	11	0.05
Transporter	23	11	0.03
Transporter. ABC transporters and multidrug resistance	4	0	0.05
not assigned	44	58	0.00

Figure 4.10 Gene Ontology (GO) groupings of down-regulated and up-regulated genes in *sat1* root tissues.

Presentation of GO groupings of up (A) and down (B) regulated root genes. Functional groupings were carried out with the MapMan software and is based on significantly down-regulated and up-regulated genes ($p < 0.05$, fold change > 2). (Table) Functional groupings were statistically compared using IDEG6 webpage and are based on the number of genes within each grouping.

Table 4.1 List of genes either down-regulated or up-regulated in both *sat1* nodules and roots

Gene ID	*Gene Name*	*Description*	*Best Arabidopsis match*	*Nodule*	*Root*
Down-regulated genes					
Glyma05g18360		Unknown		-2.61	-2.33
Glyma06g40740		Disease resistance protein (TIR-NBS-LRR class) family	AT4G12010	-2.38	-1.91
Glyma10g43850	TT5	Chalcone-flavanone isomerase family protein	AT3G55120	-1.97	-4.16
Glyma11g31530		RNA-binding KH domain-containing protein	AT3G08620	-2.95	-1.9
Glyma12g30240	FRU	FER-like regulator of iron uptake	AT2G28160	-2.1	-3.58
Glyma16g28570		Disease resistance family protein / LRR family protein	AT2G34930	-2.82	-3.26
Glyma17g03860		Unknown		-2.62	-4.21
Glyma17g14190	AGL14	AGAMOUS-like 14	AT4G11880	-1.81	-3.09
Glyma18g52250		NAD(P)-linked oxidoreductase superfamily protein	AT1G59950	-1.95	-1.95
Glyma19g35270	PDR12	Pleiotropic drug resistance 12	AT1G15520	-2.08	-2.07
Glyma19g42040	SWEET17	Nodulin MtN3 family protein	AT4G15920	-1.87	-2.16
Glyma20g28500		Protein of unknown function (DUF581)	AT3G22550	-1.87	-1.81
Up-regulated genes					
Glyma04g39860		Peroxidase superfamily protein	AT5G05340	3.41	2.19
Glyma05g03920		Protein of unknown function (DUF1442)	AT2G45360	2.06	2.31
Glyma05g36100	MIOX4	Myo-inositol oxygenase 4	AT4G26260	1.84	2.38
Glyma06g08100		Unknown	AT5G24890	1.8	2.51
Glyma06g42890	MBF1B	Multiprotein bridging factor 1B	AT2G42680	2.08	2.2
Glyma10g30560		Winged-helix DNA-binding transcription factor family protein	AT1G06760	2.39	2.33
Glyma13g12190		Unknown		3.65	2.99
Glyma13g25520		Unknown		1.97	2.22
Glyma16g02200	EMB2301	NAC (No Apical Meristem) domain transcriptional regulator superfamily protein	AT2G46770	1.86	2.35
Glyma17g03850		Unknown		2.64	2.41
Glyma18g05160	OXS3	Oxidative stress 3	AT5G56550	2.29	3.22

Table 4.2 List of genes showing opposite expression patterns between nodules and roots

Gene ID	*Gene Name*	*Description*	*Best Arabidopsis match*	*Nodule*	*Root*
Glyma02g38920		Histone superfamily protein	AT3G53730	4.83	-2.44
Glyma02g47880	FLA2	FASCICLIN-like arabinogalactan 2	AT4G12730	2.53	-2.41
Glyma06g02650	TUB1	tubulin beta-1 chain	AT1G75780	2.08	-2.23
Glyma08g19290		UDP-Glycosyltransferase superfamily protein	AT2G22590	2.28	-4.63
Glyma08g46520	CYP93D1	cytochrome P450, family 93, subfamily D, polypeptide 1	AT5G06900	1.81	-6.9
Glyma11g12810		Bifunctional inhibitor lipid-transfer protein	AT5G48485	1.95	-1.92
Glyma12g08990	CSLA02	cellulose synthase-like A02	AT5G22740	2.42	-2
Glyma12g36290	RHM1	rhamnose biosynthesis 1	AT1G78570	1.88	-1.92
Glyma14g07810	CPuORF5	conserved peptide upstream open reading frame 5		1.84	-3.29
Glyma14g09510		N-terminal nucleophile aminohydrolases (Ntn hydrolases) superfamily protein	AT3G16150	2.96	-2.46
Glyma15g06240		Unknown	AT5G09520	2.29	-1.87
Glyma15g11930	EFE	ethylene-forming enzyme	AT1G05010	1.87	-3.08
Glyma16g04950	XTH5	xyloglucan endotransglucosylase hydrolase 5	AT5G13870	2.01	-7.74
Glyma17g14850		Bifunctional inhibitor lipid-transfer protein	AT1G62510	3.76	-2.02
Glyma17g14860		Bifunctional inhibitor lipid-transfer protein	AT1G62510	2.13	-3.14
Glyma17g17970	AGP18	arabinogalactan protein 18	AT4G37450	2.08	-2.98
Glyma18g06220		Peroxidase superfamily protein	AT5G05340	2.65	-3.56
Glyma18g51880		Disease resistance-responsive (dirigent-like protein) family protein	AT2G39430	1.91	-2.11
Glyma20g24670		Pectin lyase-like superfamily protein	AT5G04310	2.18	-1.81
Glyma20g27280	TUA4	tubulin alpha-4 chain	AT1G04820	2.31	-3.42
Glyma01g00980	NRPC2	nuclear RNA polymerase C2	AT5G45140	-7.03	3.02
Glyma01g42030	PME1	pectin methylesterase inhibitor 1	AT4G12390	-2.34	7.38
Glyma04g40640	PRR5	pseudo-response regulator 5	AT5G24470	-2.86	1.82
Glyma05g09130		---	AT3G01640	-2.46	2.34
Glyma06g05280	BCAT-2	branched-chain amino acid transaminase 2	AT1G10070	-2.4	10.9
Glyma06g13280	GLT1	NADH-dependent glutamate synthase 1	AT5G53460	-2.22	2.44
Glyma09g36210	RTFL12	ROTUNDIFOLIA like 12		-1.91	2.38
Glyma10g02210	SAG21	senescence-associated gene 21	AT4G02380	-3.04	3.05
Glyma17g05920	NF-YA8	nuclear factor Y, subunit A8	AT1G17590	-2.53	8.36
Glyma17g06560			AT5G57123	-3.25	3.49
Glyma17g14680	BETA-VPE	beta vacuolar processing enzyme	AT1G62710	-2.72	2.43
Glyma17g18040	BRU6	Auxin-responsive GH3 family protein	AT4G37390	-3.2	1.96
Glyma19g32850		myb-like HTH transcriptional regulator family protein	AT5G06800	-1.92	2.49
Glyma20g24510		F1F0-ATPase inhibitor protein, putative	AT5G04750	-2.08	2.38

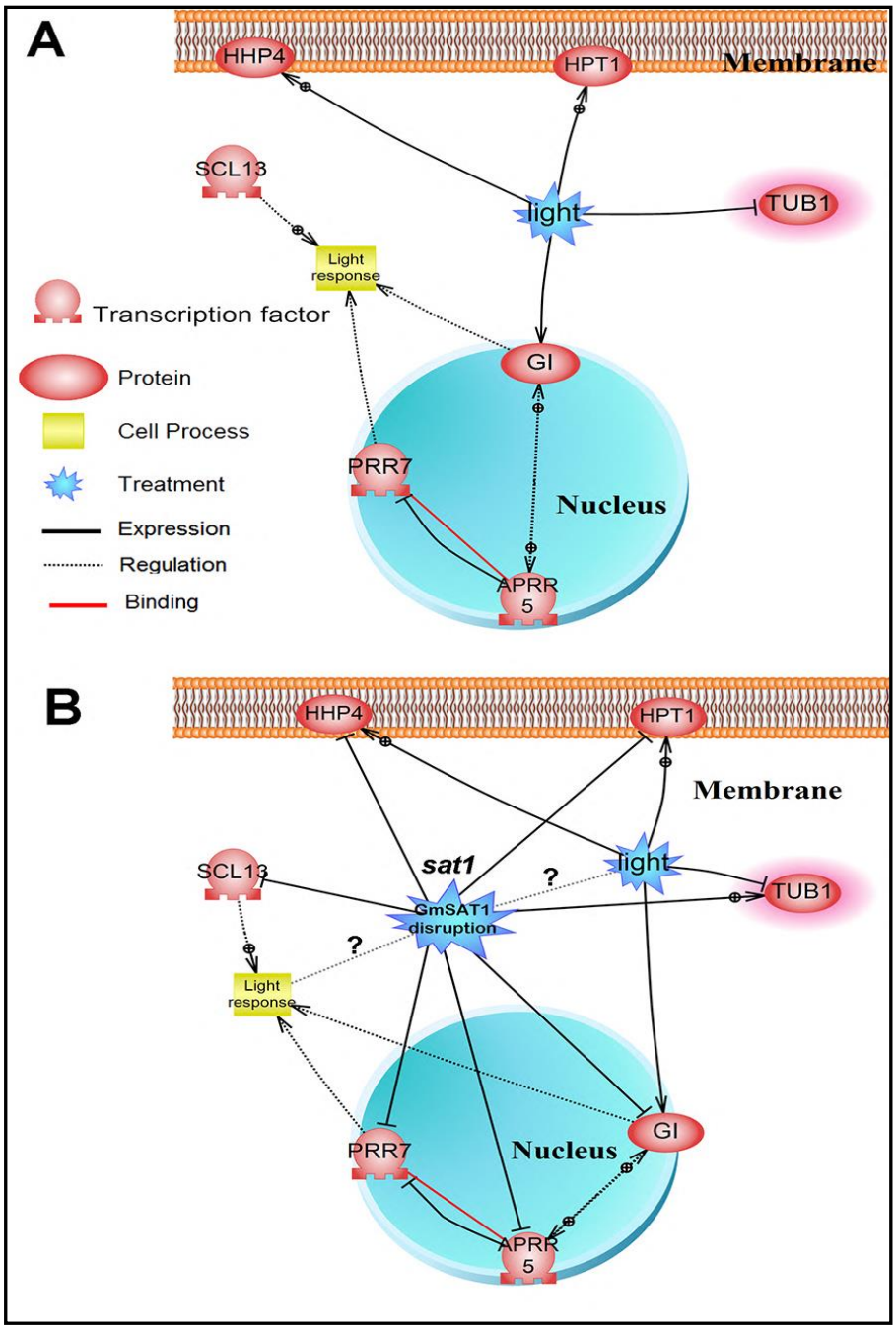


Figure 4.11 A hypothetical light responsive networks identified based on altered gene expression in *sat1* nodules.

A) The network is based on the light mediated interaction of two membrane proteins (*HPT1* and *HHP4*) three transcription factors (*PRR7*, *PRR5*, *SCL13*), a nuclear localized membrane protein *GI* and a tubulin protein *TUB1* . *PRR5*, *PRR7* and *GI* are involved in circadian clock regulation. B) The network shows how *GmSAT1* disruption has affected the expression of all these genes. All light responsive genes in networks have down-regulated in *sat1* nodule. Expression of *TUB1*, the only up-regulated gene in the network (highlighted in pink), inhibited by light.

Table 4.3 List of modulated light responsive genes in nodule and their identified relations.

* Wherever indicated --+> means up-regulation or positive regulatory effect, ---> required for regulation or expression ---| down-regulation or negative effect, ----DNA binding

** Presented references can be found in this website:

http://www.ncbi.nlm.nih.gov/pubmed/

Gene ID	***Fold change***	***Name***	***Best Arabidopsis Match***	***Type of gene interaction (Relation)****	***MedLine references****
Glyma10g44170	-3.45	HPT1	AT2G18950	light --+> HPT1	14512521, 15665245
Glyma20g30980	-3.00	GI	AT1G22770	GI --+> APRR5	18562312
				APRR5 --+> GI	
Glyma09g07240	-1.99			GI ---> light response	17098855, 16006578, 20864385
				light ---> GI	11402160, 16908503
Glyma04g40640	-2.86	PRR5	AT5G24470	APRR5 ---| PRR7	20354196
Glyma12g07860	-2.22	PRR7	AT5G02810	PRR7 ---> light response	15705949, 17102803
				APRR5 ---- PRR7	20233950
Glyma11g05030	-2.3	HHP4	AT4G37680	light --+> HHP4	16263907
Glyma06g02650	2.08	TUB1	AT1G75780	light ---| TUB1	8718628
Glyma07g39650	-1.9	SCL13	AT4G17230	SCL13 --+> light response	16680434

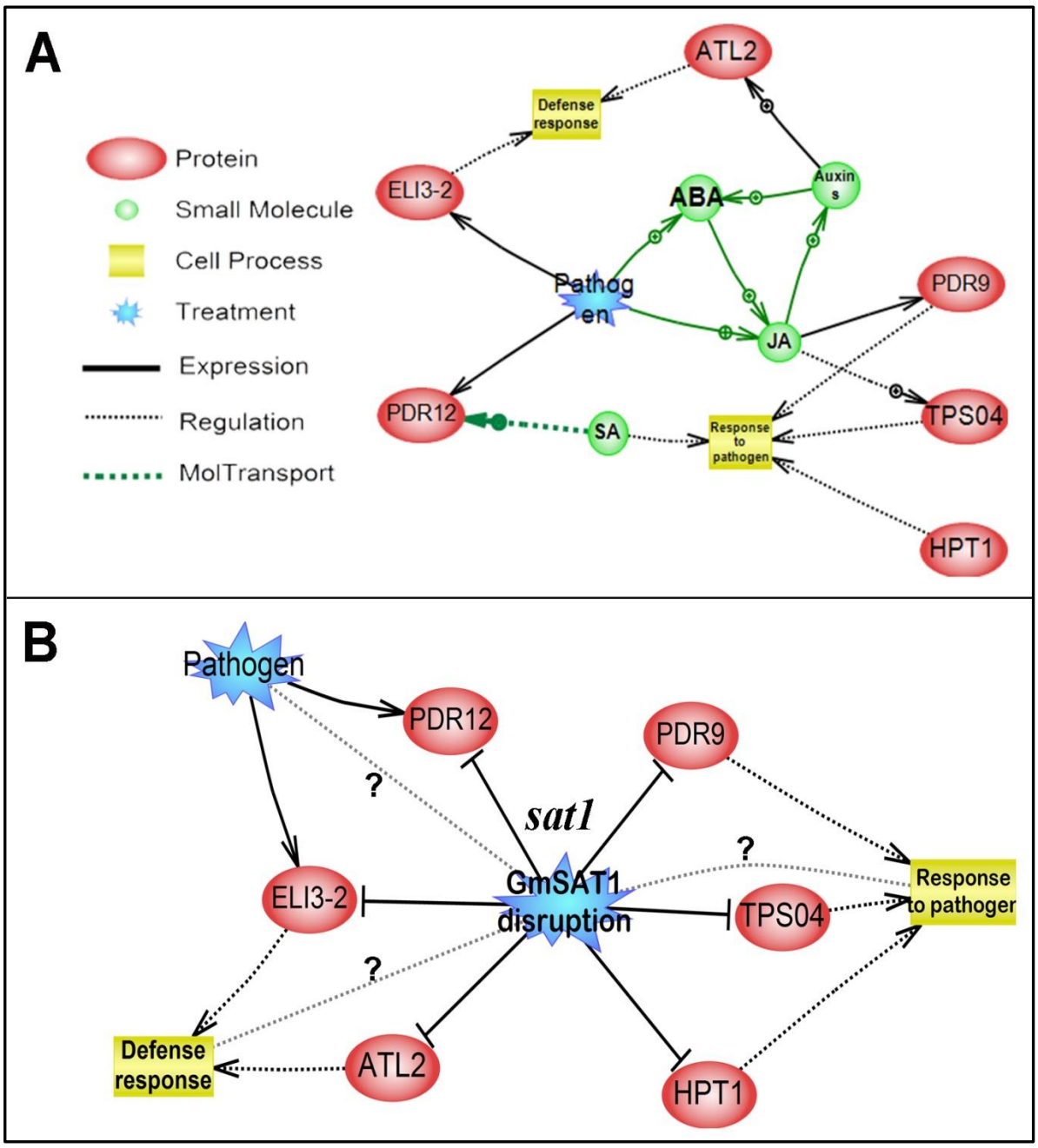


Figure 4.12 Defense response network with down-regulated genes in *sat1* nodules.

Each of these genes respond positively to either pathogen recognition (*ELI3-2* and *PDR12*) or by through generalized biotic stress response hormones including JA and SA (*PDR9*, *TPS04*, *PDR12*). B) *GmSAT1* disruption have down-regulated all these genes in nodule.

Table 4.4 List of down-regulated defense responsive genes in nodule and their identified relations.

* Wherever indicated --+> Up-regulation or Positive regulatory effect, ---> required for regulation or expression, ---| Down-regulation or negative effect, ---+> Positive effect on Molecular synthesis, **….**+>Molecular transport induction

** Presented references can be found in this website: http://www.ncbi.nlm.nih.gov/pubmed/

Gene ID	***Fold change***	***Name***	***Best Arabidopsis Match***	***Type of gene interaction (Relation)****	***MedLine references****
Glyma13g25270	-2.28	TPS04	AT1G61120	JA --+> TPS04	17965175
				TPS04 ---> Response to pathogen	17965175
Glyma19g35270	-2.08	PDR12	AT1G15520	Pathogen --+> PDR12	14526118
				SA **….**+> PDR12	16720608
Glyma17g04360	-2.2	PDR9	AT3G53480	PDR9 --> Response to pathogen	16506311, 14572653
				JA --+> PDR9	14572653
Glyma18g38670	-5.34	ELI3-2	AT4G37990	Pathogen --+> ELI3-2	
				ELI3-2 ---> Defense response	11038530
Glyma09g04750	-2.2	ATL2	AT3G16720	Auxins --+> ATL2	8914520, 10852940
				ATL2 ---> Defense response	15238540
Glyma10g44170	-3.43	HPT1	AT2G18950	HPT1 ---> Response to pathogen	23137278, 17194769

Other relations

Relation	***MedLine references****
Auxins --+> ABA	17317672, 20354195
ABA --+> JA	9161035, 8702864, 17513501, 18390489, 9880363, 18252700
Pathogen --+> ABA	18815384
Pathogen --+> JA	17475618, 20348210, 18216250, 16829584, 16935989, 19535475, 17998535, 12913177, 12615947, 17616737 <more data available...>
JA --+> Auxins	19435934, 17616737, 18390489, 14605232
SA ---> Response to pathogen	16829584, 17998535

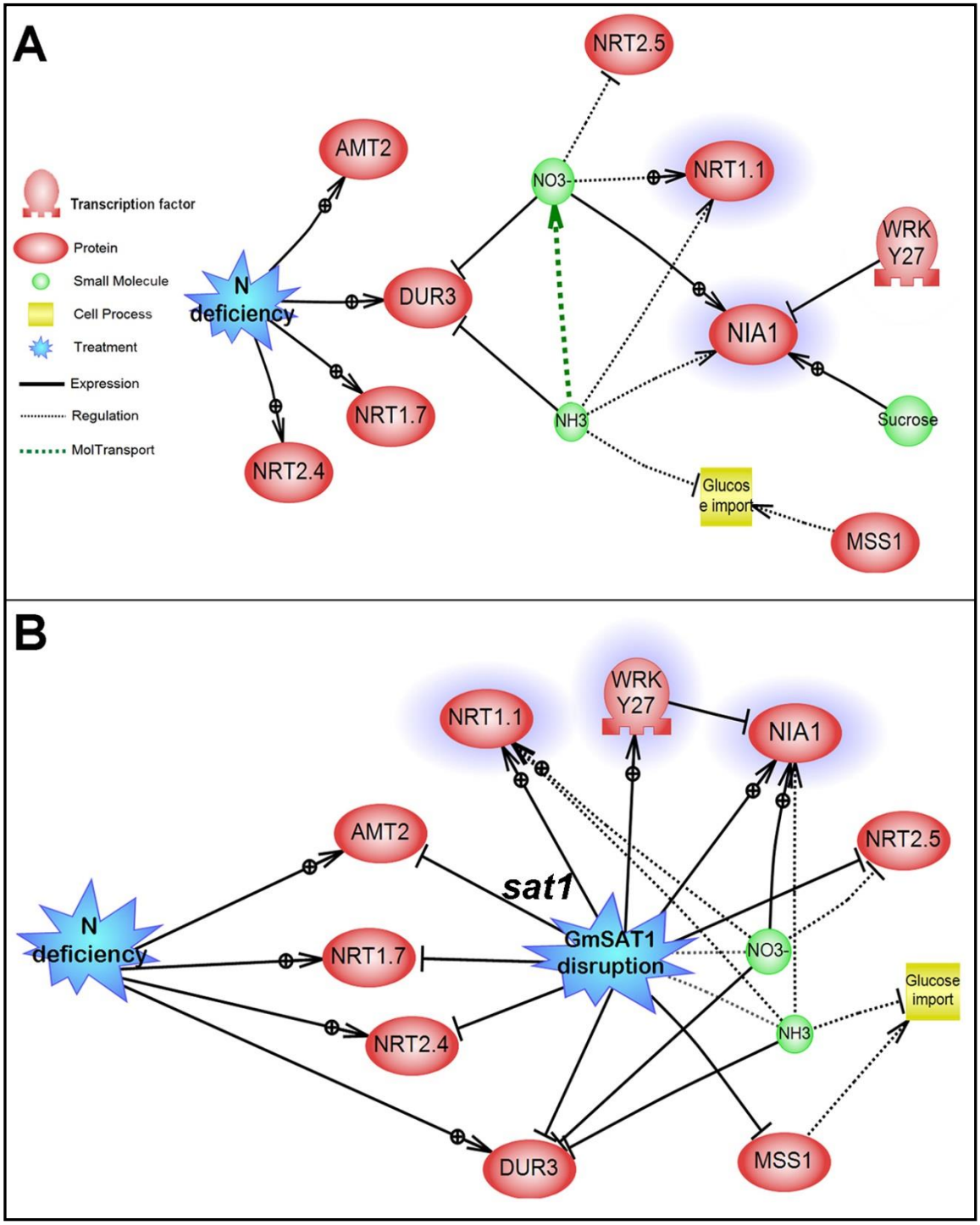


Figure 4.13 Nitrogen-linked pathways.

A) This network includes 9 genes which directly or indirectly participate in N transport. Highlighted in purple induced by nitrogen (*NIA1*, and *NRT1.1*) B) This network shows *GmSAT1* disruption down-regulated 5 genes (*NRT2.5*, *DUR3*, *AMT2*, *NRT1.7*, *NRT2.4*) which are induced by nitrogen deficiency but up-regulated expression of other genes which directly or indirectly induce by nitrogen (Highlighted in purple). *MSS1* is a member of Major facilitator superfamily protein which has a role in glucose transport and down-regulated in *sat1* root.

Table 4.5 List of modulated genes in root involved in N transport pathway and their identified relations.

* Wherever indicated --+> Up-regulation or Positive regulatory effect, ---> required for regulation or expression, ---| Down-regulation or negative effect,

** Presented references can be found in this website: http://www.ncbi.nlm.nih.gov/pubmed/

Gene ID	*Fold change*	*Name*	*Best Arabidopsis Match*	*Type of gene interaction (Relation)**	*MedLine references***
Glyma13g02510	3.1	NIA1	AT1G77760	NH3 ---> NIA1	20442374, 12644691
				nitrate --+> NIA1	19633234, 21454300, 8024571, 20668061, 20375110
				sucrose --+> NIA1	15273295
Glyma20g30290	2.01	WRKY27	AT5G52830	WRKY27 ---| NIA1	18702671
Glyma01g41930	3.11	NRT1.1	AT1G12110	NH3 ---> NRT1.1	15319483
Glyma11g03430	2.48			nitrate --+> NRT1.1	18563586, 10948265, 20088899, 11487691, 15319483
Glyma18g20510	-2.02	NRT2.5	AT1G12940	nitrate ---| NRT2.5	21151904
Glyma04g14790	-2.02	DUR3	AT5G45380	nitrate ---|DUR3	17672841
				NH3 ---| DUR3	
				Starvation --+> DUR3	17705847
Glyma05g33010	-1.95	AMT2	AT2G38290	Starvation --+> AMT2	10675553,12481062
Glyma02g02680	-2.03	NRT1.7	AT1G69870	Starvation ---> NRT1.7	19734434
Glyma11g00710	-2.17	MSS1	AT5G26340	MSS1 ---> glucose import	22041897
Glyma11g20090	-5.4	NRT2.4	AT5G60770	Starvation --+> NRT2.4	22880003, 22227893
Glyma13g39850	-2				
Other relationships					
NH3 ---| glucose import		17158605			
NH3 **….**+> nitrate		17615410, 18508958, 10677431, 14990621, 14671012, 11553754, 17172286, 17204540,12493871, 10806247, 10712542, 14730065			

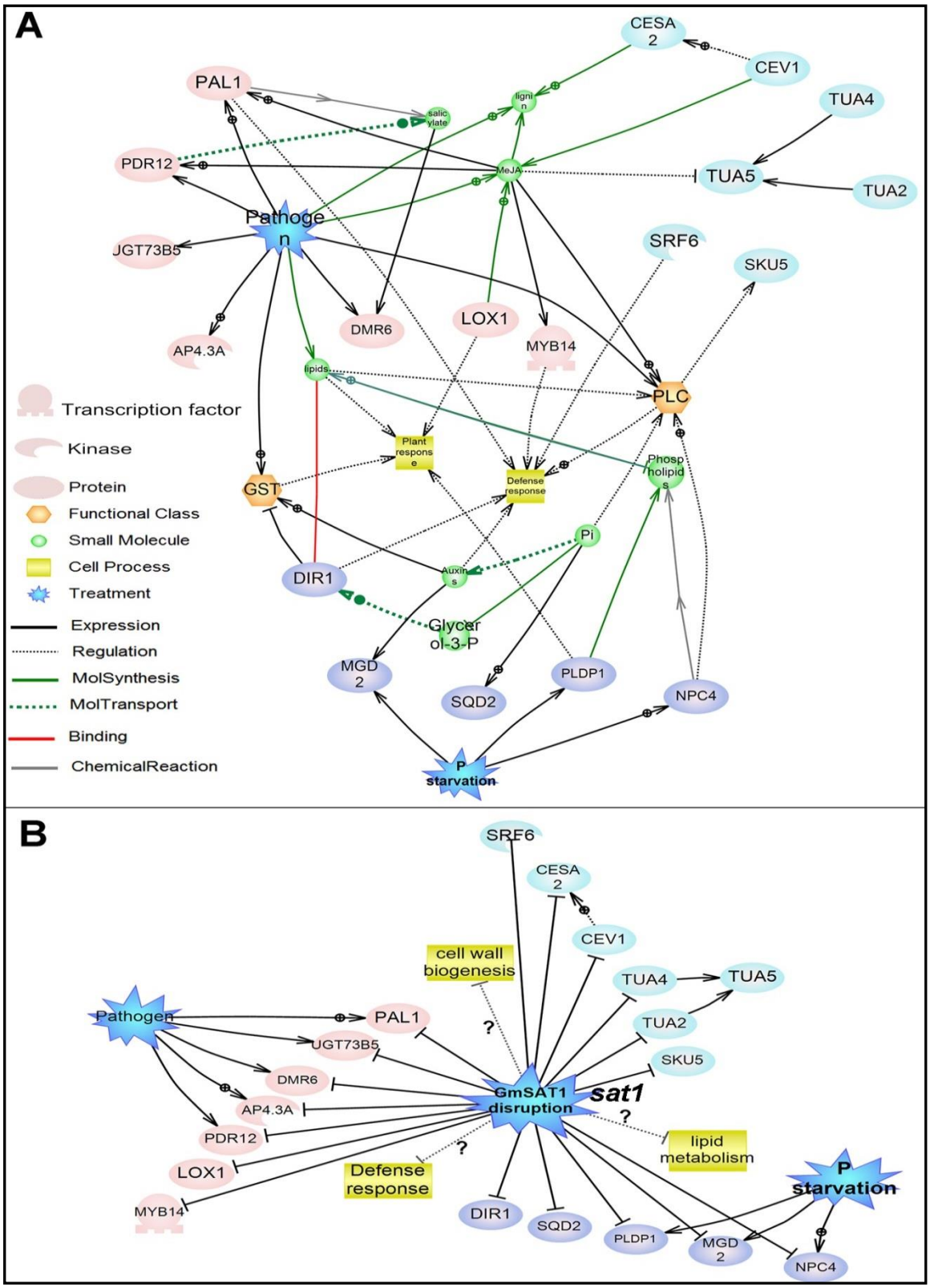


Figure 4.14 Defense response pathways.

A) Putative defense response pathway involving down-regulated genes in *sat1* roots. This network includes 3 sub-networks: Biotic stress (light pink color), Cell wall metabolism (light blue color) and lipid metabolism (purple color). Interactions between the sub-networks involve JA and Pi. B) In *sat1* roots, three individual sub networks involved in defense response, Cell wall metabolism and lipid metabolism, have down-regulated.

Table 4.6 List of down-regulated genes in root involved in biotic stress, cell wall and lipid methabolism pathway.

GST: glutathione transferase, PLC: Phospholipase C

* Wherever indicated --+> Up-regulation or Positive regulatory effect, ---> required for regulation or expression, ---| Down-regulation or negative effect, ---- binding, ---+> Positive effect on Molecular synthesis, ---| negative effect on Molecular synthesis, **….**+>Molecular transport induction, ->-> Chemical interaction

** Presented references can be found in this website: http://www.ncbi.nlm.nih.gov/pubmed/

GO	*Gene ID*	*Fold change*	*Name*	*Best Arabidopsis Match*	*Type of gene interaction (Relation)**	*MedLine references***
Defense and biotic stress	Glyma03g25380	-2.19	AP4.3A	AT2G32800	Pathogen --+> AP4.3A	9742960
	Glyma19g04280	-3.63	DMR6	AT5G24530	SA --+> DMR6	18248595
					Pathogen --+> DMR6	
	Glyma07g00920	-2.31	LOX1	AT1G55020	LOX1 --+> JA	11891244
	Glyma07g00900	-1.97			LOX1 ---> Plant response	23526882
	Glyma08g20230	-1.87				
	Glyma06g45550	-2.37	MYB14	AT2G31180	MYB14 ---> defense response	20732878
					MYB14 --->JA	
	Glyma19g36620	-2.7	PAL1	AT2G37040	JA --+> PAL1	11038546
					PAL1 ---> defense response	18434604
					PAL1 ->-> SA	18245336, 10449586
					Pathogen --+> PAL1	12239383, 18434604, 17158583
	Glyma19g35270	-2.07	PDR12	AT1G15520	JA --+> PDR12	14526118
					Pathogen ---> PDR12	
					PDR12**…**+>SA	16720608
	Glyma10g42680	-2.17	UGT73B5	AT2G15480	Pathogen ---> UGT73B5	16553894
Cell wall	Glyma07g05230	-2.45	SRF6	AT1G53730	SRF6 ---> defense response	17397538
	Glyma19g45130	-2.35				
	Glyma20g27280	-3.42	TUA4	AT1G04820	TUA4 ---> TUA5	8111033
	Glyma06g09500	-2.16	TUA2	AT1G50010	TUA2 ---> TUA5	
	Glyma04g09350	-1.94			MeJA ---| TUA5	11027363
	Glyma02g08920	-2.66	CESA2	AT4G39350	CESA2 --+> lignin	17233901
					CEV1 --+> CESA2	18349153
	Glyma15g43040	-1.93	CEV1	AT5G05170	CEV1 ---> JA	12119374, 11340179
	Glyma05g04270	-2.86	SKU5	AT4G12420	PLC ---> SKU5	12119380
	Glyma17g14730	-2.11				
Lipid metabolism	Glyma11g12810	-1.92	DIR1	AT5G48485	DIR1 ---- lipids	18552128
					DIR1 ---| GST	14673031
					DIR1 ---> defense response	
					glycerol-3-P**…**+>DIR1	22067992
	Glyma13g23150	-3.9	MGD2	AT5G20410	Auxins ---> MGD2	16762032, 15590685
					Pi starvation ---> MGD2	19179086, 14730084
	Glyma03g22860	-2.73	NPC3	AT3G03530	NPC4 --+> PLC	15618226
					Pi starvation --+> NPC4	
					Phospholipids <-<- NPC4	20699393, 15618226
	Glyma20g38200	-2.71	PLDP1	AT3G16785	Pi starvation --+> PLDP1	16891548
					PLDP1 ---> Phospholipids	

GO	*Gene ID*	*Fold change*	*Name*	*Best Arabidopsis Match*	*Type of gene interaction (Relation)**	*MedLine references***
					PLDP1 ---> Plant response	18364466
	Glyma03g14200	-2.93	SQD2	AT5G01220	Pi ---| SQD2	11960029

Other relations in biotic stress, cell wall and lipid methabolism pathway

MedLine references*	***Relation***
JA ---> lignin	21546454, 18803390
Pathogen --+> JA	17475618, 16829584, 17998535, 12615947, 17616737, 11038546
Pathogen --+> lignin	16332414, 20509918
Pathogen ---> lipids	17475618
lipids ---> Plant response	16891548, 22275366, 22589465
lipids ---> PLC	15927962, 11090221
PLC --+> defense response	22684579
Pi ---> PLC	15927962, 10922040
Pi**…**+>Auxins	19106375
Pi ---| glycerol-3-P	12226504
Auxins ---> defense response	10759534, 18192436
GST ---> Plant response	17692078, 12226515
Pathogen --+> GST	15012285, 12090627

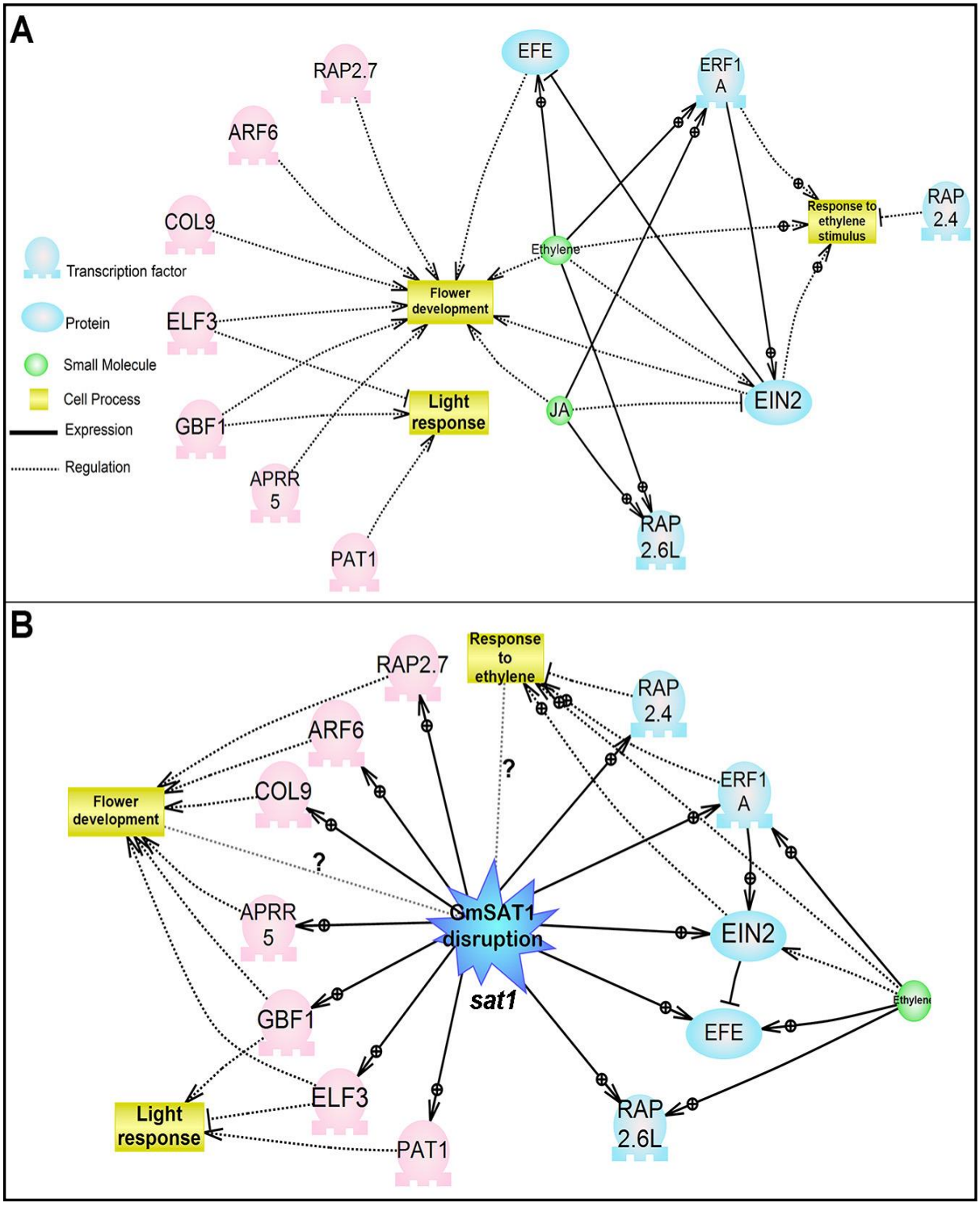


Figure 4.15 Hypothetical flowering network identified amongst up-regulated genes in *sat1* roots. Transcription factors which positively regulate flower development are identified in the pink network. Associated ethylene responsive genes are identified as the blue network

Table 4.7 List of up-regulated ethylene and flowering responsive genes in root and their identified relations.

* Wherever indicated --+> Up-regulation or Positive regulatory effect, ---> required for regulation or expression, ---| Down-regulation or negative effect

GO	*Gene ID*	*Fold change*	*Name*	*Best Arabidopsis Match*	*Type of gene interaction (Relation)**	*MedLine references***
Flowering responsive genes	Glyma11g06960	3.96	GBF1	AT4G36730	GBF1 ---> flower development	18930926, 16638747
	Glyma16g25600	1.84			GBF1 ---> light response	22692212
	Glyma04g05280	3.69	ELF3	AT2G25930	ELF3 ---| light response	20133619, 15781708, 10759510, 11402162, 12045273, 11402161
					ELF3 ---> flower development	16258016, 11402160, 11804815, 19187043, 19061637, 20838594
	Glyma04g40640	1.82	PRR5	AT5G24470	APRR5 ---> flower development	17504813, 12461138
	Glyma13g11590	2.14	COL9	AT3G07650	COL9 ---> flower development	16115071
	Glyma12g34420	2.12	SCL5 / PAT1	AT5G48150	PAT1 ---> light response	23109688
	Glyma15g09750	2.02	ARF8	AT1G30330	ARF6 ---> flower development	16107481, 19458116, 16461383
	Glyma12g07800	1.8	RAP2.7	AT2G28550	RAP2.7 ---> flower development	17595297
Ethylene responsive	Glyma08g28820	2.44	RAP2.6L	AT5G13330	Ethylene --+> RAP2.6L	
					JA --+> RAP2.6L	
	Glyma07g39420	2.35	EFE	AT1G05010	EFE ---> flower development	19560230, 14555699
					Ethylene --+> EFE	15349780, 16665167
	Glyma04g11290	2.13	RAP2.4	AT1G78080	RAP2.4 ---| response to ethylene stimulus	20031913
	Glyma03g33850	2.01	EIN2	AT5G03280	EIN2 --+> response to ethylene stimulus	15784879, 18713391, 20826954, 15466231, 19196655,
					EIN2 ---> flower development	21911380, 19168715
					Ethylene ---> EIN2	12857828, 17977152, 19196655, 12628921, 12953109,
					JA ---| EIN2	17977152
	Glyma01g41520	1.95	ERF-1	AT4G17500	ERF1A --+> EIN2	12509529
					ERF1A --+> response to ethylene stimulus	9851977, 11910004, 12045274,
					Ethylene --+> ERF1A	12509529, 14555690, 16531464, 15282545, 20974735, 17114278,
					JA --+> ERF1A	12509529, 18567832, 18467450,
Other relations						
Ethylene --+> response to ethylene stimulus				19836254, 20181754, 18692429, 19293381, 17525078		
Ethylene ---> flower development				20030751, 18367517, 17389366, 18565620, 12869518		
JA ---> flower development				19131630, 11595796, 19211701		

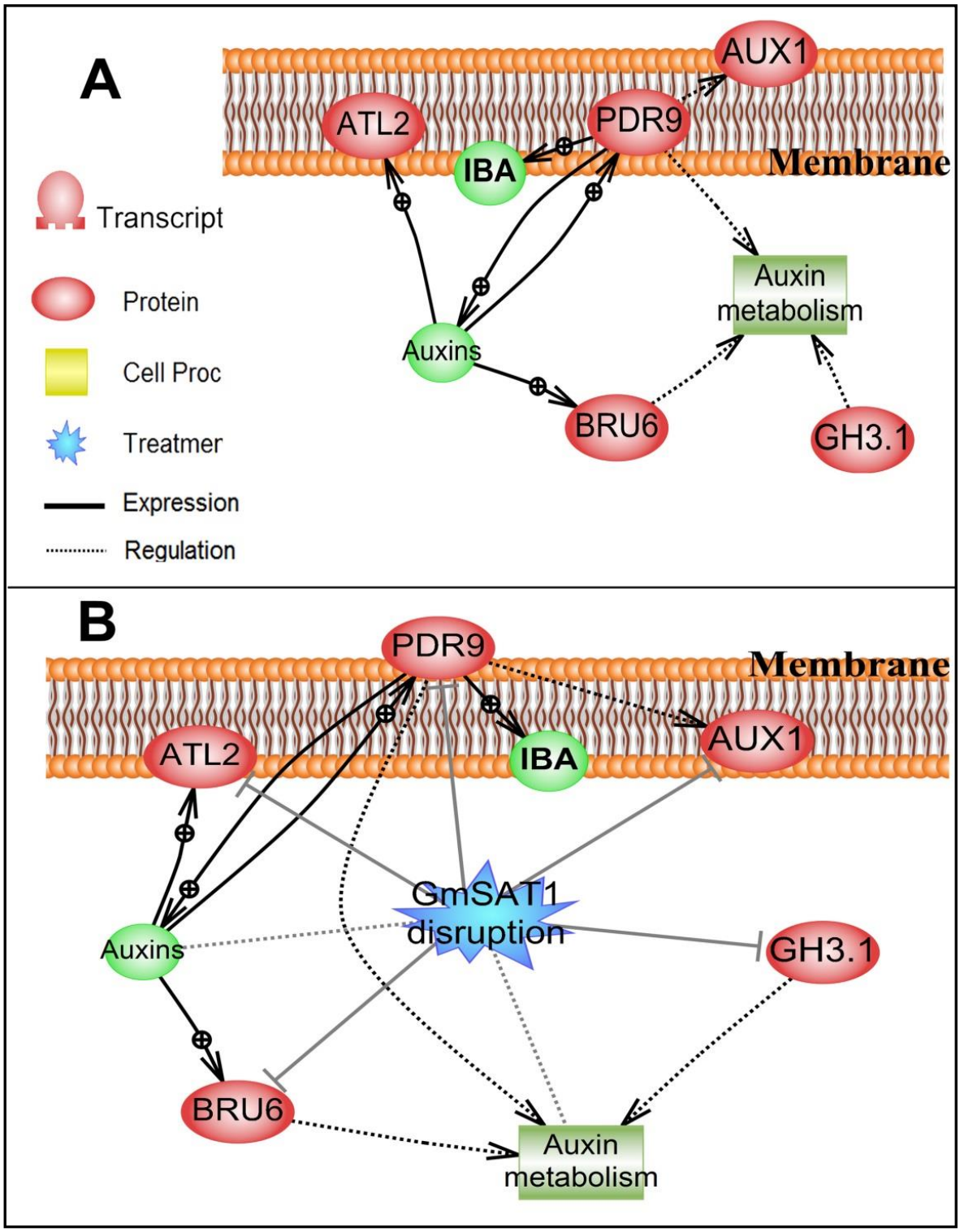


Figure 4.16 Hypothetical auxin network identified amongst down-regulated genes in *sat1* nodules.

Table 4.8 List of down-regulated auxin responsive genes in root and nodule and their identified relations.

Tissue	*Gene*	*Name*	*Best Arabidopsis Match*	*Explanation*	*Fold change*	*P-value*	*References*
Nodule	Glyma05g21680	*BRU6*	AT4G37390	Involve in auxin homestasis	-4.75	0.0002	(Fu et al., 2011)
	Glyma17g18040				-3.18	0.001	
Nodule	Glyma11g05510	*GH3.1*	AT2G14960	Involve in auxin homestasis	-3.46	1.30E-06	(Li et al., 2007)
Nodule	Glyma17g04360	*PDR9*	AT3G53480	Involve in auxin homestasis	-2.2	0.0006	(Růžička et al., 2010)
Nodule	Glyma09g04750	*ATL2*	AT3G16720	ATL2 is an early-response gene and induced by auxin in *Arabidopsis*	-2.2	0.03	(Martínez-García et al., 1996)
Root	Glyma14g36390	*IAA9*	AT5G65670	*IAA9* is an auxin responsive gene which promote plant growth and involve in defense responses	-2.68	0.0003	(Fujita et al., 2012)
Root	Glyma02g16080	*IAA7*	AT3G23050	*IAA7* acts as repressor of auxin-inducible gene expression and involve in defense responses	-2.24	0.0001	(Devoto et al., 2005; Cui et al., 2013)
Root	Glyma10g35480	*ARF16*	AT4G30080	Auxin responsive factor controls (*ARF16*) expression of *ABSCISIC ACID INSENSITIVE 3* (*ABI3*) during seed germination	-2.6	3.83E-08	(Liu et al., 2013)

Chapter 5

5. Involvement of *GmSAT1* in root phosphorus homeostasis and arbuscular mycorrhizal symbiosis

5.1 Abstract

The transcriptional regulatory controls underlying two vital symbioses that influence nutrient acquisition, including the bacterial (*Rhizobium*) and fungal (*arbuscular mycorrhiza* (AM)) partnerships are mainly undiscovered. More importantly, up to now, there is only one transcription factor (TF) identified with a positive regulatory effect on both symbioses. Previous experiments have shown that *GmSAT1* has an important role in nodulation and loss of its activity was associated with alteration of several nitrogen transporters (Chapter 4). Furthermore transcriptomics analysis of *sat1* plants revealed that disruption in expression of *GmSAT1* down-regulates a number of P-related genes including a pyridoxal phosphate phosphatase, apyrase, and a P transporter (*PHT1;7*), suggesting an interaction between P concentration and expression of *GmSAT1*. The fact that *GmSAT1* has orthologs in non-legume plants increases the possibility that it may have an involvement with AM symbioses. In this chapter, the effects of phosphorus (P), nitrogen (N), and interaction between P and N were examined in shoot, root, and nodule tissues of soybean. Although all tissues showed positive responses to P supply, nodule tissue showed the highest percentage weight increase in response to exogenous application of P. In the next part of this study, I found a link between the expression of *GmSAT1* homologues (*GmSAT1;1* and *GmSAT1;2*) and P. A significant increase in *GmSAT1;1* and *GmSAT1;2* expression was observed in P deficient conditions (low level of applied P, 25 µM). An interesting result was observed when we compared root

colonization between *GmSAT1*-silenced (*sat1*) and control (empty vector) plants. *sat1* plants showed a reduction in AM colonization at low P. As P supply increased from low (25 µM) to high (150 µM), *sat1* plants maintained their AM colonization levels, showing a potential loss of P uptake regulatory control and or internal P homeostasis. Furthermore, on average, *sat1* plants had a significantly higher internal P concentration in the shoots. The findings of this study showed that *GmSAT1* is a TF with a broad transcriptional imprint, which influences both rhizobial and AM symbioses and might play a role in P homeostasis.

5.2 Introduction

The regulatory mechanisms underpinning the AM symbiosis in plants are poorly understood. As discussed in Chapter 1, shared symbiotic genes facilitating both AM fungi and rhizobia infection have been identified in many legumes. These include the common SYM genes: *DMI2/SYMRK*, *NUP133*, *NUP85*, *CASTOR/POLLUX/DMI1*, *CCaMK/DMI3* and *CYCLOPS/DMI3* (Oldroyd, 2013). As *GmSAT1* is a rhizobia enhanced TF, questions have been raised on whether this activity also extends to the AM symbiosis and P nutrition. In Chapter 4, it was revealed using microarray analysis, that in *sat1* roots and or nodules, loss of *GmSAT1* expression identified a number of putative signaling pathways involving *GmSAT1*. One pathway identified in roots showed a clear link to phosphate uptake and metabolism (Figure 5.1). Prior to this study, Mazurkiewicz (2008) showed that the expression of *GmSAT1;1* in yeast, caused a significant up-regulation of P-related genes, including *PHO84* (~39-fold), *PHM6* (~14-fold), *VTC3* (~3.5-fold), *PHO5* (~3-fold), *PHO3* (~2.5-fold), *PHM8* (~2.2-fold), *PHO11;PHO12* (~2.2-fold), and *PHO8* (~2-fold). In *sat1* roots, there was strong down-regulation of P responsive genes including *HAD1*, *SPX2*, *PHT1*;7, *MGD2*, *RNS2* and the ecto-apyrase *GS52* (Table 5.2). *HAD1* is a root expressed pyridoxal phosphate phosphatase belonging to the haloacid dehalogenase (HAD) superfamily. HAD1 has been shown to participate in the signaling processes activated under Pi starvation (Baldwin et al., 2001; Stenzel et al., 2003; Baldwin et al., 2008; Liu et al., 2010; May et al., 2011). *SPX2* genes are identified by their conserved *SPX2* domain and in many cases their relationship to P

homeostasis in plants (Duan et al., 2008; Secco et al., 2012). *PHT1;7* is a phosphate transporter which is linked to Pi transport in pollen and is highly expressed in Pi-deprived roots (Bariola et al., 1999; Mudge et al., 2002). Monogalactosyldiacylglycerol synthase (*MGD2*) is a main constituent of chloroplastic membrane lipids and are essential for membrane lipid remodelling during Pi starvation (Kobayashi et al., 2004; Kobayashi et al., 2009). *RNS2* is a S-RNase, involved in gametophytic self-incompatibility in plants and its expression is induced in Pi-deprived roots (Bariola et al., 1999). *GS52* is an ecto-apyrase, which are a class of membrane-localized proteins that hydrolyse ATP to AMP and the release of inorganic phosphate. Loss of *GS52* in soybean, disrupts nodule development (Govindarajulu et al., 2009) and recently in lotus, the *GS52* ortholog, *LNP*, was shown to be critical in establishing both AM and rhizobium symbioses (Roberts et al., 2013). *GmSAT1* silencing was also associated with the down-regulation of *GmNF-YA1a*, a positive regulator of the AM symbiosis (Schaarschmidt et al., 2013). Interestingly, *NF-YA* TF's are a direct target of microRNA169, which is also linked to nodule development (Combier et al., 2006; Zanetti et al., 2010) and P and N starvation responses (Hsieh et al., 2009; Zhao et al., 2011). Collectively the transcriptional responses associated with *GmSAT1* expression highlight a potential involvement in P nutrition in soybean. To confirm whether *GmSAT1* participates in an AM and or P signaling cascade a preliminary set of experiments were designed that explored P-mediated transcriptional changes in both *GmSAT1;1* and *GmSAT1;2* and whether the symbiotic relationship observed with rhizobia also extended to the AM fungal symbiosis.

The first set of experiments examined the impact of N and P supply on *GmSAT1* expression in roots, shoots, and nodules. In the second experiment, the influence of *GmSAT1* on AM colonization and root P homeostasis was examined using *GmSAT1* RNAi and empty vector transformed hairy roots .

5.3 Materials and Methods

5.3.1 Plant growth and experimental design

Effects of different levels of P supply on nodule development and growth of shoot and roots of soybean with and without nitrogen

This experiment was designed to identify how different levels of P effect nodule development in soybean. A factorial experiment was designed based on a completely randomized design with 5 replications (5 plants) per treatment. The first factor was nitrogen (N) source provided at two concentrations (0 and 2.5 mM NH_4NO_3). The second factor was phosphorus (P) supplied as combination of K_2HPO_4 and KH_2PO_4 at five concentrations (0 µM, 25 µM, 100 µM, 150 µM, and 250 µM).

N Treatments	P Treatments	KH_2PO_4 (1M) (µl /1L)	K_2HPO_4 (1M) (µl /1L)
0mM NH_4NO_3	0 µM	0	0
	25 µM	20	5
	100 µM	80	20
	150 µM	120	30
	250 µM	200	50
2.5mM NH_4NO_3	0 µM	0	0
	25 µM	20	5
	100 µM	80	20
	150 µM	120	30
	250 µM	200	50

Table 5.1 Phosphorus and N combinations used in the P treatments

Soybean seeds (*Glycine max* cv. Djakal) were surface-sterilized as previously described by Kereszt et al., (2007) and then grown in 1 kg pots, filled with coarse Waikerie sand and inoculated with *Bradyrhizobium japonicum* strain USDA110 (inoculum density of 10^5 –10^6 cells/ml (Jitacksorn and Sadowsky, 2008) on the day of planting and again on the following day. Plants were watered 3 times per week with nutrient solution described previously (Chapter 3 Table 3.1) supplemented with different concentrations of P and or N and the P source was a combination of K_2HPO_4 and KH_2PO_4 in ratio of 5 to 1 (Table 5.1). Twenty-eight

days after planting, shoot and roots were harvested and numbers of nodules counted. Dry weights of root, shoot, and nodules were determined after 48 hours at 80˚C. For analysis of variance (ANOVA), MINITAB 16 package was used to determine the effects of P levels on nodule number, dry weight of shoot, root, and nodule tissues. A Tukey test was used for mean separation and statistical comparison of means at $P \leq 0.05$. This experiment was carried out twice and the result of both experiments was found to be consistent. The result of the second replication is presented.

The relationship between different concentrations of P supply and expression of GmSAT1;1 and GmSAT1;2

The aim of this experiment was to determine the expression response of *GmSAT1;1* and *GmSAT1;2* under different concentrations of P. Soybeans were grown and inoculated as described in Experiment 1 (Section 5.3.1). Plants were watered 3 times per week with N free nutrient solution containing modified P (25 µM, 100 µM, 150 µM, and 250 µM) (Table 5.1). Twenty-five days after planting, root and nodule samples were harvested for RNA extraction and mRNA expression analysis (3 plants), as well as P measurement analysis (5 plants). Total RNA was extracted from nodules with a Spectrum Plant Total RNA kit (Sigma-Aldrich). After treating with Turbo DNase (Ambion), 1 µg of total RNA was used for cDNA synthesis with Superscript III (Invitrogen). Transcript abundance of *GmSAT1;1* and *GmSAT1;2* in each cDNA pool was determined by a Light Cycler (Bio-Rad) qPCR machine using IQ SYBR Green (Bio-Rad). Relative expression measured by the mean C_T value of three biological replicates (3 plants) was used to generate a ΔCT value using *cons6* (Glyma12g05510) as an internal control (Libault et al., 2008). The relative expression was then calculated by using the formula $2^{-\Delta\Delta C_T}$ (Gallou et al., 2012). For each sample of root and nodule tissues, one-way ANOVA was used for statistical analysis based on a completely randomized design. For measurements of plant P concentration, plant materials (shoot, root and nodule) were dried for 48 hours at 80˚C. Dry materials were then digested as described in Section 5.3.3.

AM colonization in hairy roots and adventitious roots in response to different P applications

The aim of this experiment was to first examine if transformed hairy roots influenced the P response and AM symbiosis in soybean and to secondly determine two suitable concentrations of P that provide contrasting growth responses. Since comparison of AM colonization between transformed hairy root and non-transformed root has not examined before, to start this experiment performing this comparison was essential. In this experiment, soybean seeds were germinated and transformed by *A. rhizogenes* K599 harboring an empty vector with GFP reporter gene (pK7GWIWG2D(II)) according to method described in Chapter 3.

For comparison of transformed versus non-transformed roots, only one transformed root and one non-transformed root were retained on each plant. Transformed roots were selected based on GFP expression as described in Chapter 3. All plants with two root systems, were transferred to an inoculated soil mixture (described in Section 5.3.2). For the shoot and each of the roots, the effect of different P concentrations in the medium (0 µM, 25 µM, 100 µM, 150 µM, and 250 µM), on dry weight, and P concentration were determined using a one-way anova (completely randomized design) with 5 replications (5 pots with one plant per pot). The percentage of root length colonized on each of the transformed and non-transformed roots was measured. To do this, a weighed sub-sample of roots was taken randomly from each root system 28 days after inoculation. The rest of the transformed or non-transformed roots were oven-dried at 80°C for 48 h and used for dry weight and P measurement. Paired t-tests were used to compare the effects of treatments between transformed and non-transformed roots.

Effects of GmSAT1 on the AM symbiosis and internal P concentration

The aim of this experiment was to determine whether the loss of *GmSAT1* expression influences AM colonization and internal P concentrations in either high or low P supply. Hairy roots were transformed with the empty vector (control) and *GmSAT1* RNAi constructs

via *A. rhizogenes* according to the protocol described in Chapter 3. A high (150 µM) and low (25 µM) level of P was selected based on the growth responses from Experiment 3.

For each transformation treatment (empty vector or *GmSAT1* RNAi) and each level of P (25 µM or 150 µM), 12 soybean plants were transformed (48 plants in total). In each plant, we screened transformed hairy roots by GFP where only one transformed hairy root was grown per plant. To be sure *GmSAT1* expression in the RNAi plants was repressed, *GmSAT1* expression was measured using qPCR (data not shown). Percentage of root length colonized, shoot P concentration, root length, and shoot dry weight were measured. To measure the percentage of root length colonized by AM, total fresh weight of roots (28 days after inoculation) was first measured. A small subsample of the roots were selected and weighed and stained. Stained arbuscules were counted under a light microscope using a grid-based procedure as outlined below in section (5.3.2). The rest of the roots were frozen with liquid nitrogen and stored at -80°C for total RNA extraction. Paired t-tests were used to compare the effects of *GmSAT1* silencing on AM colonization of root and root length of *sat1* and *vector* plant as well as shoot dry weight and shoot P concentration and P content.

5.3.2 AM inoculation and root staining

For AM inoculation, *Glomus intraradices* Schenk and Smith (DAOM 181602 WFVAM 10, Import Permit No. 99302429) was used. Plants with transformed hairy roots were cultured in pots containing 1.2 kg of soil containing a homogenous mixture of (90%) autoclaved Waite Arboretum soil and Waikerie sand (1:9 ratio, respectively) mixed with (10%) root inoculum (*Glomus intraradices* grown on subterranean clover (*Trifolium subterraneum)* roots in 1:9 Waite Arboretum soil:fine sand mix with a mean of 75% colonization). Soybean pots were watered 3 times per week with 2.5 mM NH_4NO_3 and a designed P modified nutrient solution (Table 5.1). AM colonization was determined by the percentage root length colonized method involving a weighed sub-sample of roots taken randomly from each root sample. Root segments were cleared and stained using vinegar and ink according to method described by Vierheilig (1998). Small sections of root were placed into 10% KOH at 90°C for 3 min. After

rinsing with water they were rinsed with acidified water (5% white vinegar) for 3 min followed by staining with 5% Schaeffer black ink on 90°C for 2 min (Vierheilig et al., 1998). Roots were de-stained with acidified water overnight and percentage root length colonized determined under dissecting microscope (Giovannetti and Mosse, 1980).

5.3.3 Plant phosphate concentration and content

The P concentrations were determined by the acid hydrolysis method. Approximately 500 mg of dried and ground material was mixed with 7 ml of HNO_3 (70%). Samples were placed in a digestion block and heated for 2 h at 100ºC, followed by 140ºC until the volume of HNO_3 was reduced to 1ml. Digested material was diluted with 20 ml of Reverse osmosis (RO) water. An aliquot of 0.1 ml of clear sample was mixed with 2.75 ml water and 0.25 ml of P Colour Reagent containing [0.25% ammonium vanadate (NH_4VO_3); 5.0% ammonium molybdate ($(NH_4)_6MO_7O_{24}$); concentrated nitric acid (1:1:1)]. After 30 min, absorbance was measured by spectrophotometer at 390 nm. P concentration was calculated using standard carve with a concentration range between 0-18 µg P mL^{-1} (Hanson, 1950; Zarcinas and Cartwright, 1983; Zarcinas et al., 1987).

5.3.4 Calculation of root length colonized and total root length

For root length measurement a weighed subsample of root was spread on a grid petri dish and the grid intersect method applied (Giovannetti and Mosse, 1980). Length of root subsample was calculated according to the following equations (Marsh, 1971):

1- The length of the subsample (cm/g root) = Total number of intersects in the subsample × 11/14 × the dimensions of the grid (cm).
2- The length of whole root (cm) = The length of the subsample (cm/g root) × total weight of the roots.

For calculation of percentage root length colonization following equations were used:

1- Mycorrhizal root length in the subsample (cm/g) = Number of mycorrhizal intersects in the subsample × 11/14 × the dimensions of the grid (cm).
2- The length of colonized root (cm) = Mycorrhizal root length in the subsample (cm/g) × total weight of the roots.
3- The percent root length = Colonized Mycorrhizal length/total length × 100.

5.4 Results

5.4.1 The effects of different levels of phosphorus (P) on dry weights of nodules, shoots and roots in presence and absence of nitrogen (N) source

The result of the first experiment showed that generally the supply of N increased the dry weight of soybean shoots and roots while it decreased the shoot/root ratio, number of nodules and their dry weights (Figure 5.2). Nodule dry weight was strongly affected by the availability of extra P, as the highest nodule weight was obtained in the highest concentration of P (250 µM) regardless of the presence of N (Figure 5.2 A). There was no significant difference between plants grown with different concentrations of P with respect to nodule number in the absence of N (Figure 5.2 B), while P significantly increased the mean dry weight per nodule (Figure 5.2 A). In contrast, in the presence of 2.5 mM NH_4NO_3, lower concentrations of P had no effect on either nodule number or dry weight, but both were significantly increased at the higher concentrations (150 µM and 250 µM) (Figure 5.2 A-B).

Higher concentrations of P (150 µM, and 250 µM) resulted in significant increases in shoot dry weights in both N treatments. Regardless of N level, the lowest shoot dry weight was observed when P level was low (0 and 25 µM P) (Figure 5.2 C). In the presence of 2.5 mM NH_4NO_3, there were more statistically significant differences between P levels. For example, there was no significant difference in shoot dry weight between 100 and 250 µM P in the absence of N; while the effect of 250 µM P on shoot dry weight was statistically different with 100 µM, in the presence of 2.5 mM NH_4NO_3. Plants grown on 250 µM P produced more shoot dry weight than 100 µM (Figure 5.2 C). It can be concluded that adding P increased

shoot weight; however, this increase in more obvious when adequate amounts of N is available.

The pattern of root response to the added P in absence/presence of N was obviously different from that of the shoot. In absence of N, the highest dry weights of roots were found in 100 and 150 µM P, while in presence of N, the highest dry weights were found in 250 µM P (Figure 5.2 D). Despite these differences, the root/shoot ratios follow a similar pattern in the presence or absence of nitrogen, and in both treatments lower root/shoot ratios were observed in higher concentrations of P (250 and 150 µM). However N supply reduced overall root/shoot ratios in all treatments (Figure 5.2 E).

Taken together the result of this experiment showed the significant impact of P to nodule development and growth of soybean in a N dependent manner.

5.4.2 Expression of GmSAT1;1 and GmSAT1;2 is influenced by the availability of P.

The result of Experiment 2 revealed that *GmSAT1;1* and *GmSAT1;2* are expressed differently in nodules and roots across different concentrations of P supply. At lower concentrations of P (25 µM), both *GmSAT1;1* and *GmSAT1;2* showed increased expression of root and nodule tissues; while their expression both gradually decreased with application of higher levels of P (Figure 5.3 A & B). The expression of *GmSAT1;1* and *GmSAT1;2* across different P levels was more responsive in roots than in nodules. There was a negative correlation between the expression of *GmSAT1;1* and *GmSAT1;2* in roots with supplied P and with the P concentration in roots, shoots and nodules (Table 5.3). The correlation between expression (Figure 5.3 A&B) and internal P concentration (Figure 5.4 A, B & C) was tissue-dependent (Table 5.3). Expression of *GmSAT1;1* and *GmSAT1;2* in root but not nodule tissues was negatively correlated ($P \leq 0.01$) with P concentration of root, shoot, and nodule tissues (Table 5.3).

5.4.3 AM colonization in hairy roots and adventitious roots in response to different P applications.

In experiment 3, AM colonization of transformed hairy roots and untransformed adventitious roots were evaluated across different external P levels in plant grown in the presence of 2.5 NH_4NO_3. When P supply in the soil was increased, shoot dry weight and P concentration also increased where the highest shoot dry weight and tissue P concentration were found at 250 µM external P (Figure 5.5 A and B). Comparison of hairy roots and adventitious roots revealed that both type of roots respond similarly to P levels and have similar internal P concentrations (Figure 5.5 C and D). In both transformed and non-transformed roots, increasing external P resulted in significant increases in root dry weights and P concentrations (Figure 5.5 C and D).

Paired T-tests showed the percentage of root length colonized by the AM fungus *G. intraradices* (percent AM colonization) was the same in both hairy and adventitious roots. In both root systems, a low level of added soil P (25 µM) resulted in the highest rate of root colonization (Figure 5.6). A positive impact of lower levels of P on increasing AM colonization in soybean and other plants has been reported before (Asimi et al., 1980; Habte et al., 1987; Manjunath et al., 1989). The lowest percent AM colonization was observed at the highest level of applied P (250 µM) (Figure 5.6).

5.4.4 The effect of the loss of GmSAT1 activity on AM symbiosis and P concentration.

The comparison between shoot dry weight, P concentration and total P content of *sat1* and *vector* plants showed that both have a similar response to low (25 µM) and high (150 µM) levels of applied P. In both plants, higher levels of applied P resulted in higher shoot dry weight as well as P concentration and total content (Figure 5.7 A, B and C). Growth of both plants appeared similar with no specific symptoms (deficiency or toxicity) observed (Figure 5.7 D and E). However the comparison between *sat1* and *vector* controls showed that *sat1* plants had a higher dry weight, P concentration and total P content than the empty vector

controls ($p \leq 0.05$) (Figure 5.7 A, B and C). There were no significant differences in root length between *sat1* and *vector* plants (Figure 5.8A); however, they responded differently with respect to AM colonization. While percent AM colonization was significantly different at high and low levels of applied P (25 and 150 µM) in vector controls ($p \leq 0.05$), there was no significant difference in *sat1* plants (Figure 5.8 B). In fact, control plants were similar to wild type plants and showed a significantly higher percentage of root colonization ($P \leq 0.05$) at low levels of P (25 µM), whereas there was no significant difference between high (150 µM) and low (25 µM) levels of applied P in *GmSAT1*-silenced plants. No abnormality in growth of hyphae or colonization was observed in *sat1* plants (Figure 5.8 D and F), however it seems these plants have lost their sensitivity to P supply and behave similarly at both P levels.

5.5 Discussion

5.5.1 Changes in growth and internal P in soybean tissues with applied P

Increasing P application significantly influenced the dry weight of shoot, root, and nodule tissues. This response was dependent on external N (2.5 mM NH_4NO_3) availability. In contrast when N was also supplied to nodulated roots, root growth became less responsive to the increased P supply. In the absence of N, nodule growth flourished as external P supply increased without changing nodule numbers. Interestingly, elevated levels of P were effective in restoring nodule growth and nodule numbers in the NH_4NO_3 treated plants. This suggests a potential limitation of nodule growth by P when the plant has access to an alternative N source that N_2-fixation in nodule. Overall, the data strongly indicates and confirms a generally held view that plants respond favourably to applied P. However, the data highlights tissue-specific interactions (roots and nodules) where co-availability of both P and N (N through fertiliser application or N_2-fixation) can enhance growth and in the case of legume nodules may partially compensate for the negative influence of N on nodulation and nodule growth. This result is consistent with previous reports indicating a positive interaction between P and

N in nodulation of soybean and other plants (Israel, 1987; Almeida et al., 2000; Gentili and Huss-Danell, 2002; Gentili et al., 2006).

5.5.2 The influence of GmSAT1 on the AM symbiosis through regulation of P homeostasis

The availability of P in the soil solution will modulate symbiotic interactions with AM fungi (Smith and Smith, 2011). At low P, AM colonization is often promoted while at high P, colonization often decreases. AM inoculated *sat1* plants displayed a reduction in root AM colonization at low P relative to the empty vector controls. As P concentrations increased, colonization in the empty vector controls decreased while colonization in *sat1* tissues remained unchanged. Thus it would appear that loss of *GmSAT1;1* altered the sensitivity of roots to increasing P concentrations and associated AM colonization. Interestingly, shoot total P content and P concentration increased ($p \leq 0.001$) in the *GmSAT1* silenced plants relative to the control plants, indicating a potential mis-regulation of P homeostasis in the *sat1* roots. As both *GmSAT1;1* and *GmSAT1;2* expression responded to external P supply (high at 25 µM, low at 250 µM) (Figure 5.3), it would appear both AM responsiveness and root P homeostasis or signaling are possibly linked through *GmSAT1* activity.

As discussed previously in Chapter 4, microarray analysis of gene expression in *sat1* roots indicated a significant down-regulation of many P-related genes (Table 5.2). These included three pyridoxal phosphate phosphatase-related proteins (*HAD1*), two SPX domain genes (*SPX2*), a phosphate transporter (*PHT1;7*), an ecto-apyrase (*GS52*), a monogalactosyldiacylglycerol synthase (*MGD2*) and a ribonuclease (*RNS2*). All these genes have been shown to be induced by P starvation (Stenzel et al., 2003; Baldwin et al., 2008; Liu et al., 2010; May et al., 2011). It is possible these and potentially other genes are involved in the changes we have observed in AM colonization and P responsiveness. In summary, the data supports the hypothesis that *GmSAT1* is an important regulator of soybean P homeostasis which influences both the AM and *Rhizobium* symbioses. Questions remain on where

GmSAT1 sits within the P signaling pathway and its genetic relationship with AM fungi in soybean and other plant species.

5.5.3 Possible involvement of GmSAT1 in both symbioses through crosstalk between N and P starvation related microRNAs

Known regulatory networks that govern both N and P homeostasis in plants are poorly understood. Analytical approaches based on literature mining using the Pathway Studio bioinformatic platform (see Chapter 4), led to the association of a number of potential microRNAs (particularly microRNA169 and microRNA156) and microRNA-regulated TF's, which may be involved in the coordinated regulation of N and P starvation responses in soybean (Hsieh et al., 2009; Gu et al., 2010; Devers et al., 2011; Zhao et al., 2011). The hypothetical interaction network between *GmSAT1* and the microRNAs 169 and 156 is illustrated in Figure 5.1. The network was constructed based on *sat1* altered expression patterns of P responsive genes in roots including *HAD1, SPX2, PHT1; 7, MGD2, GS52* and *RNS2* (Chapter 4). The network shows that the loss of *GmSAT1* activity results in the down regulation genes have been shown to be induced by P starvation while *GS52* is required for both rhizobial and AM symbiosis. The role of microRNA169 and microRNA156 in phosphate and nitrogen starvation is well documented. *NF-YA3* and *NF-YA8* are targets of microRNA169 while microRNA156 probably interacts with *GmSAT1*. Based on our microarray analysis result and published literature we suggest that the *GmSAT1* is probably involved in N and P starvation by regulating selected *NF-YA* family members and interaction with microRNA156. Taken together this study indicates a putative link between *GmSAT1* and regulation of P homeostasis and AM colonization. Based on hypothetical network analysis, (Figure 5.1), the impact of *GmSAT1* to AM colonization might be indirect and influenced by microRNA169 and microRNA156. However future experiments such as RNAseq analysis of *sat1* plants will be required to define whether *GmSAT1;1* influences *NF-YA* expression and if so, determine if it is linked to microRNAs involved in P and or N regulation such as microRNA169 and microRNA156.

Figure 5.1 Hypothesized interaction network of *GmSAT1* and P responsive genes.

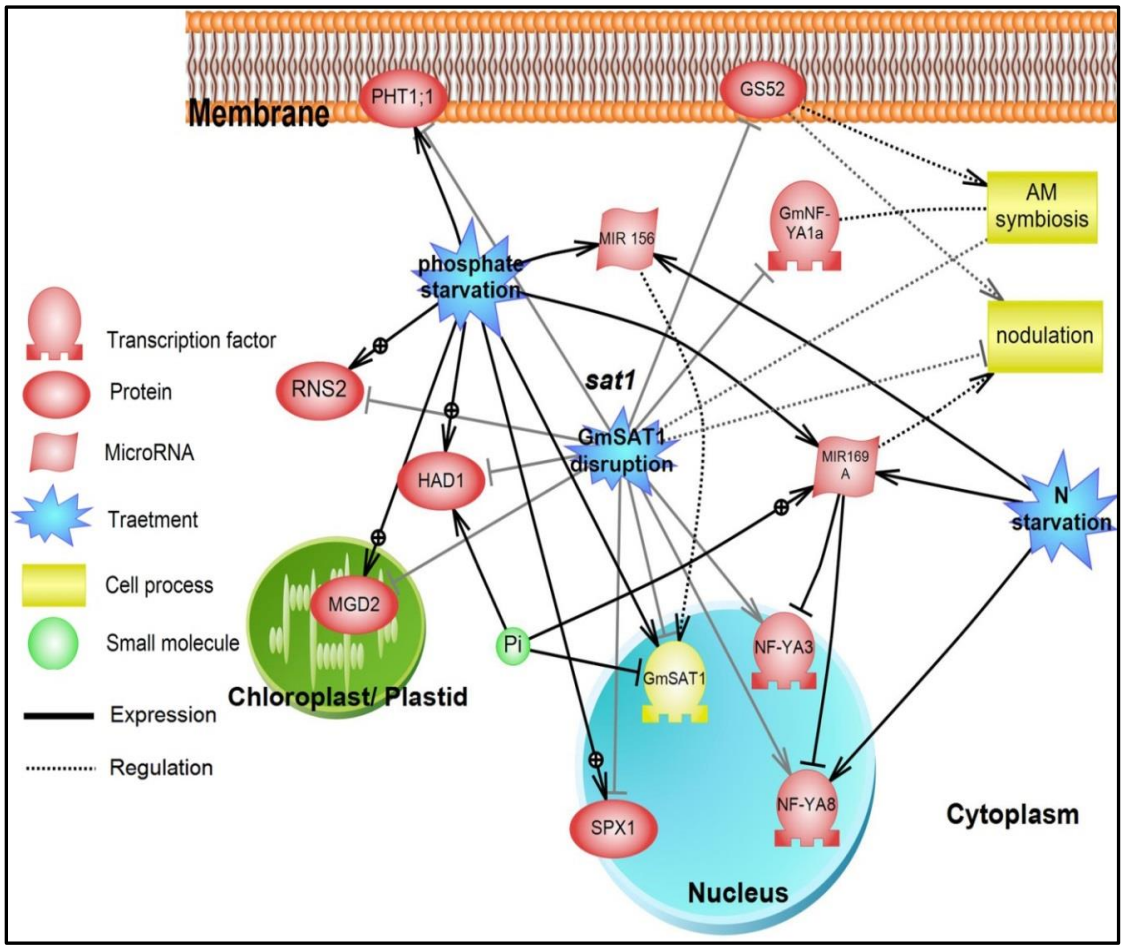


Table 5.2 List of putative P and N starvation related genes that changed their expression pattern in the *GmSAT1*-silenced roots and nodules relative to the empty vector control.

issue	Gene	Gene assignment	Fold change	P-value	References
Root	Glyma08g20810	HAD1	-10.01	2.43E-13	(Liu et al., 2010)
Root	Glyma16g04750	GS52 (apyrase)	-7.74	5.02E-10	(Govindarajulu et al., 2009; Tanaka et al., 2011)
Root	Glyma13g23150	*MGD2*	-3.90	2.40E-07	(Kobayashi et al., 2004; Kobayashi et al., 2009)
Root	Glyma06g07260	*SPX2*	-2.73	3.68E-06	(Duan et al., 2008; Secco et al., 2012)
Root	Glyma17g12340		-1.97	0.00578	
Root	Glyma03g31950	*PHT1;7*	-2.32	2.47E-07	(Mudge et al., 2002)
Root	Glyma07g06520	*RNS2*	-2.04	6.43E-06	(Bariola et al., 1999)
Root	Glyma03g36140	*GmNF-YA1a*	-1.93	1.97E-07	(Schaarschmidt et al., 2013)
Root	Glyma17g05920	*NF-YA8*	8.35	9.13E-09	(Zhao et al., 2011)
Root	Glyma13g16770	*NF-YA3*	4.35	1.61E-06	(Combier et al., 2006)
Nodule	Glyma17g05920	*NF-YA8*	-2.53	0.004	(Zhao et al., 2011)

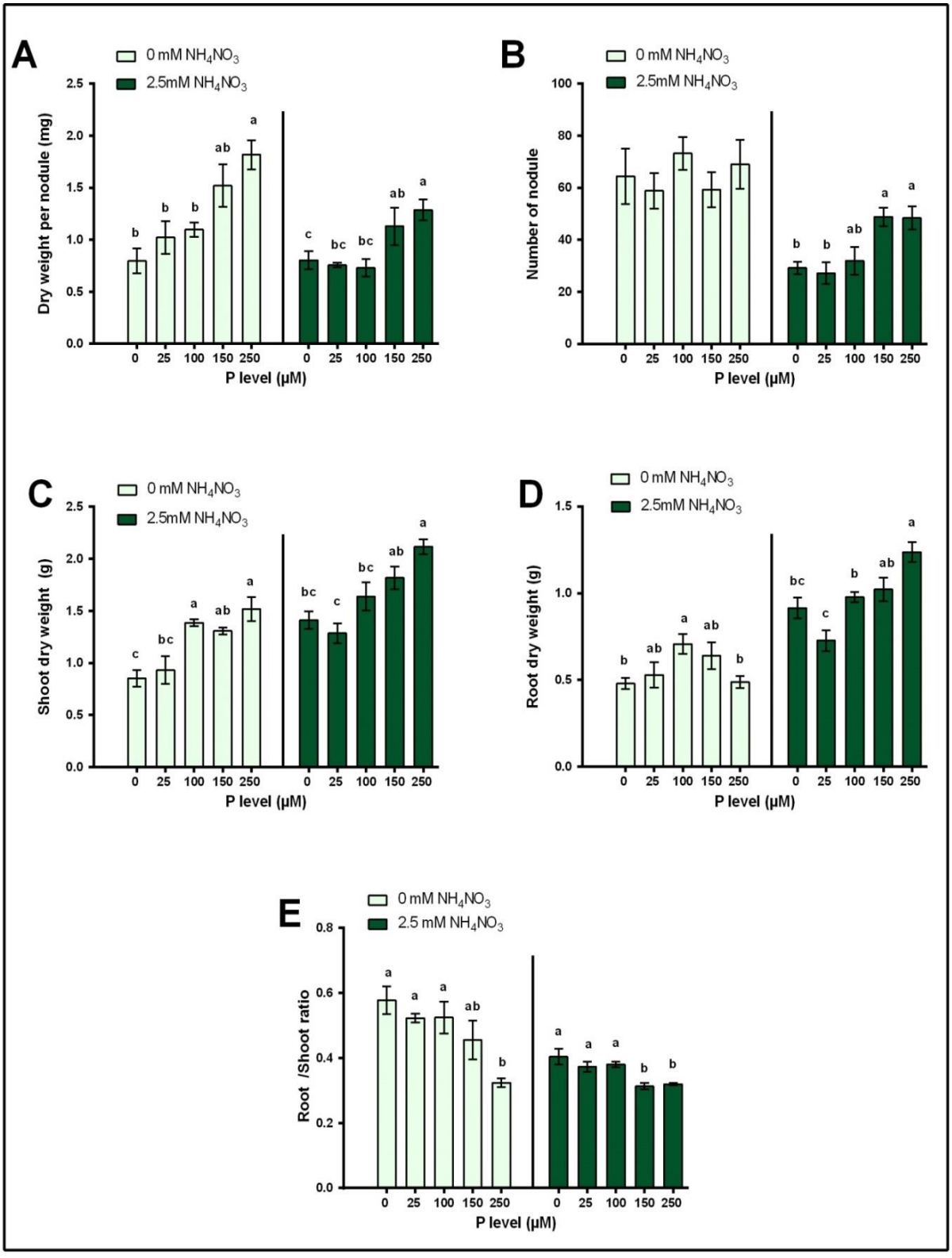


Figure 5.2 The effects of P and N application on nodule, root and shoot growth.

(A) Dry weights per nodule, (B) numbers of nodules, (C) shoot dry weight, (D) and root dry weight after growth with different P levels (0, 25, 100, 150, 250 μM) and in presence of 2.5 mM NH_4NO_3 and absence of N. Plants were harvested 28 days after planting. Values are means ±SE (n=5). Values with different letters indicate significant differences between the P treatments based on Tukey test ($P < 0.05$).

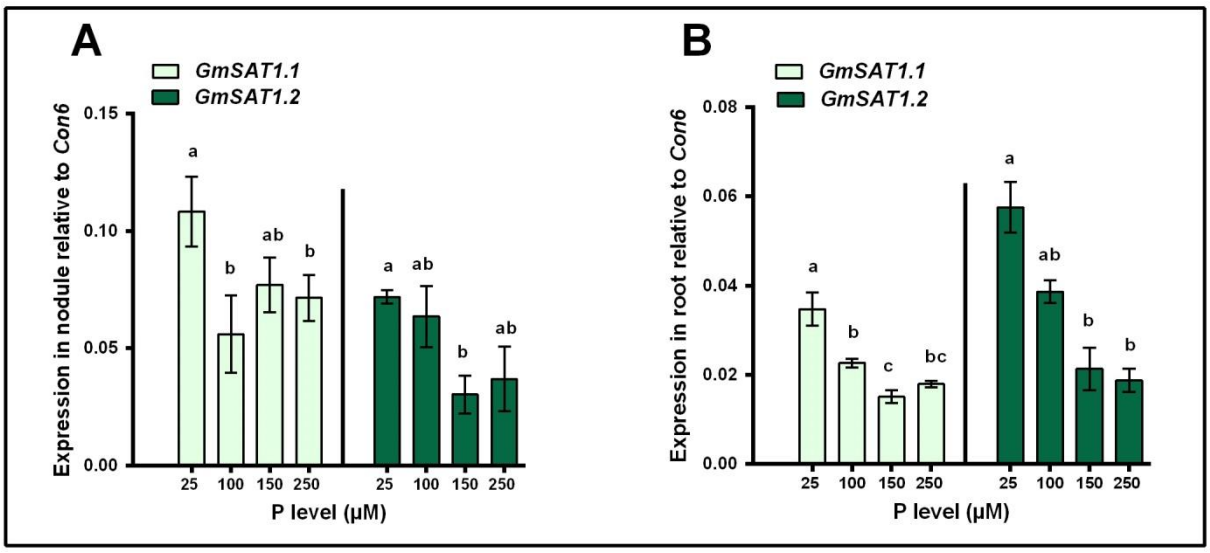


Figure 5.3 Expression of *GmSAT1;1* and *GmSAT1;2* in response to P supply.

Expression of *GmSAT1;1* and *GmSAT1;2* relative to *Con6* in response to P supply in nodules (A) and roots (B) of soybean 25 days after planting. How much N was supplied. Values represent the means ± SE (n=3). Values with different letters are significantly different between P treatments base on Tukey test ($P < 0.05$).

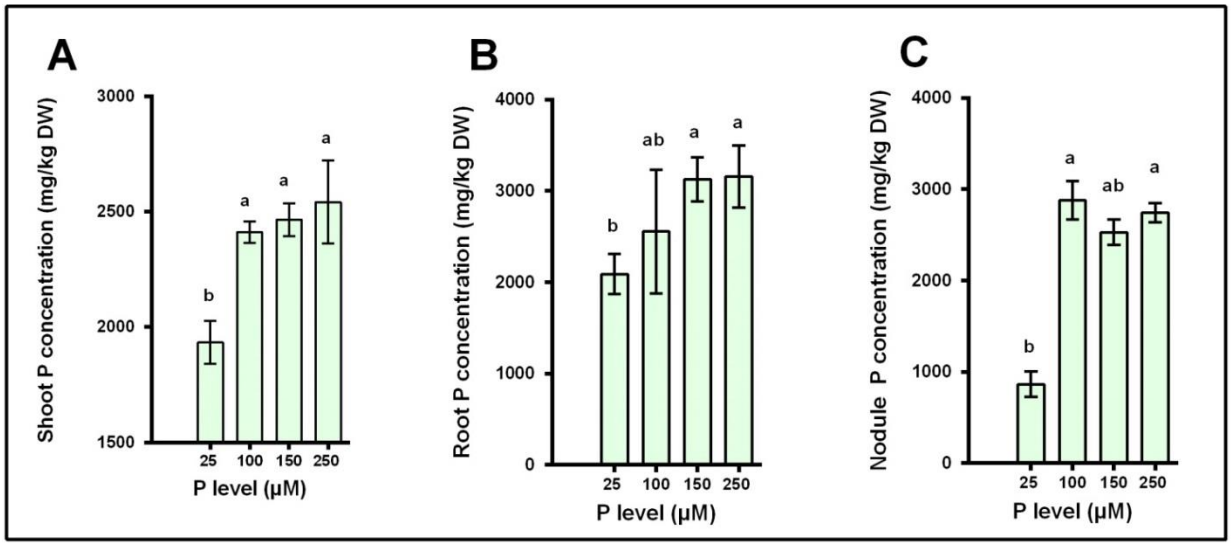


Figure 5.4 Effect of different P levels on P concentration.

Effect of different P levels on P concentration of (A) shoots, (B) roots and (C) nodules of soybean 25 days after planting. Values represent the means ± SE (n=5). Values with different letters are significant different between P treatments base on Tukey test ($P < 0.05$).

Table 5.3 Correlation analysis

Correlation analysis of *GmSAT1;1* and *GmSAT1;2* expression and supplied P level as well as P concentration of shoot, root and nodule tissues. Correlations were made across P concentrations used (25-250 µM) to grow the plants.

	Nodule		**Root**	
	GmSAT1;1	***GmSAT1;2***	***GmSAT1;1***	***GmSAT1;2***
P treatments (25-250 µM P)	-0.402 (NS)	-0.647 (P= 0.032)	-0.75 (P=0.005)	-0.863 (P= 0.000)
Shoot P concentration	-0.396 (NS)	-0.67 (P = 0.024)	-0.833 (P = 0.001)	-0.784 (P = 0.003
Root P concentration	-0.024 (NS)	-0.475 (NS)	-0.695 (P = 0.012)	-0.612 (P = 0.034)
Nodule P concentration	-0.605 (P = 0.037)	-0.537 (NS)	-0.827 (P = 0.001)	-0.689 (P = 0.013)

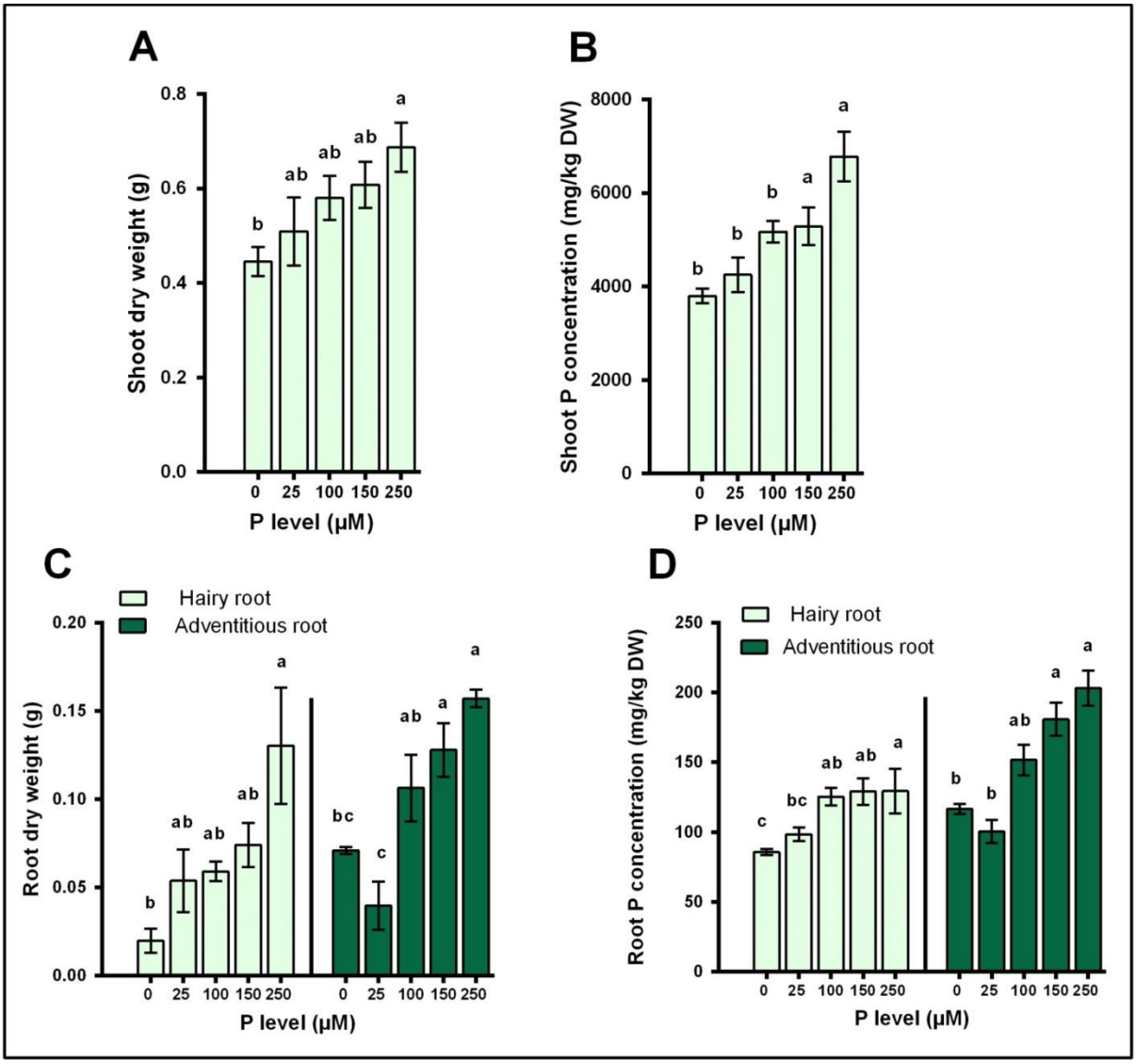


Figure 5.5 Shoot and root dry weight and P concentration in empty vector control with adventitious and hairy roots inoculated with AM fungus.

Plants were grown with different P levels (0, 25, 100, 150, and 250) and inoculated with *Glomus intraradices*. (A) Shoot dry weight, (B) Shoot P concentration, (C) Hairy root and adventitious root dry weight, and (D) Hairy root and adventitious root. Values represent mean ±SE (n=5). Values with different letters mean significant differences between the P treatments base on Tukey test ($P \leq 0.05$).

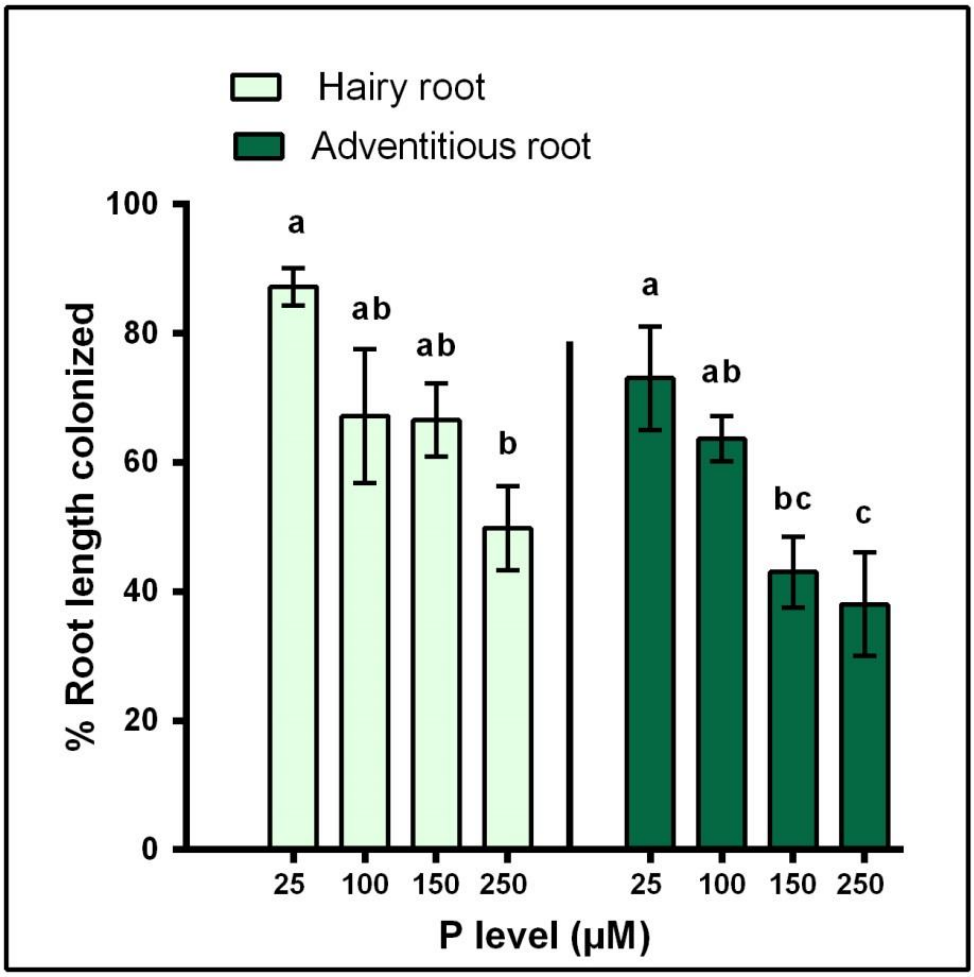


Figure 5.6 Colonization of hairy and adventitious roots.

Values represent mean ±SE (n=5). Values with different letters indicate significant differences between P treatments based on Tukey test ($P \leq 0.05$). There was no significant difference between hairy roots and adventitious roots based on t-test comparison.

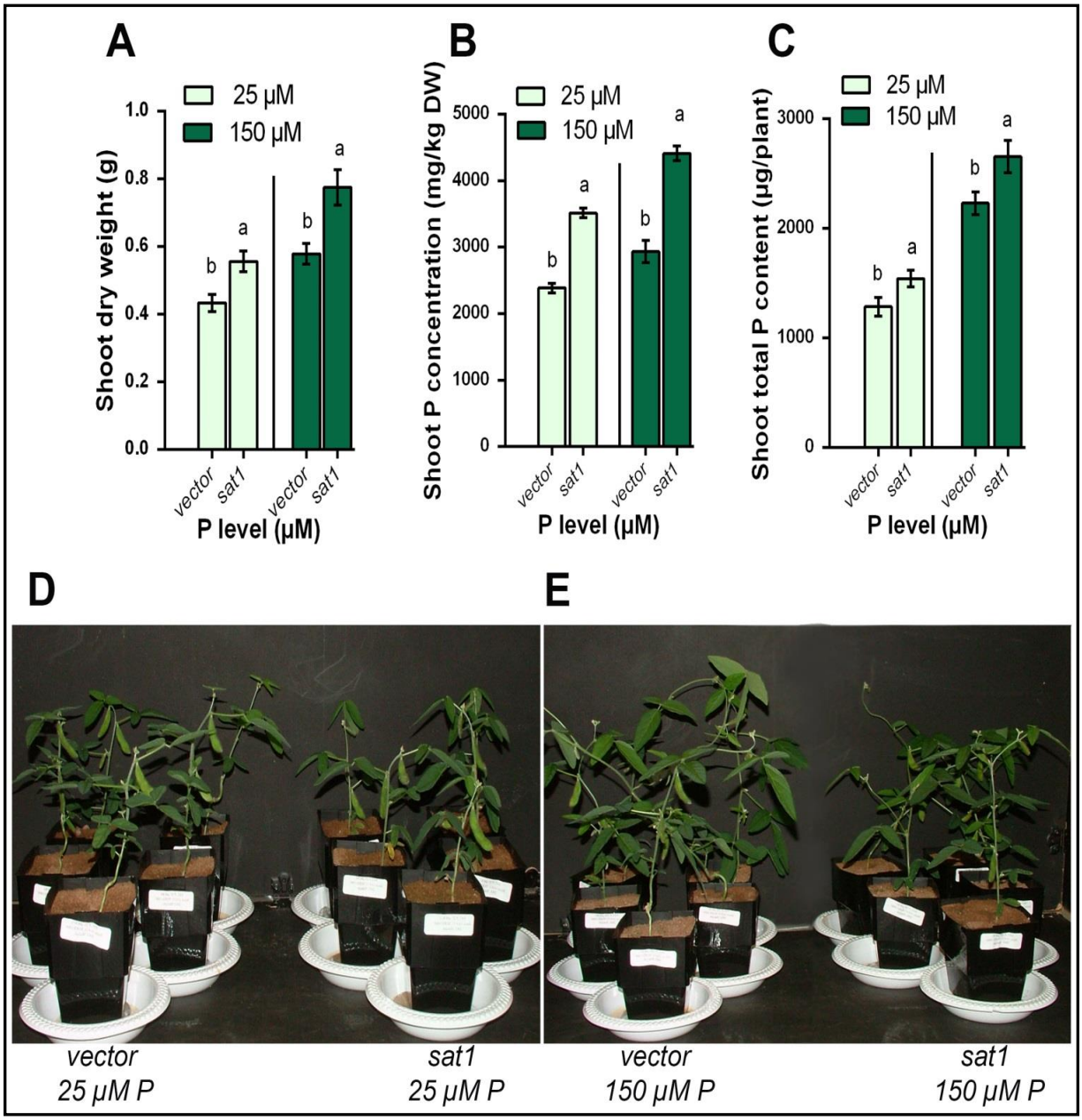


Figure 5.7 ***Experiment 4:*** **Comparison of shoot dry weight and P concentration of** ***GmSAT1*****RNAi silenced and empty vector control plant.**

(A) Shoot dry weight (B) Shoot P concentration and (C) total P content of *vector* and *sat1* plants grown at either 25 µM (D) or 150 µM P (E). Different letters indicate significant differences between *vector* and *sat1* plants based on t-test analysis. Values are means ± SE (n=10).

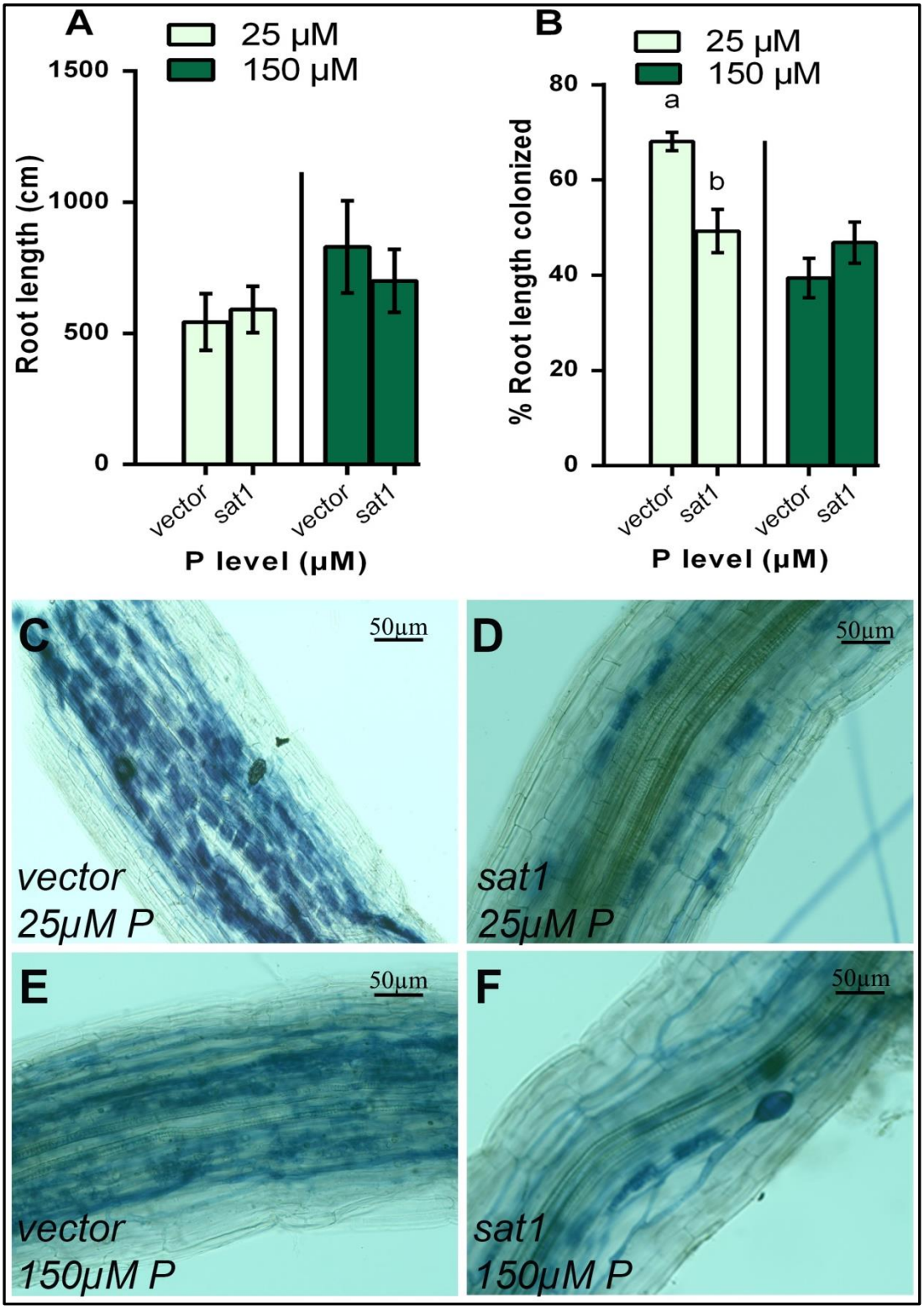


Figure 5.8 *Experiment 4*: Impact of AM colonization and P supply on *GmSAT1*RNAi-silenced roots.

Comparison of (A)root length and (B) percentage of root length colonized by *Glomus intraradices* in *vector* plant (C, E) and *sat1* plants (D, F) 28 days after inoculation. Different letters indicate significant differences between *vector* and *sat1* plants based on t-test analysis. Values are means ± SE (n=10).

Chapter 6

6. Effect of gibberellin on the transcriptional activity of the soybean nodule transcription factor *GmSAT1*

6.1 Abstract

The regulation of *GmSAT1* activity is poorly understood despite its key role in legume nodulation where loss of activity causes a reduction in nodule number and development. *In silico* promoter analysis revealed a relatively high frequency of gibberellin (GA)-responsive regulatory elements on the promoter region of *GmSAT1;1* and *GmSAT1;2*, which led me to examine whether signaling exists between *GmSAT1* and GAs. For this I have used transcriptomic analysis, promoter cloning and expression, qPCR analysis of gene expression and network analysis to help define the link between gibberellins and *GmSAT1*. In this study, different concentrations of bioactive GA_3 (0, 10^{-7} and 10^{-5} M) were applied two times per week following germination and inoculation with *Bradyrhizobium japonicum* USDA110. The results showed that expression of *GmSAT1;1* in roots and nodules, the number of nodules and their fresh weight significantly decreased with application of GA_3. In the next part of this study, the promoter of *GmSAT1;1* and *GmSAT1;2* were cloned and their activities examined in different concentrations of GA_3 using a promoter:GUS reporter gene construct. In line with previous parts of this investigation, the results confirmed a negative effect of GA_3 on *GmSAT1;1* and *GmSAT1;2* expression.

6.2 Introduction

6.2.1 Gibberellin biosynthesis

The majority of genes involved in gibberellin (GA) biosynthesis have already been characterized (Yamaguchi, 2008; Maekawa et al., 2009). GAs are synthesized from the C20 precursor of diterpenoids, geranylgeranyl diphosphate (*GGDP*) (Yamaguchi, 2008). Three classes of enzymes are involved in the biosynthesis of bioactive GAs from *GGDP* in plants, including plastid-localized terpene synthases (*TPSs*), endoplasmic reticulum (ER) membrane-bound cytochrome P450 monooxygenases (*P450s*), and cytosolic soluble 2-oxoglutarate–dependent dioxygenases (*2ODDs*) (Helliwell et al., 2001; Yamaguchi, 2008). TPSs are activated during the first stage of GA biosynthesis converting geranylgeranyl diphosphate to ent-kaurene inside plastids (Sun and Kamiya, 1997). P450s are active in the second stage of GA biosynthesis, where they are involved in several oxidation steps including the oxidation of ent-kaurene to form GA_{12} or GA_{53} in the ER (Helliwell et al., 2001; Morrone et al., 2010). Recently a number of P450s have shown an involvement in GA homeostasis by deactivating bioactive GAs (Zhang et al., 2011; Magome et al., 2013; Nomura et al., 2013). The final stage of GA biosynthesis is completed by sequential reactions of cytosolic *2ODDs*, which converts both GA_{12} and GA_{53} to the bioactive forms of GA (GA_4 and GA_1) respectively (Toyomasu et al., 1997; Hedden and Phillips, 2000). The role of *2ODDs* in GA homeostasis is important. In *Arabidopsis*, *GA20ox* and *GA3ox* (members of *2ODDs* cluster) are both down-regulated in response to high endogenous GA levels or with the application of exogenous GA. Both genes are up-regulated under GA deficiency conditions (Martin et al., 1996; Hedden, 1999; Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000; Hedden and Thomas, 2012). In contrast, high endogenous levels of GA up-regulate expression of *GA2ox*, restoring GA homeostasis (Thomas et al., 1999; Elliott et al., 2001; Sakamoto et al., 2003).

6.2.2 Effect of GA in organogenesis

The relationship between bioactive GAs and plant growth are well known (Yamaguchi, 2008; Davière and Achard, 2013). GAs are involved in many plant developmental processes, including seed germination, stem and shoot elongation, leaf and fruit expansion and flowering (Achard and Genschik, 2009). The response to bioactive GAs in plants is tempered by the activity of the regulatory GA-sensitive DELLA proteins. In the absence of GA, DELLAs inhibit GA-mediated transcriptional regulators. When in the presence of GA, DELLA proteins become earmarked for proteasome degradation and the GA-regulated transcriptional networks become active. DELLAs can also act as transcriptional activators in the absence of GA or alternatively participate in GA-mediated transactivation pathways involving direct interactions between GA, GA receptors (*GID1* proteins) and DELLAs (Davière and Achard, 2013).

In many cases, cell elongation and tissue expansion is enhanced under elevated GA availability. In both wheat and rice, internode elongation has been correlated with increased levels of bioactive GA content, which can be positively influenced by N supply (Zhang et al., 2007; Jang et al., 2008). In cereals, such as wheat and rice, modification to the GA-signaling pathway has been a successful strategy to help produce dwarfing phenotypes (Hedden and Phillips, 2000). Shorter stature crops are less prone to lodging, particularly when grown with excess nitrogen fertilisers (Jang et al., 2008). In wheat, the reduced height (dwarfing) alleles *Rht-D1a* and *Rht-D1b* encode truncated DELLA proteins that lack conserved GA binding domains (DELLA/TVHYNP), which decreases GA sensitivity. This technological approach has been a fundamental driver of increased yields associated with the green revolution over the last 50 years (Sasaki et al., 2002).

In addition to dwarfism, increased number of buds per inflorescence, reduced fertility, reduced apical dominance, and delayed senescence are other phenotypes present in GA mutants (Kawaguchi et al., 1996; Hedden and Phillips, 2000; Maekawa et al., 2009). Interestingly, the role of bioactive GAs in promoting the shift from meristem identity to

organogenesis (organ differentiation) is based on the association between *KNOX* (*KNOTTED1*-like homeobox) proteins and GA metabolism (Yamaguchi, 2008). Ectopic expression of *KNOX* will reduce endogenous GA levels and cause dwarfism in plants (Kusaba et al., 1998; Tanaka-Ueguchi et al., 1998; Sakamoto et al., 2001). This process involves *KNOX* directly binding to an intron within *GA20ox*, causing a repression of GA biosynthesis (Sakamoto et al., 2001). Consequently, elevated levels of *KNOX* expression in the corpus of the shoot apical meristem (SAM) will prevent *GA20ox* expression in this region. In contrast, the lack of *KNOX* expression in the flanks of both the SAM and the sub-apical tissues, enhances GA biosynthesis in these regions. Thus the suppression and or activation of GA biosynthesis is an important mechanism in maintaining the indeterminate state of corpus cells and stem cell proliferation stages in plant tissues.

6.2.3 Gibberellin and nodule development

In the symbiosis between Rhizobia and legumes, plant hormones play important roles in the regulation of nodule development and the establishment of a nitrogen-fixing symbiosis (Venable and Coggeshall, 1965; Harrison, 2005; Ding et al., 2008; Maekawa et al., 2009). However, the specific roles of hormones in regulating root nodule formation are largely unknown. Auxins and cytokinins have positive roles in the nodulation process while ABA, ethylene, jasmonate acid, salicylic acid, and brassinosteroid tend to play negative roles (Reviewed Ryu et al., 2012). In the case of GA, studies have shown that GA levels increase in developing nodules, suggesting a positive role of GAs during the nodulation process (Ferguson and Mathesius, 2003). In contrast, GA-deficient pea mutants show decreased nodule numbers (Ferguson et al., 2005). Additional reports indicated that nodule-like structures on *L. japonicus* roots can develop in the absence of *Rhizobium* when low concentrations of exogenous GA are applied (Kawaguchi et al., 1996). The nodule-like structures were blocked by the application of 15 mM KNO_3 or NH_4NO_3 (Kawaguchi et al., 1996). There has been a suggestion that the presence of cytochrome P450 genes in *B. japonicum* genome may regulate nodule GA levels (Tully et al., 1998). In a recent study in

soybean, RNA-seq analysis has shown that two genes involved in GA biosynthesis, *GA20oxa* (Glyma04g42300) and *GA3ox1a* (Glyma15g01500), were up-regulated in roots, 12 h after inoculation with wild type *B. japonicum,* while a nod factor mutant (nodC$^-$) showed no effect on either *GA20oxa* or *GA3ox1a* expression (Hayashi et al., 2012). This result is consistent with a previous experiment with *Sesbania rostrata*, where *SrGA20ox1* was found to be transiently up-regulated at the site of bacterial infection and nodule initiation in lateral roots (Lievens et al., 2005). In contrast, exogenously applied GA has an inhibitory effect on nodulation, preventing root hair curling, infection thread formation and nodule development in both *S. rostrata and L. japonicus* (Lievens et al., 2005; Maekawa et al., 2009). Recently, GA application has been linked to the reduced expression of the *L. japonicus* nodulation transcription factors *NSP2* and *NIN* (Maekawa et al., 2009). The literature suggests both positive and negative influences of GA on nodulation and nodule development. This variability in the GA response may be related to alternative GA signaling networks and nodulation processes operating across different legumes or to different levels of GA availability (Fletcher et al., 1959; Ryu et al., 2012).

6.2.4 cis-regulatory elements and their function

A useful approach to define regulatory mechanisms of novel genes is through promoter analysis (Deihimi et al., 2012). Transcription factor binding sites (TFBs or cis-regulatory elements), which determine the specific timing and location of transcriptional activity are regularly positioned on upstream 'promoter regions' of designated genes. Diverse cis-regulatory modules are required for specific expression patterns and or involvement of genes in different pathways (Su et al., 2010). Consequently, the identification of cis-regulatory elements and their organization modules are important steps to develop an understanding of gene expression and its regulation. Previous studies have identified a number of recognized hormone-related cis-regulatory elements in plants (Table 6.1). Two GA elements (P-Box and TATAC) have been identified previously. *In silico* promoter analysis of *GmSAT1;1* and *GmSAT1;2* revealed a relatively high frequency of gibberellin-responsive regulatory elements

across the promoter regions of both genes. In addition microarray transcriptomic analysis of RNAi *GmSAT1*–silenced plant (Chapter 4) showed that GA-responsive genes such as *GAMA-TIP*, *CLE2*, *MTO3*, *GIP1*, *TPS11*, *GBF1* (G-box binding factor 1) and *GASA6* (GA-Stimulated Arabidopsis6) were all stimulated in nodules and roots with the loss of *GmSAT1* activity. These result led me to examine whether signaling exists between *GmSAT1* and GA by promoter cloning, gene expression analysis (qPCR) *in silico* network analysis and physiological responses to GA on nodulation and expression of *GmSAT1;1* and *GmSAT1;2*.

Table 6.1 Name and function of known regulatory elements

Function	Name of regulatory elements	Sequence	Reference
Abscisic acid-responsive element	ABRE	TACGTG CACGTG	(Yamaguchi-Shinozaki et al., 1990)
Gibberellins-responsive element	P-box	CCTTTTG	(Schneider et al., 1992; Sun and Kamiya, 1994; Pastuglia et al., 1997)
	TATC-box	TATCCCA	
Auxin-responsive element	TGA-1	AACGAC	(Sakai et al., 1996; Pastuglia et al., 1997; Hedden and Phillips, 2000; Seo et al., 2006)
Jasmonic acid-responsive element	CGTCA-motif	CGTCA	(Matton et al., 1993; Rouster et al., 1997)
	TGACG-motif	TGACG	
Salicylic acid responsive element	TCA-element	GAGAAGAATA	(Goldsbrough et al., 1993; Bustamante et al., 2009)
Ethylene-responsive element	ERE	ATTTCAAA	(Itzhaki et al., 1994)
Defense and stress responsiveness	TC-rich repeats	ATTCTCTAAC	(Diaz-De-Leon et al., 1993)
Fungal elicitor responsive	Box-W1	TTGACC	(Rushton et al., 1996)
Involved in drought-inducibility	MBS	CAACTG	(Urao et al., 1993)
Wound-responsive	WUN-motif	AAATTTCCT	(Matton et al., 1993)
Heat stress responsive	HSE	AAAAAATTTC	(Pastuglia et al., 1997)
Light-responsive element	Box 4	ATTAAT	(Lois et al., 1989)
	Box 1	TTTCAAA	(Green et al., 1987)
	3-AF1 binding site	TAAGAGAGGAA	(Lam and Chua, 1989)
	ACE	ACGTGGA	http://bioinformatics.psb.ugent.be/webtools/plantcare/html/
	CATT-motif	GCATTC	(Dickey et al., 1998)
	G-Box	CACGTA	(Williams et al., 1992)
	Sp1	CC(G/A)CCC	http://bioinformatics.psb.ugent.be/webtools/plantcare/html/
	TCT-motif	TCTTAC	http://bioinformatics.psb.ugent.be/webtools/plantcare/html
	I-box	ATGATATGA	(Donald and Cashmore, 1990)
	GT1-motif	GGTTAA	(Green et al., 1987)
	circadian	CAANNNNATC	(Argüello-Astorga and Herrera-Estrella, 1998)
	AT1-motif	ATTAATTTTACA	http://bioinformatics.psb.ugent.be/webtools/plantcare/html
	Box II	GTGAGGTAATAT	(Gilmartin et al., 1992)
	chs-CMA1a	TTACTTAA	http://bioinformatics.psb.ugent.be/webtools/plantcare/html
	AE-box	AGAAACAA	http://bioinformatics.psb.ugent.be/webtools/plantcare/html

6.3 Materials & Methods

6.3.1 In silico promoter analysis

GmSAT1 homologs in soybean and their orthologs in *Arabidopsis*, *Phaseolus vulgaris*, *Medicago truncatula*, *Prunus persica*, *Zea mays*, *Oryza sativa*, *Brassica rapa* were determined by BLAST search using the phytozome (http://www.phytozome.net/) database. This search resulted in the identification of *GmSAT1;1* (Glyma15g06680), *GmSAT1;2* (Glyma13g32650), *GmSAT2;1* (Glyma05g23530), and *GmSAT2;2* (Glyma17g16720.1) in soybean, AT4G37850 and AT2g22750 in *Arabidopsis*, Phvul.006G198400 and Phvul.002G216700 in *P. vulgaris*, Medtr2g010450 and Medtr4g092700 in *M. truncatula*, *ppa008130m.g* in *P. persica*, GRMZM2G120021 in *Z. mays*, LOC_Os03g51580 in *O. sativa* and Bra011790 in *B. rapa*. The promoters were extracted from the Phytozome (http://www.phytozome.net/) databank by extracting 1.5-1.8 kb sequences before translation start site of the sequences.

Promoter analysis of *GmSAT1;1* homologs was done using the Plant-CARE database [http://bioinformatics.psb.ugent.be/webtools/plantcare/html/; (Lescot et al., 2002)]. In PlantCare, data concerning the transcription sites are extracted mainly from the literature, supplemented with an increasing number of *in silico* predicted data (Lescot et al., 2002). Apart from a general description for specific transcription factor sites, levels of confidence for the experimental evidence, functional information and the position on the promoter are provided as well. Regulatory elements are represented by positional matrices, consensus sequences and individual sites on particular promoter sequences (Lescot et al., 2002).

6.3.2 The effect of different concentrations of GA_3 on GmSAT1;1 and GmSAT1;2 expression, nodule number, and nodule dry weight

To examine the possible relationship between *GmSAT1;1* and *GmSAT1;2* and the effects of the GA_3 on nodulation, different concentration of GA_3 (0, 10^{-7} and 10^{-5} M) were applied

twice per week (by watering the soil) from 5 d after inoculation. Nodules and roots of 26 day-old plants were harvested for analysis. RNA extraction and qPCR were done using a similar procedure to that described in Chapter 2.

The experiment was carried out in an one-way ANOVA experimental plan. Three pots containing 4 plants were used as replications for each of 3 GA_3 treatments (0, 10^{-7} and 10^{-5} M). Pots were inoculated with *B. japonicum* USDA 110 two times, first after sowing seeds and the second, 2 d later. Germinated seeds were supplied with nutrient solution 6 d after germination three times per week. Five days after the second inoculation, plants were treated with GA_3 two times per week by watering with 50 ml of GA_3 solution per pot. Expression of *GmSAT1;1* and *GmSAT1;2* in root and nodule tissues consisted of two biological replications with 3 plants used in each replication.

6.3.3 The effects of short term and long term application of GA_3 on GmSAT1 expression and nodulation

The impact of GA_3 supply on *GmSAT1* expression was tested using four individual treatments: T1 treatment, water control; T2 treatment, 10^{-5} M GA_3 two times per week from 5 d after inoculation; T3 treatment, 10^{-5} M GA_3, 5 h before harvest at day 15 and 26; T4 treatment with 10^{-5} M GA_3 a week before harvest at day of 26. Expression of *GmSAT1;1* and *GmSAT1;2* in root and nodule tissue were tested in two biological replications consisting of 3 plants each.

6.3.4 GmSAT1;1 and GmSAT1;2 promoter cloning, Agrobacterium rhizogenes-based transformation and expression of the cloned promoter

The promoters of *GmSAT1;1* and *GmSAT1;2* where cloned from soybean genomic DNA and transferred to *A. rhizogenes* (strain K599) as described in Chapter 2 (2.3.3). Soybean seedlings were transformed using an optimized *A. rhizogenes*-based genetic transformation protocol (see Chapter 3). Transformed plants were treated with 3 concentrations of GA_3 (0, 10^{-7}, 10^{-5} M) two times per week after the fifth day of inoculation. At day 26, six plants per treatment were harvested for analysis.

For GUS staining, one entire hairy root with nodules from the soybean plants was placed in a 50 ml falcon tube and filled with ice cold 90% acetone. Samples were then rinsed twice in sodium phosphate buffer at room temperature for 5 min. Samples were covered in GUS staining buffer (0.1 M sodium phosphate buffer (pH 7), 3% sucrose, 50 mM sodium, 0.5 mM EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.05% (w/v), and X-Gluc (5-bromo-4-chloro-3-indolyl-β-d-glucoronic acid) dissolved in 0.5 ml dimethyl formamide) and infiltrated under vacuum for 30 min before incubated at 37°C for 5 h. Samples were rinsed 3 times with MQ water and used for analysis.

6.3.5 Effect of GmSAT1;1 silencing on shoot height (in both nodulated and non-nodulated conditions)

Glycine max cv. Djakal was transformed using a *GmSAT1* RNAi construct (see Chapter 4) and empty vector (control) according to the methods described in Chapter 3. Plants with hairy roots were transferred to individual 1 L pots containing sand to develop roots and or nodules. A total of 22 *GmSAT1 RNAi* plants and 10 empty vector controls were used in each treatment. Those plants inoculated with *B. japonicum* USDA 110 (2 days after transferring to new pots) were supplied nitrogen-free nutrient solution (Table 3.1). Non-inoculated plants were supplied nutrient solution supplemented with 2.5 mM NH_4NO_3. After 26 d, plant height was measured from the site of hairy root emergence to the highest axillary bud of the developing shoot.

6.3.6 Building SAT-gibberellin interaction networks

Gibberellin-induced genes detected in microarray experiments presented in Chapter 4 were used to build a *SAT1*-gibberellin interaction network using Pathway Studio software (version 9) and its protein-protein interaction database, as discussed in Chapter 4. A literature review based of the relations of the predicted network is presented in Table 6.2.

6.4 Results

6.4.1 The effect of exogenous application of GA_3 on nodulation (nodule number and nodule dry weight) and expression of GmSAT1;1 and GmSAT1;2

The effect of GAs in legume nodulation is wide ranging and it depends on both external as well as internal concentrations of GAs. To determine if GAs have an impact on nodulation of soybean, we applied three different concentrations of GA_3 to inoculated soybean plants. This exogenous application of GA_3 inhibited nodulation, with number of nodules and nodule dry weight (10^{-5} M) significantly decreased by application of GA_3 ($P \leq 0.05$). Lower concentrations of GA_3 (10^{-7} M) had no significant effect on nodulation, although plant height was significantly increased with GA_3 (Figure 6.2 A-C).

qPCR analysis of GA_3 treated roots and nodules revealed that expression of both *GmSAT1;1* and *GmSAT1;2* were significantly decreased ($P \leq 0.05$) with the treatment (Figure 6.2 D, E). Interestingly, while *GmSAT1;1* expression was less in root and nodules across all concentrations of GA_3, *GmSAT1;2* was only repressed at the higher concentration (10^{-5} M). This suggests that *GmSAT1;1* is more sensitive to GA_3 than *GmSAT1;2.*

6.4.2 The effect of short term and long term application of GA_3 on GmSAT1;1 and GmSAT1;2 expression and nodulation

The expression pattern of *GmSAT1;1* and *GmSAT1;2* was explored in young (15 day old) and mature (26 day old) nodules. GA_3 (10^{-5} M) applied twice weekly from planting (treatment 2), significantly reduced *GmSAT1;1* expression at days 15 and 26 (Figure 6.3A). For *GmSAT1;2*, the effect of GA_3 was only evident on 26 day nodules (Figure 6.3B). Prior application of GA_3 (5 h) before harvest (treatment 3) at days 15 and 26, decreased *GmSAT1;1* expression across both days (Figure 6.3 A) but had no effect on *GmSAT1;2* expression (Figure 6.3 B). A GA_3 treatment 7 days prior to harvest (day 26 only) did not influence *GmSAT1;1* expression but did inhibit *GmSAT1;2* expression (Figure 6.3 A, B).

6.4.3 The effect of GmSAT silencing on plant height

Loss of *GmSAT1;1* expression (*sat1* roots) resulted in a significant increase in overall plant height when plants were not nodulated and grown with external N (Figure 6.4). This response was not replicated in shoots of plants containing nodulated *sat1* roots and grown solely on –N nutrient solution. In light of the fix- phenotype associated with *sat1* nodules (Loughlin 2007), we assume the nodulated plants may have been N deficient and may have prevented a parallel increase in shoot height. However, the empty vector controls, which retain *GmSAT1* activity and do develop N_2-fixing nodules, suggest that the lack of *GmSAT1* (*sat1*) in combination with a suitable N supply may be linked with changes in shoot growth. Collectively this highlights a possible synergy between N nutrition, *GmSAT1* expression and shoot elongation in soybean. The increased shoot height observed with *sat1*, +N grown plants was similar to the response in N_2-fixing nodulated wild-type plants treated with GA_3 (Figure 6.2C).

6.4.4 In silico analysis of regulatory elements on the promoter regions of SAT1 genes

To shed light on the regulatory mechanisms and possible signaling pathways that *GmSAT1* may be involved in, we exploited the Plantcare database (Table 6.3) to identify putative regulatory elements across the two *SAT1* promoters in soybean. These elements were then evaluated against other cis-acting regulatory elements identified on promoter regions of *SAT1* homologues found in other plants (Table 6.3). The total number of regulatory elements across these promoters in response to hormones, light and stress are listed in Table 6.3. Light-responsive elements were the most common across all *SAT1* homologs. Stress responsive elements are the second most common element in all *SAT1* homolog promoters. A number of other stress-responsive elements were also identified including, ABRE (cis-acting element involved in abscisic acid responsiveness) (Yamaguchi-Shinozaki et al., 1990), CGTCA and TGACG-motifs (Jasmonic acid-responsive elements) (Matton et al., 1993; Rouster et al., 1997).

Analysis of hormone regulatory elements on the promoter regions of different *SAT* homologs can help to provide valuable information regarding potential signal transduction pathways. The GA responsive elements, *P-Box* ('5-CCTTTTG-3') (Schneider et al., 1992) and TATC-Box ('5-TATCCCA-3') (Sun and Kamiya, 1994) sequences, were both located on the promoters of *GmSAT1;1* and *GmSAT1;2* (Figure 6.5 A and 6.6A). GA responsive elements were more frequent in *GmSAT1;1* and *GmSAT1;2* than in other *SAT1* homologues (Table 6.3). Two *P-Box* and one *TATC-Box* elements were identified on promoter of *GmSAT1;1* and *GmSAT1;2* (Figure 6.5A and 6.6A). The auxin-responsive element (TGA – AACGAC) (Hedden and Phillips, 2000; Seo et al., 2006) was also identified, and was more frequent in *GmSAT1;1* than in *GmSAT1;2* and other *SAT1* homologues (Table 6.3) (Figure 6.5A and 6.6A). This may be related to the enhanced expression of *GmSAT1;1* in nodules over that of roots.

The result of *in silico* promoter analysis was consistent with the result of microarray analysis where loss of *GmSAT1* activity were resulted in significant alteration of stress, light, auxin and gibberellin responsive genes in root and nodule.

6.4.5 GmSAT1;1 and GmSAT1;2-promoter based transient expression of the GUS reporter in soybean root and nodule tissues

One of the best laboratory-based approaches for functional analysis of regulatory elements within a promoter is through cloning of the promoter sequence into a vector harboring *GUS/GFP*, and transforming that construct into the plant. The activity of the desired elements can then be monitored by adding the agent related to the regulatory element within the promoter to the plants.

Promoter fragments (~1.8 kb) 5' upstream of the start codon of *GmSAT1;1* and *GmSAT1;2* were cloned from soybean genomic DNA and inserted into the destination vector pKGWFS7 (Chapter 2, Figure 2.1) (Karimi et al., 2002). Transformation of the plasmids via *A. rhizogenes* K599 induced hairy roots revealed that both *GmSAT1;1* and *GmSAT1;2* promoters

were active and increased GUS expression in both nodules and roots (Figure 6.5 B-C, 6.6 B-C). Both promoters were active in soybean nodules when treated with water, but decreased with higher concentrations of applied GA_3 (10^{-5} M) (Figure 6.5 B-C, 6.6 B-C). The performed promoter cloning and expression in this study documents a putative negative association between GAs and *GmSAT1;1* and *GmSAT1;2* expression. However these studies need to be expanded to quantify the expression of the promoter and the individual roles of GA responsive elements in GA signaling.

6.5 Discussion

The underling mechanism by which the loss of *GmSAT1;1* reduces nodule growth and development is unclear. From this work, the data suggests that bioactive GAs may play a role in the activation of *GmSAT1* at a level most likely downstream of the GA/DELLA signaling pathway. There is a clear link between N availability and the impact of *GmSAT1* activity on shoot elongation. Shoot height of *sat1* plants increased significantly relative to the empty vector controls in non-inoculated plants supplied with external nitrogen (2.5 mM NH_4NO_3). Surprisingly there was no change in shoot growth when *sat1* plants were nodulated and grown in the absence of external N. This inconsistency is most likely due to the fact that *sat1* nodules grow poorly and are less effective in delivering fixed N to the plant (Loughlin, 2007). We assume these plants were also N deficient. The impact on shoot height in the noninoculated +N grown plants is also surprising in that only the root tissues are transgenic (*sat1*), while the phenotype was displayed primarily in non-transgenic stems. The increase in plant height in the *sat1* +N treatment is similar to the response observed in nodulated soybean, which received exogenous GA_3 applications. Interestingly, this exposure to GA_3 reduces nodule number and growth but also causes a reduction in *GmSAT1;1* and *GmSAT1;2* expression in both nodules and roots. Hence, the synergy between shoot elongation and *GmSAT1* activity could be related to tissue N status. This is consistent with the fact that internode elongation in rice is correlated with increased levels of bioactive GA content promoted by a positive influence of N supply (Zhao et al., 2007; Jang et al., 2008).

Based on the microarray analysis completed in Chapter 4, loss of *GmSAT1* activity in soybean roots and nodule was associated with the derepression of a number of known GA-responsive genes (*GIP1, CEL2, MTO3, GASA6* and *GBF1* - Table 6.2). This suggests a role of *GmSAT1* as a putative GA-mediated transcriptional repressor. Therefore changes in plant height in +N grown *sat1* plants may be associated with a change in root-derived GA signaling cascades perturbed with the loss of *GmSAT1* expression. Furthermore, the presence of three GA-responsive elements on the promoters of both *GmSAT1;1* and *GmSAT1;2* combined with the negative effect of GA_3 on the transcription of both genes with GA_3 treatment suggests GA signaling is an important regulatory component of *GmSAT1* mediated transcriptional signaling.

A hypothetical model for a *GmSAT1*-GA_3 interaction network is presented in Figure 6.1 and Table 6.2. Transcriptomic analysis of *sat1* nodules and roots revealed an up-regulation of several GA-inducible genes, including *GASA6*, Gamma-tip, *CLE2*, *MTO3*, *GIP1*, *GBF1*, *TEM1* and *TPS11* (Mudd, 1962; Mathur et al., 1992; Mathur et al., 1993; Phillips and Huttly, 1994; Lee and Kende, 2002; Ben-Nissan et al., 2004; Sehnke et al., 2005; Wieczorek et al., 2008; Lin et al., 2011; Hong et al., 2012; Osnato et al., 2012). *TEM1* is a transcription factor that links the photoperiod and GA-dependent flowering pathways and represses expression of GA synthesis enzymes *GA3OX1* and *GA3OX2* which are involved in GA homeostasis (Osnato et al., 2012). Expression of *GA3OX1* in pea was down-regulated in response to high endogenous GA levels or application of exogenous GA (Martin et al., 1996; Hedden, 1999; Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000; Hedden and Thomas, 2012). *GIP1* and *GASA6* are involved in cell wall elongation (Lee and Kende, 2002; Ben-Nissan et al., 2004). Up-regulation of these genes is one of the possible explanations of the shoot height increase in *sat1* plants. As shown in Figure 6.1, *GBFs* (a G-box cis-acting DNA transcription factor) interact with *GIP1* protein (Sehnke et al., 2005). Interestingly, the presence of *GIP1* is accompanied by a tenfold increase in *GBF* DNA binding activity (Sehnke et al., 2005).

As presented in Chapter 4 loss of *GmSAT1* activity was associated with down-regulation of a select number of auxin-responsive genes (Figure 4.16, Table 4.8). Auxin responsive regulatory elements was also identified on the promoters of *GmSAT1;1* and *GmSAT1;2* in this study. These were more frequent on promoter of *GmSAT1;1* (Figure 6.5A and 6.6A). Based on these observations it seems there is a tentative link between the function of *GmSAT1* and the plant hormones GA and auxin. Whether *GmSAT1;1* controls nodule development through regulation of these hormones needs more investigation.

This study was initially based on promoter analysis of *GmSAT1;1* and *GmSAT1;2* but it uncovered possible negative interaction between these genes and GA_3, where application of exogenous GA_3 down-regulates expression of *GmSAT1;1* and *GmSAT1;2*. It should be noted that the number of cis-acting regulatory elements is often assumed as an index of expression control (Deihimi et al., 2012). Higher frequencies of gibberellin and auxin-responsive regulatory elements on the promoter of *GmSAT1;1* and *GmSAT1;2* compared to the other homologs may suggest an level of interaction of this homolog with GA. These studies need to be expanded to quantify the expression of the promoter and the individual roles of the GA_3 elements in GA signaling and measuring endogenous GAs in *sat1* tissues.

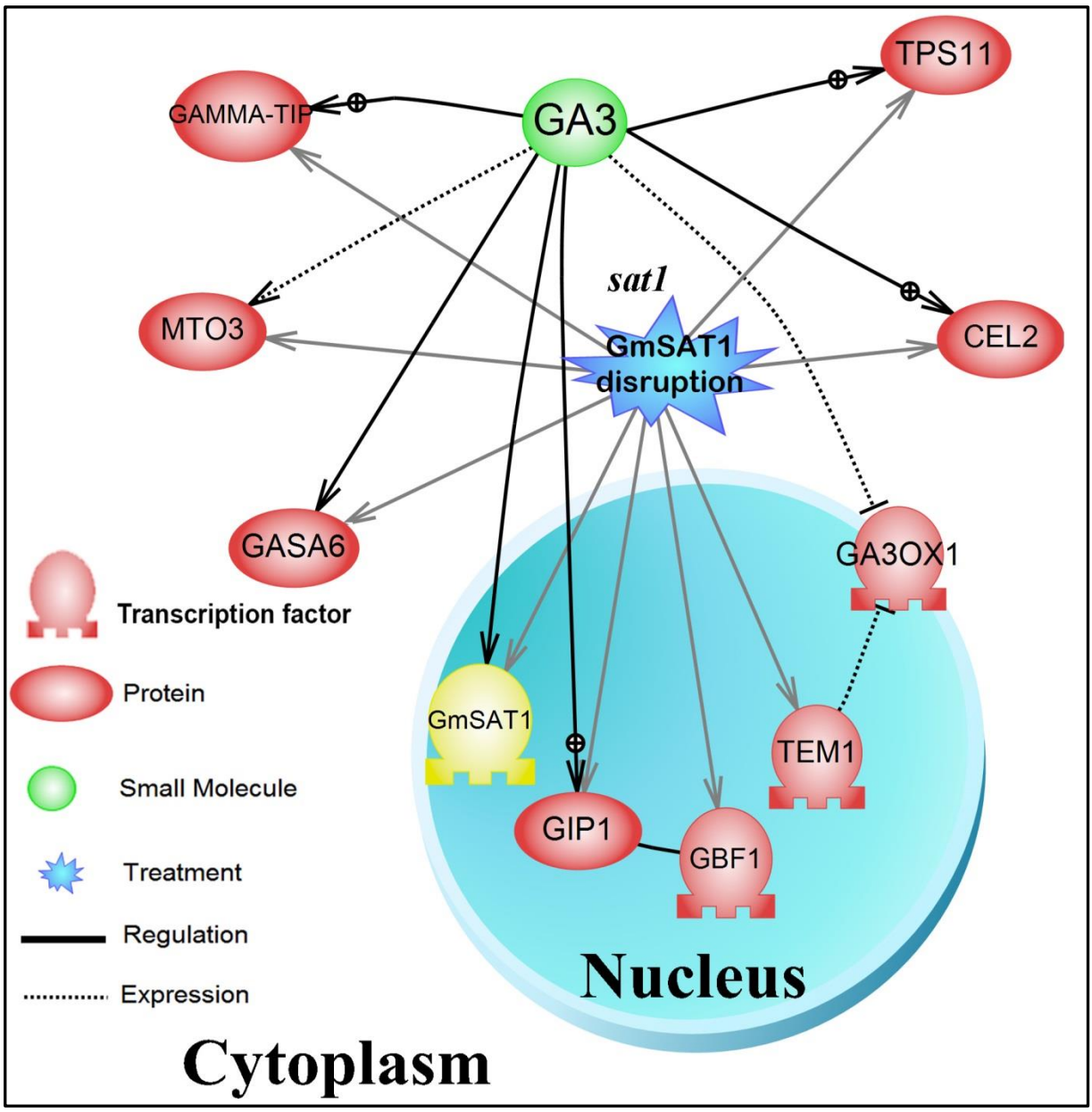


Figure 6.1 Hypothetical model for a *GmSAT1*-GA_3 interaction network.

Arrows indicate positive regulation while terminal ends indicate repression. Known transcription factors are identified in the nucleus, while cytosolic and membrane localised proteins are presented in the cytoplasmic/microsomal compartment.

Table 6.2 Gibberellin-induced soybean genes significantly over-expressed (p = 0.05, bayesian T-test) after silencing of *GmSAT1;1* in nodule and root tissues.

Tissue	Gene	Name	Best Arabidopsis Match	Explanation	Fold change	P-value	References
Nodule	Glyma19g31480	GASA6	AT1G74670	Known as a GA-inducible gene and involve in cell elongation	5.73	4.68E-05	(Lee and Kende, 2002; Lin et al., 2011)
Nodule	Glyma03g34310	GAMMA-TIP	AT2G36830	Isolated from a GA3 induced cDNA library in a GA-deficient dwarf mutant of the *Arabidopsis* (*gal*) where its expression highly increased 24hr after GA3application.	3.44	2.37E-05	(Phillips and Huttly, 1994)
	Glyma19g37000				2.07	0.001	
Nodule	Glyma18g03470	CEL2	AT1G02800	*CLE2* carry GA-responsive elements on the promoter region, cyst nematodes use this protein to induce syncytial and to depredate cells.	2.87	0.028	(Wieczorek et al., 2008)
Nodule	Glyma03g12150	GIP1	AT4G09550	*GIP1* is GA-induced cysteine-rich protein which possibly is involved in shoot elongation and transition to flowering and localized in nucleus.	2.40	0.01	(Ben-Nissan et al., 2004)
Nodule	Glyma17g04330	MTO3	AT3G17390	*MTO3* catalyzes the formation of S-adenosylmethionine from methionine and ATP and simulated by GA_3 in wheat aleurons.	2.22	0.0008	(Mudd, 1962; Mathur et al., 1992; Mathur et al., 1993)
Root	Glyma11g06960	GBF1	AT4G36730	*GBFs* is a G-box cis-acting DNA transcription factor which interacts with *GIP1* protein.	3.96	2.76E-06	(Sehnke et al., 2005)
Root	Glyma17g07530	TPS11	AT2G18700	*TPS11* is responsible for the formation of almost all *Arabidopsis* floral volatile sesquiterpenes and stimulate by GA_3.	2.65	5.21E-09	(Hong et al., 2012)
Root	Glyma01g22260	TEM1	AT1G25560	*TEM* (*TEMPRANILLO*) directly regulate the expression of the GA4 biosynthetic genes (*GA3OX1* and *GA3OX2*) and represses their expression.	1.90	0.007	(Osnato et al., 2012)

Table 6.3 Comparison of regulatory elements related to hormones, stress, and light between *GmSAT1* homologs in soybean and other plants.

Regulatory elements	Soybean				*Arabidopsis*		*Phaseolus vulgaris*		*Medicago truncatula*		*Prunus persica*	*Zea mays*	*Oryza sativa*	*Brassica rapa*
	GmSAT1;1 Glyma15g06680	*GmSAT1;2* Glyma13g32650	*GmSAT2;1* Glyma05g23530	*GmSAT2;2* Glyma17g16720	AT4g37850	AT2g22750	Phvul.006G198400	Phvul.002G216700	Medtr2g010450	Medtr4g092700	ppa008130m.g	GRMZM2G120021	LOC_Os03g51580	Bra011790
Abscisic acid-responsive element	2	2	4	4	1	0	0	0	2	0	3	1	1	1
Gibberellins-responsive element	3	3	0	1	1	0	1	1	0	1	1	0	1	0
Auxin-responsive element	2	1	0	0	0	0	0	0	0	1	0	0	0	0
Jasmonic acid-responsive element	2	4	2	4	4	0	3	2	2	0	4	2	4	0
Salicylic acid responsive element	0	0	1	0	0	2	1	1	0	1	0	1	0	1
Ethylene-responsive element	1	1	0	0	0	0	1	0	0	0	0	1	1	0
defense and stress responsiveness	2	0	2	2	4	1	2	2	2	4	2	1	1	3
fungal elicitor responsive	1	1	0	0	0	2	2	2	1	0	3	0	0	0
involved in drought-inducibility	3	4	0	0	0	0	4	1	0	1	2	1	0	2
wound-responsive	1	1	0	0	0	0	0	0	0	0	1	0	1	0
heat stress responsive	0	2	3	1	1	1	1	0	1	2	2	0	0	1
Total stress-responsive element	7	8	5	3	5	4	10	5	4	7	12	2	2	6
Light-responsive element	17	14	27	24	18	17	17	20	16	13	19	13	27	24

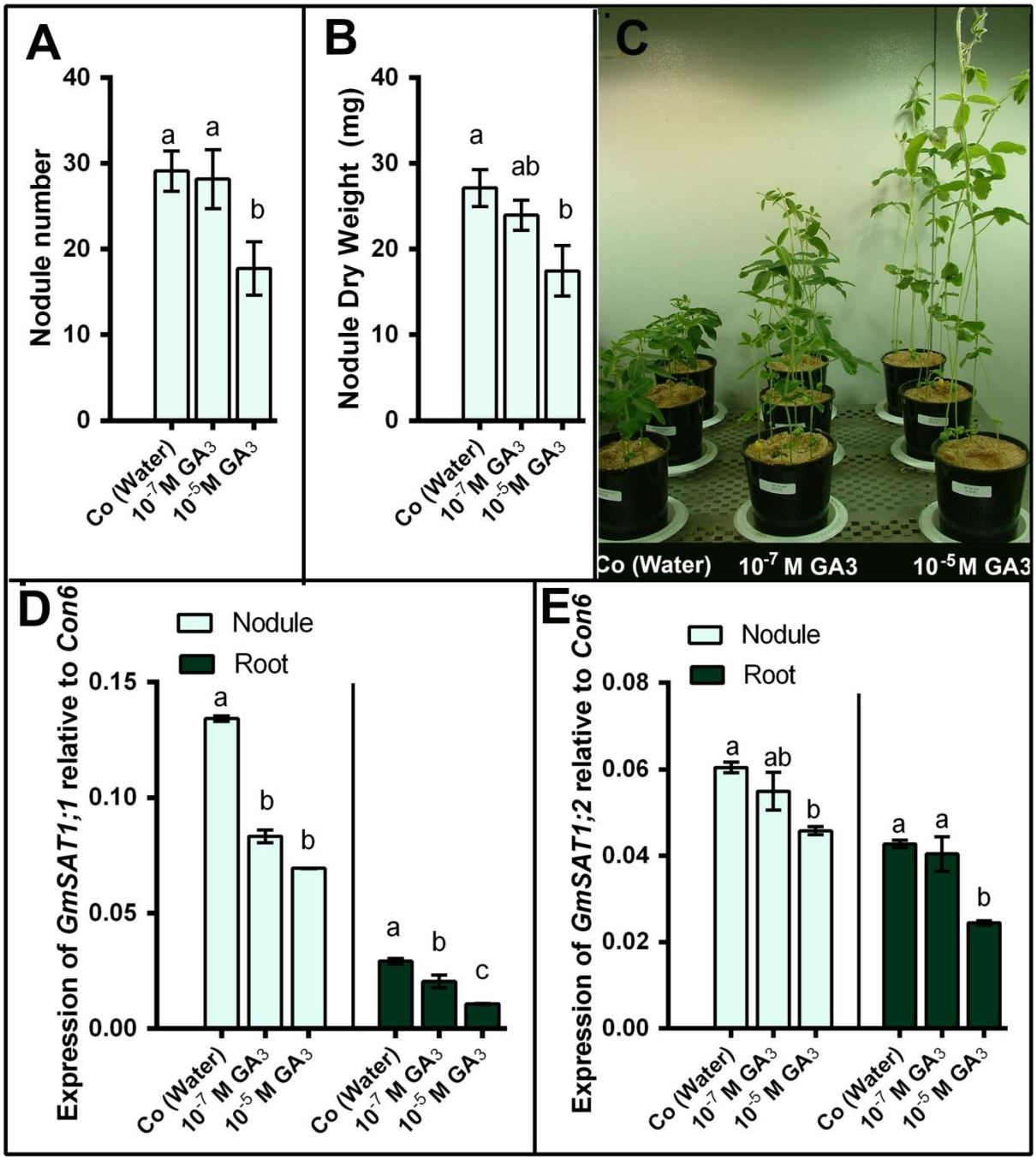


Figure 6.2 The effect of GA_3 application (0, 10^{-7} and 10^{-5} M) on *GmSAT1;1* and *GmSAT1;2* expression.

GA3 was applied 2 times per week from 5 d after inoculation with *B. japonicum* USDA 110. (A) The number of nodules on control and GA_3 treated roots; (B) Nodule dry weight (C) Plant growth with or without GA_3 treatments. (D, E) Expression of *GmSAT1;1* and *GmSAT1;2* in nodules and roots of ± GA_3 treated 26 day-old plants. Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) with *Con6* (Glyma12g05510) reference genes (Libault et al., 2008).

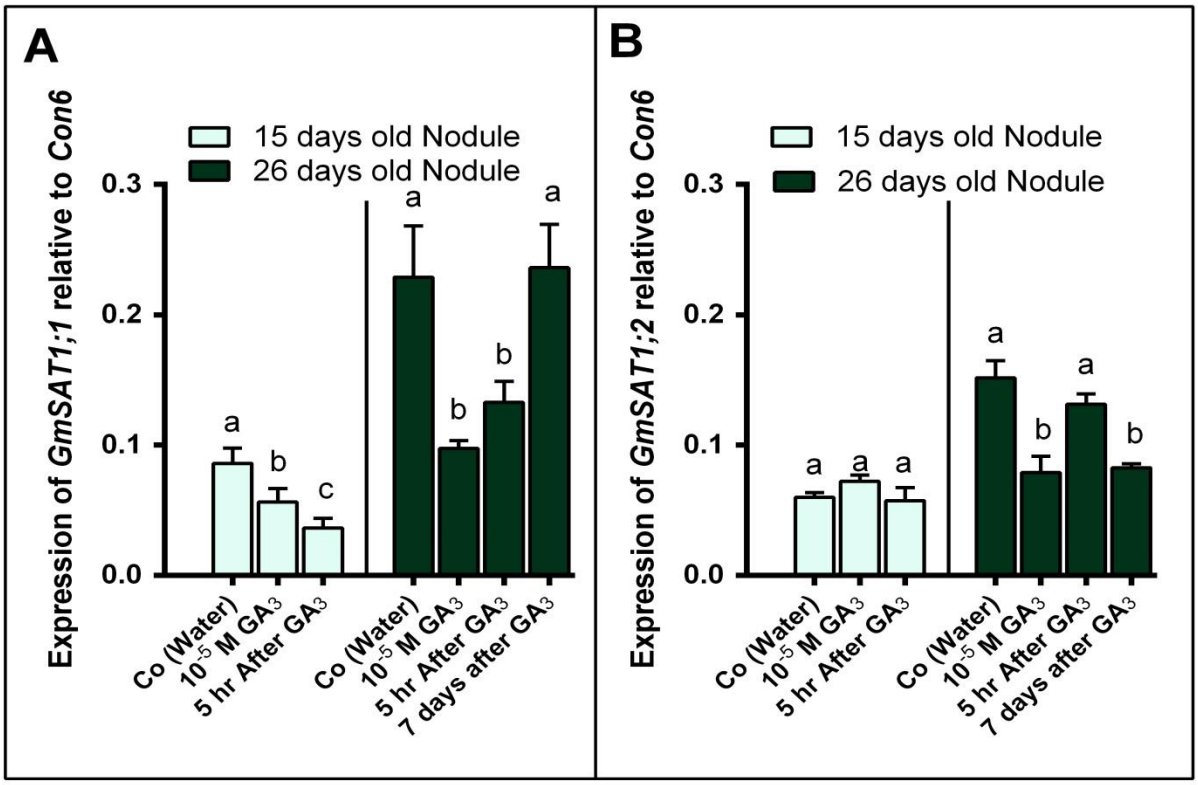


Figure 6.3 The effect of long-term and short time exposures of GA_3 on expression of *GmSAT1;1* and *GmSAT1;2*.

(A, B) Relative expression of *GmSAT1;1* and *GmSAT1;2* in response to varied GA_3 treatments. Treatments consisted of a water control (T1), repeated exposure to 10^{-5} M GA_3 (two times/week) (T2), treatment with 10^{-5} M GA_3 5 h before harvest at day of 15 and 26 (T3), and a sole treatment with 10^{-5} M GA_3, one week before harvest at 26 d. Data represents mean ± SE (n=3 plants) where relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) with *Con6* (Glyma12g05510) reference genes(Libault et al., 2008).

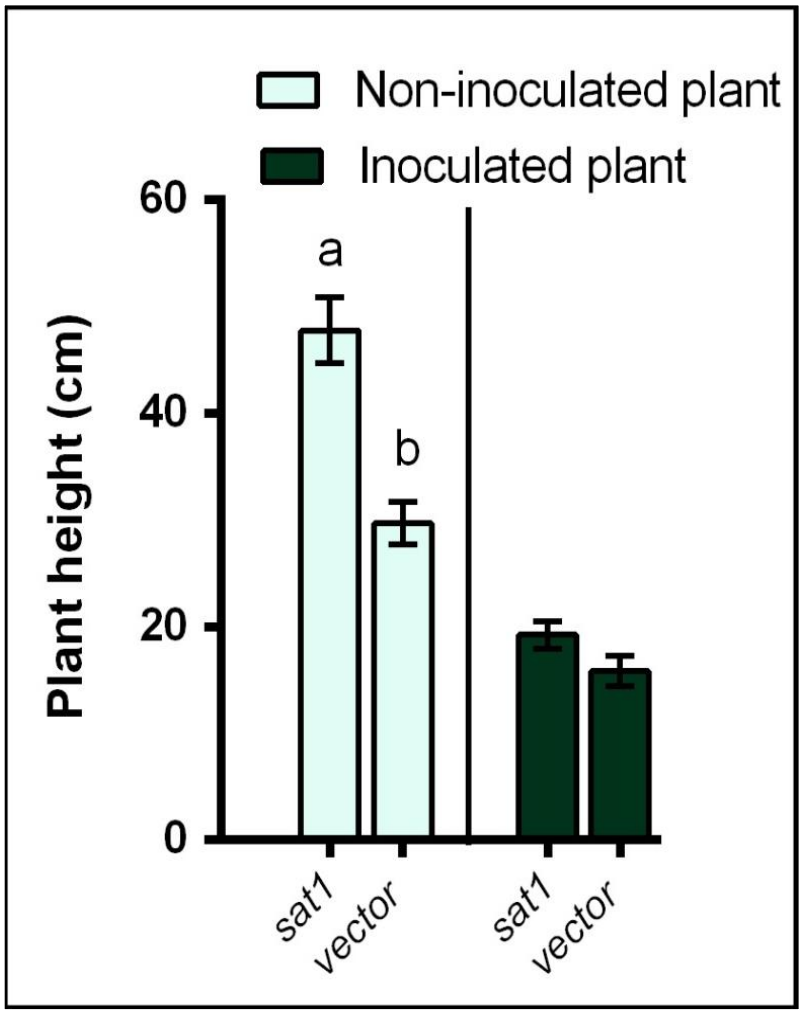


Figure 6.4 The effects of *GmSAT1* silencing on shoot height of inoculated plants and non-inoculated plants (supplemented with nitrogen).

Data represents mean ± SE (n=10-22 plants)

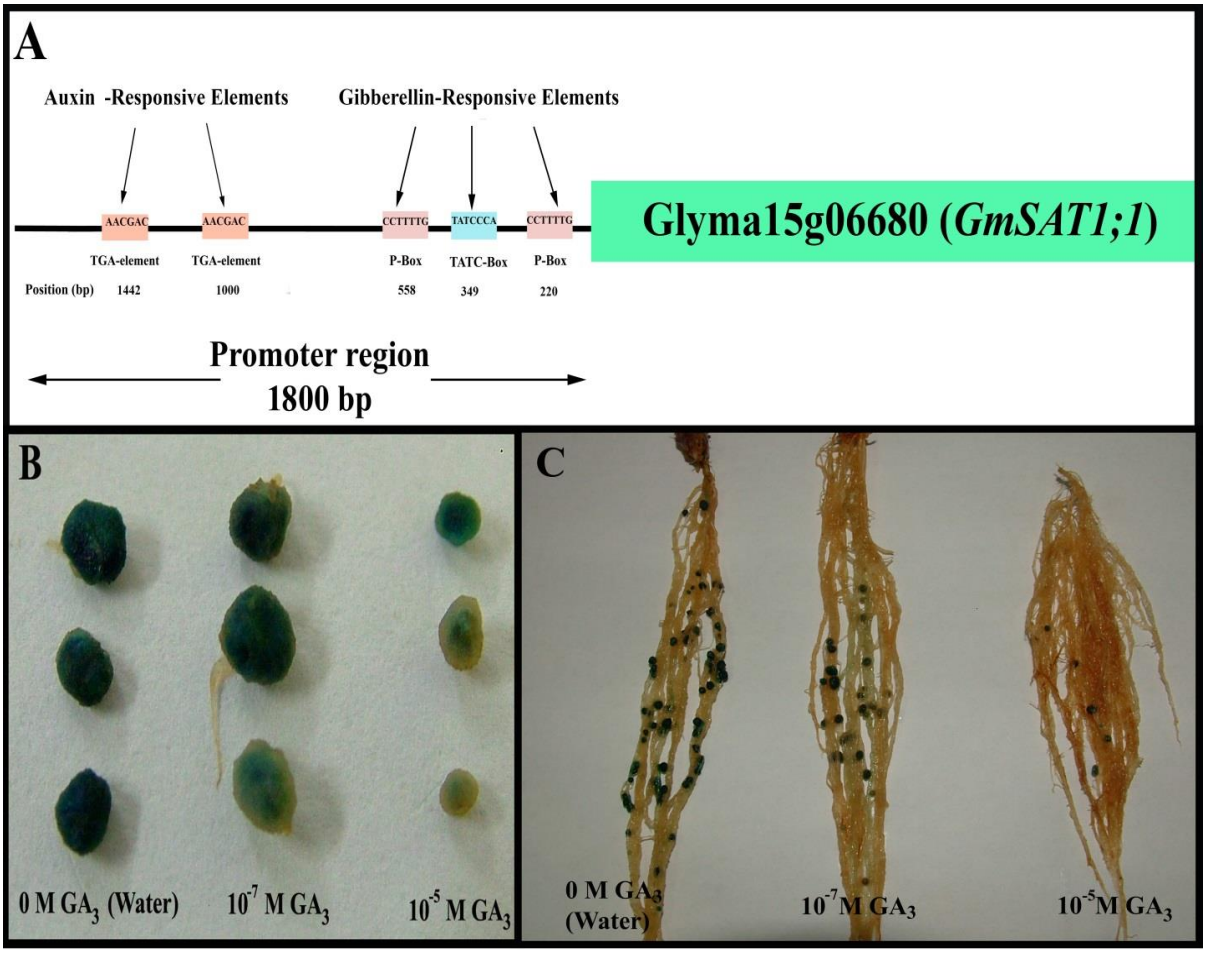


Figure 6.5 *In silico* and *in planta* analysis of *GmSAT1;1* promoter structure or activity.

The promoter for *GmSAT1;1* was evaluated using the Plant-CARE database to identify ibberellin (TATAC box and P-Boxes) and auxin (TGA-element) responsive elements. (B and C) Representative tissues displaying the activity of *GmSAT1;1* promoters fused to the GUS reporter gene. Transgenic plants were treated with different concentrations of GA_3 (0, 10^{-7} and 10^{-5} M) for 2 d prior to staining. Whole roots of 6 plants from each treatment were harvested for analysis 26 d after inoculation.

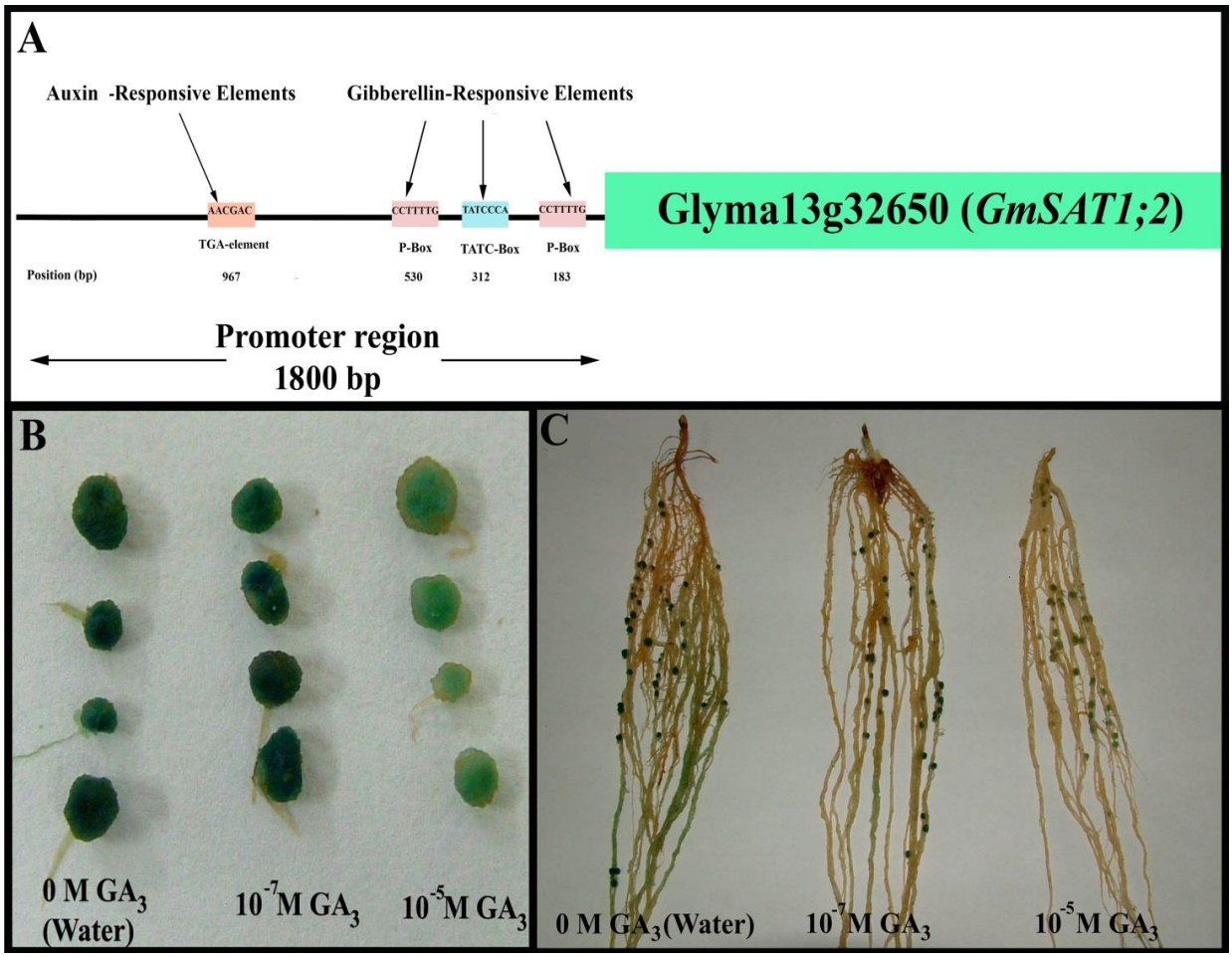


Figure 6.6 *In silico* and *in planta* analysis of *GmSAT1;2* promoter structure or activity.

Promoter for *GmSAT1;1* were evaluated using the Plant-CARE database to identify gibberellin (TATAC box and P-Boxes) and auxin (TGA-element) responsive elements. (B and C) Representative tissues displaying the activity of *GmSAT1;2* promoters fused to the GUS reporter gene. Transgenic plants were treated with different concentrations of GA_3 (0, 10^{-7} and 10^{-5} M) for 2 d prior to staining. Whole roots of 6 plants from each treatment were harvested for analysis 26 d after inoculation.

Chapter 7

7. General discussion

7.1 Introduction

The aim this study was to shed light on the function of *GmSAT1* and answer the question does *GmSAT1* only functions in the legume/rhizobium symbiosis or is it a multifunctional TF, active across both symbiotic and non-symbiotic tissues. Previous studies were only focused on the role of *GmSAT1* in nodulation and its importance for nodule development and activity (Kaiser et al., 1998; Loughlin, 2007). When *GmSAT1* is silenced, using an RNAi approach, nodules grow poorly and become ineffective in delivering reduced N to the plant. Its expression pattern, which increases with the onset of nitrogen fixation, would suggest its activity is predominantly linked with nodule activity post infection and after the release of bacteria into the infected cells. Unfortunately, very little is known about the regulatory mechanisms regulating the post-infection/nodulation period in the legume/rhizobium symbiosis. To help better understand *GmSAT1*'s role at this stage of nodule activity, I completed a series of experiments, which investigated at different levels the transcriptional activity of *GmSAT1* in both nodules and roots.

Preliminary experiments in yeast (Mazurkiewicz 2008) showed that *GmSAT1* was an active TF and was capable of modifying the expression pattern of a number of yeast genes. It was subsequently shown by Chiasson (2012), that *GmSAT1* did this by physically binding, through its bHLH DNA binding motif, selected promoter regions of genes, particularly two involved in ammonium and phosphate transport (Mazurkiewicz 2008, 2013). However, deeper analysis of the perturbed yeast transcriptome identified a number of genes differentially expressed. Many of these showed clear relationships to separate pathways

involved in P homeostasis, cell wall biogenesis and membrane transport (Mazurkiewicz, 2008). With this in mind and the potential diversity of genetic signaling pathways *GmSAT1* may participate in soybean, a series of experiments were conducted to develop a broader understanding of the transcriptional activities of *GmSAT1* in soybean and in particular its role in supporting legume nodule growth and activity. These experiments were then supported by transcriptomic analysis of *sat1* nodules and *sat1* roots as well as *in silico* promoter analysis of *GmSAT1* and its homologues in soybean and other plants. The results have provided an important first look at *GmSAT1*'s potential role in supporting symbiotic relationships and identified a number of potential activities when the plant is not in symbiosis.

7.2 *GmSAT1* expression is linked to N and P status.

Initial identification of *GmSAT1* was linked to N, where it was characterized as an ammonium transporter based on its ability to complement a yeast ammonium transport mutant (26972c) on low ammonium concentrations (Kaiser et al., 1998). Further studies demonstrated that expression of *GmSAT1;1* in yeast (strain 26972c) up-regulates the expression of *MEP3*, which normally is not active in this strain, under low ammonium conditions (Mazurkiewicz, 2008). In this study, the expression pattern of *GmSAT1;1* and *GmSAT1;2* during nodule development (Chapter 2) showed that expression of both genes was N linked, with higher expression detected in the absence of external N. Application of N decreased expression of both genes particularly, *GmSAT1;1* which is a nodule enhanced gene.

Further expression analysis showed that *GmSAT1;1* and *GmSAT1;2* expression responded to external P supply, where expression increased at low P (25 µM) and decreased at high P (250 µM) (Chapter 5). Interestingly expression of both genes were negatively correlated to internal P concentrations (Table 5.3). These studies suggest that *GmSAT1;1* and *GmSAT1;2* might act as a dual P and N sensor, and thus regulate P and N status. In support of this hypothesis, an *in silico* analysis of interacting microRNAs with *GmSAT1* homologs predicted that 3 members of microRNA156 (f, p, t) would interact with *GmSAT1;1*. These microRNAs have been shown to be expressed in response to N and P starvation in soybean and *Arabidopsis* (Hsieh et

al., 2009; Wang et al., 2013; Xu et al., 2013). MicroRNA169 is another P and N responsive microRNA which also interacts with members of the NF-Y's TF family. The microarray analysis completed in Chapter 4, identified that *GmSAT1* disruption modified the expression of several members of the NF-Y's TF family (*NF-YA3, NF-YA8, NF-YA10*) in both roots and nodules. This suggest that the *GmSAT1* is probably also involved in N and P starvation through an association with selected *NF-YA* family members and an interaction with microRNA169.

7.3 GmSAT1 deficiency highlights multiple signaling cascades.

Transcriptome analysis of *GmSAT1* RNAi silenced root and nodule tissues, showed that *GmSAT1* is a TF with multiple targets and involved in a range of different signaling pathways including nitrogen and phosphorous homeostasis, hormone interactions, stress responses and the circadian regulatory network.

7.3.1 Nitrogen homeostasis

Transcriptomic analysis of *sat1* roots showed that a number of nitrogen-related genes have altered expression with the loss of *GmSAT1* expression. Network analysis showed that nitrogen-starvation induced genes (*NRT1;7 AMT2, DUR3, NRT2;4*) were all down-regulated in the *sat1* root tissues while, the nitrate induced transceptor (*NRT1.1*) and nitrate reductase (*NIA1*) were up-regulated (Figure 4.13). This contrasting genetic response may indicate roots lacking *GmSAT1* are no longer capable of sensing external or internal nitrogen levels. It may also indicate that *GmSAT1* activates selected pathways important to the transport of nitrogen under starvation conditions.

7.3.2 Phosphorus homeostasis and AM symbiosis

Loss of *GmSAT1* in soybean was also associated with a significant down regulation of many P-related root genes including *HAD1*, *SPX2*, *PHT1;*7 (phosphate transporter), *GS52*, *MGD2* and *RNS2*. Previous studies had shown that overexpression of *GmSAT1* in yeast resulted in

up-regulation of a large number of P responsive genes including, *PHO84* (~39-fold), *PHM6* (~14-fold), *VTC3* (~3.5-fold), *PHO5* (~3-fold), *PHO3*(~2.5-fold), *PHM8* (~2.2-fold), *PHO11;PHO12* (~2.2-fold), and *PHO8* (~2-fold) (Mazurkiewicz, 2008). These results show that *GmSAT1* may have different targets in soybean and yeast but in both its activity is associated with activation of P responsive signaling pathways. The positive role of *GmSAT1* in P homeostasis was more evident when AM colonization of *sat1* versus *vector* root was examined under low and high concentrations of P supply. Colonization of *sat1* roots did not change when external P supply was provided while P concentrations in the shoot in *sat1* plants was found to be higher than the controls indicating a potential misregulation of Pi homeostasis in *sat1* roots. This is an exciting outcome as it suggests an alternative mechanism by which *GmSAT1* influences nutritional status of the plant and potentially identifies a genetic link that helps mediate P-signaling when plants are subjected to a P-sensitive symbiotic partnership. Since the AM symbiosis pre-dates that of *Rhizobium*, the relationship to P may be an underlying component of its activity in soybean and other plant species. In preliminary results obtained in Maize (Kaiser, personal communication), a P-responsive QTL promoting shoot growth under low P availability has been identified in the location where a *SAT1* homolog resides. It will be important to further investigate these relationships as they may be important in how plants manage their P homeostasis.

7.3.3 Hormone interactions

The loss of *GmSAT1* expression unexpectedly identified a relationship with hormone responsive genes in legume nodules and roots. The expression patterns of auxin, GA and ethylene responsive genes were modified in the absence of *GmSAT1*. Auxin is a hormone that has a known positive effect on nodulation, while ethylene has been shown to negatively control nodulation (Badenoch-Jones et al., 1984; Penmetsa and Cook, 1997; Pacios-Bras et al., 2003; Sun et al., 2006; Penmetsa et al., 2008). The effect of GA on nodulation is varied across legume species. For example GA-deficient pea mutants show decreased nodule numbers which can be recovered by exogenous application of GA (Ferguson et al., 2005). In

contrast, exogenously applied GA has an inhibitory effect on nodulation, preventing root hair curling, infection thread formation and nodule development in both *S. rostrata and L. japonicus* (Lievens et al., 2005; Maekawa et al., 2009). In soybean (Chapter 6), GA application was found to inhibit nodule formation and development.

Microarray analysis of *GmSAT1;1* RNAi silenced plants showed that a number of auxin-responsive genes including, *BRU6*, *GH3.1*, and *PDR9* were down-regulated after *GmSAT1;1* silencing. Moreover *in silico* promoter analysis showed there are at least two auxin-responsive elements located on *GmSAT1;1* promoter. The link with auxin may involve potential regulation by a member of microRNA393 family (microRNA393b), predicted by *in silico* analysis to interact with *GmSAT1;1*. microRNA393b is well-known because of its interaction with the auxin receptor *AFB3*, and its quick response to *Rhizobium* inoculation (Subramanian et al., 2008; Vidal et al., 2010; Turner et al., 2013; Vidal et al., 2013). In a recent study that profiled zonal expression patterns across *Medicago* nodules, *MtSAT1* (Medtr2g010450) and several auxin responsive genes were found co-expressed in the meristematic zone (Limpens et al., 2013). Although not conclusive that an interaction is occurring, the results do support a hypothesis that *GmSAT1* may interact with auxin during the course of nodule development.

The possible interaction of *GmSAT1;1* with GAs were also examined in this study (Chapter 6). Exposure of soybean roots to GA_3 reduced nodule number and growth and caused a reduction in *GmSAT1;1* and *GmSAT1;2* expression in both nodules and roots. These results were confirmed where *in planta* promoter analysis using GUS reporter protein that showed exogenous application of GA_3 reduces *GmSAT1* expression. This may be linked to the presence of three GA responsive regulatory elements in the promoter of *GmSAT1*. The negative effects of GA_3 on expression of *GmSAT1* were consistent with the result observed in *sat1* plants where plant height was positively affected and a number of gibberellin-responsive genes that were up-regulated after *GmSAT1* silencing (including Gamma-tip, *GIP1*, *CEL2*, *MTO3*, and *GBF1*).

Loss of *GmSAT1* activity was also associated with up-regulation of several ethylene responsive genes in roots including *EIN2, EFE, EFR, RAP2.4,* and *EBF1*. Ethylene negatively regulates nodulation probably by reducing Nod factor-induced ENOD11 expression and root-hair calcium spiking/sensitivity in response to Nod factor (Penmetsa and Cook, 1997; Sun et al., 2006; Penmetsa et al., 2008). Mutations to ethylene responsive genes, such as *EIN2*, resulted in increased infection in the AM symbiosis and nodule number in the rhizobium symbiosis (Penmetsa and Cook, 1997; Penmetsa et al., 2008).

Taken together these results suggest that the reduction in nodulation and the small size of *sat1* nodules could be linked to the collective down-regulation of auxin responsive genes and the up-regulation of GA and ethylene responsive genes. It will be interesting to identify at what stage *GmSAT1* participates in these signaling cascades and whether the interaction with hormones extends to other tissues, developmental stage of growth and environmental influences.

7.3.4 Stress response

In this study, it was also revealed that the loss of *GmSAT1* activity is associated with significant down-regulation ($P \leq 0.05$) of biotic stress functional groups in both root and nodule tissues (Chapter 4). *In silico* promoter analysis of *GmSAT1* and related homologs identified a relatively high frequency of stress responsive regulatory promoter elements (TC-rich repeats, Box-W1) (Table 6.3). This tentatively suggests an involvement of *GmSAT1* in stress-related signaling pathways. One of the best characterized *GmSAT1* homologs is *NAI1* (At2g22760), which is predicted to regulate the expression of genes involved in the synthesis of ER bodies, an endoplasmic reticulum-derived structure induced in response to biotic stress conditions (Matsushima et al., 2002; Matsushima et al., 2004). Predictive network analysis identified a root defense response pathway, that linked functional groups of down-regulated cell wall, and lipid metabolism genes in *sat1* nodules. Cell walls can signal stress responses in plants influencing the biogenesis of cell walls (Ellis et al., 2002). Interestingly, loss of

GmSAT1 in nodules was associated with the down-regulation of a significant number of genes is linked to cell wall expansion and development (Chapter 4).

7.3.5 Circadian regulatory networks

Based on the result of the *in silico* promoter analysis of *GmSAT1,* light responsive elements were the most frequent regulatory elements identified across its promoter and that of close homologs (Chapter 6). In addition, *GmSAT1* disruption was associated with down-regulation of several light responsive genes and circadian clock regulators, including the sucrose sensor *GIGANTEA* (Dalchau et al., 2011) *PRR5,* and *PRR7* (Chapter 4). Chiasson (2012) has examined the relationship between the circadian clock and *GmSAT1* which identified both that *GmSAT1;1* and *GmSAT1;2* expression in nodules peaked in the dark, while nodule expressed *GIGANTEA (GmGI1, GmGI2)* and *GmPRR7* peak in the early evening and *GmPRR5* peaked at the beginning of the dark-period (Chiasson, 2012). The circadian clock governs many physiological and developmental responses including GA and auxin signaling pathways, flowering and stress responses (Covington and Harmer, 2007; de Montaigu et al., 2010; Arana et al., 2011). The influence on clock expression in nodule tissues highlights a potential signaling cascade that could be involved laterally across many of the signaling cascades previously discussed. Defining how a nodule clock functions and its importance to nodule activity will ultimately be important in providing the context by which *GmSAT1* operates in nodules and potentially other tissues within the plant.

7.4 Conclusion and future studies

In summary, *GmSAT1* is an important TF where its activity responds to known symbiotic partnerships including rhizobia and AM fungi. Clear links to the management of N and P in soybean roots and or nodules and the consequences it has on symbiotic partnerships could suggest a role as a potential ‘sensor’ – suited to the underlying roles of these two symbioses. Its inherent membrane location provides an interesting mechanism by which a sensing role could take place. Future experiments targeted at identifying interacting proteins involved in

membrane release and DNA activity (binding) will be important to better identify and characterise the role *GmSAT1* actually plays. Furthermore the interesting relationship observed across selected hormones (GA, auxin and ethylene), suggests a primary activity linked to cell development and or a response to both biotic and abiotic stresses. Since *SAT1* homologs are present in most plant species, we expect these classes of proteins will be involved in a number of variable plant and growth specific signaling cascades. This thesis has established a putative blueprint that will help future investigations on *SAT1*-related activities.

8. References

Achard P, Genschik P (2009) Releasing the brakes of plant growth: how GAs shutdown DELLA proteins. Journal of Experimental Botany **60:** 1085-1092

Almeida JF, Hartwig UA, Frehner M, Nösberger J, Lüscher A (2000) Evidence that P deficiency induces N feedback regulation of symbiotic N_2 fixation in white clover (*Trifolium repens L.*). Journal of Experimental Botany **51:** 1289-1297

Andriankaja A, Boisson-Dernier A, Frances L, Sauviac L, Jauneau A, Barker DG, de Carvalho-Niebel F (2007) AP2-ERF Transcription Factors Mediate Nod Factor–Dependent *Mt ENOD11* Activation in Root Hairs via a Novel cis-Regulatory Motif. The Plant Cell Online **19:** 2866-2885

Arana MV, Marín-de la Rosa N, Maloof JN, Blázquez MA, Alabadí D (2011) Circadian oscillation of gibberellin signaling in Arabidopsis. Proceedings of the National Academy of Sciences **108:** 9292-9297

Argüello-Astorga G, Herrera-Estrella L (1998) Evolution of light-regulated plant promoters. Annual Review of Plant Physiology and Plant Molecular Biology **49:** 525-555

Arrighi J-F, Barre A, Ben Amor B, Bersoult A, Soriano LC, Mirabella R, de Carvalho-Niebel F, Journet E-P, Gherardi M, Huguet T, Geurts R, Denarie J, Rouge P, Gough C (2006) The *Medicago truncatula* Lysine Motif-Receptor-Like Kinase Gene Family Includes *NFP* and New Nodule-Expressed Genes. Plant Physiol. **142:** 265-279

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet **25:** 25-29

Asimi S, Gianinazzi-Pearson V, Gianinazzi S (1980) Influence of increasing soil phosphorus levels on interactions between vesicular–arbuscular mycorrhizae and *Rhizobium* in soybeans. Canadian Journal of Botany **58:** 2200-2205

Auriac M-C, Timmers AC (2007) Nodulation studies in the model legume Medicago truncatula: Advantages of using the constitutive EF1α promoter and limitations in detecting fluorescent reporter proteins in nodule tissues. Molecular Plant-Microbe Interactions **20:** 1040-1047

Badenoch-Jones J, Summons R, Rolfe B, Letham D (1984) Phytohormones,Rhizobium mutants, and nodulation in legumes. IV. Auxin metabolites in pea root nodules. Journal of Plant Growth Regulation **3:** 23-39

Baldwin J, Karthikeyan A, Cao A, Raghothama K (2008) Biochemical and molecular analysis of *LePS2;1:* a phosphate starvation induced protein phosphatase gene from tomato. Planta **228:** 273-280

Baldwin JC, Karthikeyan AS, Raghothama KG (2001) *LEPS2*, a Phosphorus Starvation-Induced Novel Acid Phosphatase from Tomato. Plant Physiology **125:** 728-737

Banasiak J, Biała W, Staszków A, Swarcewicz B, Kępczyńska E, Figlerowicz M, Jasiński M (2013) A *Medicago truncatula* ABC transporter belonging to subfamily G modulates the level of isoflavonoids. Journal of Experimental Botany **64:** 1005-1015

Bariola PA, MacIntosh GC, Green PJ (1999) Regulation of S-Like Ribonuclease Levels in Arabidopsis. Antisense Inhibition of *RNS1* or *RNS2* Elevates Anthocyanin Accumulation. Plant Physiology **119:** 331-342

Baseri S, Towhidi M, Ebrahimie E (2011) A modified efficient empirical bayes regression model for predicting phenomena with a large number of independent variables and fewer bbservations; Examples of its Application in human disease, protein bioinformatics, and microarray gene expression profiling. Advanced Studies in Biology **3:** 181-204

Ben-Nissan G, Lee J-Y, Borohov A, Weiss D (2004) *GIP*, a Petunia hybrida GA-induced cysteine-rich protein: a possible role in shoot elongation and transition to flowering. The Plant Journal **37:** 229-238

Benková E, Michniewicz M, Sauer M, Teichmann T, Seifertová D, Jürgens G, Friml J (2003) Local, Efflux-Dependent Auxin Gradients as a Common Module for Plant Organ Formation. Cell **115:** 591-602

Benning C, Ohta H (2005) Three Enzyme Systems for Galactoglycerolipid Biosynthesis Are Coordinately Regulated in Plants. Journal of Biological Chemistry **280:** 2397-2400

Bi Y-M, Wang R-L, Zhu T, Rothstein S (2007) Global transcription profiling reveals differential responses to chronic nitrogen stress and putative nitrogen regulatory components in Arabidopsis. BMC Genomics **8:** 281

Blume B, Nürnberger T, Nass N, Scheel D (2000) Receptor-Mediated Increase in Cytoplasmic Free Calcium Required for Activation of Pathogen Defense in Parsley. The Plant Cell Online **12:** 1425-1440

Boisson-Dernier A, Chabaud M, Garcia F, Becard G, Rosenberg C, Barker DG (2001) *Agrobacterium rhizogenes*-Transformed Roots of *Medicago truncatula* for the Study

of Nitrogen-Fixing and Endomycorrhizal Symbiotic Associations. Molecular Plant-Microbe Interactions **14:** 695-700

Bonfante P, Genre A (2010) Mechanisms underlying beneficial plant-fungus interactions in mycorrhizal symbiosis. Nat Commun **1**

Boot KJM, van Brussel AAN, Tak T, Spaink HP, Kijne JW (1999) Lipochitin Oligosaccharides from *Rhizobium leguminosarum* bv. viciae Reduce Auxin Transport Capacity in *Vicia sativa* subsp. *nigra* Roots. Molecular Plant-Microbe Interactions **12:** 839-844

Brown AHD, Burdon JJ, P. GJ (1990) Genetic structure of *Glycine canescens*, a perennial relative of soybean. Theor Appl Genet **79:** 729-736

Bustamante CA, Civello PM, Martínez GA (2009) Cloning of the promoter region of β-xylosidase (*FaXyl1*) gene and effect of plant growth regulators on the expression of *FaXyl1* in strawberry fruit. Plant Science **177:** 49-56

Cao D, Hou H, Song S, Wu C, Gao Y, Han T (2009) Assessment of conditions affecting *Agrobacterium rhizogenes*-mediated transformation of soybean. Plant Cell Tiss Organ Cult **96:** 45–52

Catoira R, Galera C, de Billy F, Penmetsa RV, Journet E-P, Maillet F, Rosenberg C, Cook D, Gough C, Dénarié J (2000) Four Genes of *Medicago truncatula* Controlling Components of a Nod Factor Transduction Pathway. The Plant Cell Online **12:** 1647-1665

Charpentier M, Bredemeier R, Wanner G, Takeda N, Schleiff E, Parniske M (2008) *Lotus japonicus* CASTOR and POLLUX Are Ion Channels Essential for Perinuclear Calcium Spiking in Legume Root Endosymbiosis. The Plant Cell Online **20:** 3467-3479

Cheng T-Y, Saka H, Voqui-Dinh TH (1980) Plant regeneration from soybean cotyledonary node segments in culture. Plant Science Letters **19:** 91-99

Cheon CI, Lee NG, Siddique AB, Bal AK, Verma DP (1993) Roles of plant homologs of Rab1p and Rab7p in the biogenesis of the peribacteroid membrane, a subcellular compartment formed de novo during root nodule symbiosis. Embo J **12:** 4125-4135

Chiasson D (2012) Characterization of *GmSAT1* and related proteins from legume nodules. University of Adelaide

Collier R, Tegeder M (2012) Soybean ureide transporters play a critical role in nodule development, function and nitrogen export. The Plant Journal **72:** 355-367

Combier J-P, Frugier F, de Billy F, Boualem A, El-Yahyaoui F, Moreau S, Vernie T, Ott T, Gamas P, Crespi M, Niebel A (2006) *MtHAP2-1* is a key transcriptional regulator

of symbiotic nodule development regulated by microRNA169 in *Medicago truncatula*. Genes & Dev. **20:** 3084-3088

Consortium TGO (2008) The Gene Ontology project in 2008. Nucleic Acids Res **36:** D440-444

Cooper JB, Long SR (1994) Morphogenetic Rescue of *Rhizobium meliloti* Nodulation Mutants by trans-Zeatin Secretion. The Plant Cell Online **6:** 215-225

Covington MF, Harmer SL (2007) The Circadian Clock Regulates Auxin Signaling and Responses in <italic>Arabidopsis</italic>. PLoS Biol **5:** e222

Crespi M, Frugier F (2008) De Novo Organ Formation from Differentiated Cells: Root Nodule Organogenesis. Sci. Signal. **1:** re11

Cui F, Wu S, Sun W, Coaker G, Kunkel B, He P, Shan L (2013) The *Pseudomonas syringae* Type III Effector *AvrRpt2* Promotes Pathogen Virulence via Stimulating *Arabidopsis* Auxin/Indole Acetic Acid Protein Turnover. Plant Physiology **162:** 1018-1029

Dai X, Zhao PX (2011) psRNATarget: a plant small RNA target analysis server. Nucleic Acids Research **39:** W155-W159

Dalchau N, Baek SJ, Briggs HM, Robertson FC, Dodd AN, Gardner MJ, Stancombe MA, Haydon MJ, Stan G-B, Gonçalves JM, Webb AAR (2011) The circadian oscillator gene *GIGANTEA* mediates a long-term response of the *Arabidopsis thaliana* circadian clock to sucrose. Proceedings of the National Academy of Sciences **108:** 5104-5109

Dash S, Van Hemert J, Hong L, Wise RP, Dickerson JA (2012) PLEXdb: gene expression resources for plants and plant pathogens. Nucleic Acids Research **40:** D1194-D1201

Davière J-M, Achard P (2013) Gibberellin signaling in plants. Development **140:** 1147-1151

de Billy F, Grosjean C, May S, Bennett M, Cullimore JV (2001) Expression Studies on *AUX1*-like Genes in Medicago truncatula Suggest That Auxin Is Required at Two Steps in Early Nodule Development. Molecular Plant-Microbe Interactions **14:** 267-277

de Montaigu A, Tóth R, Coupland G (2010) Plant development goes like clockwork. Trends in Genetics **26:** 296-306

Deihimi T, Niazi A, Ebrahimi M, Kajbaf K, Fanaee1 S, Bakhtiarizadeh MR, Ebrahimie E (2012) Finding the undiscovered roles of genes: an approach using mutual ranking of coexpressed genes and promoter architecture-case study: dual roles of thaumatin like proteins in biotic and abiotic stresses. SpringerPlus **1:** 1

Deihimi T, Niazi A, Ebrahimi M, Kajbaf K, Fanaee S, Bakhtiarizadeh M, Ebrahimie E (2012) Finding the undiscovered roles of genes: an approach using mutual ranking of coexpressed genes and promoter architecture-case study: dual roles of thaumatin like proteins in biotic and abiotic stresses. SpringerPlus **1:** 1-10

Devers EA, Branscheid A, May P, Krajinski F (2011) Stars and Symbiosis: MicroRNA- and MicroRNA*-Mediated Transcript Cleavage Involved in Arbuscular Mycorrhizal Symbiosis. Plant Physiology **156:** 1990-2010

Devoto A, Ellis C, Magusin A, Chang H-S, Chilcott C, Zhu T, Turner J (2005) Expression profiling reveals COI1 to be a key regulator of genes involved in wound- and methyl jasmonate-induced secondary metabolism, defence, and hormone interactions. Plant Molecular Biology **58:** 497-513

Diaz-De-Leon F, Klotz KL, Lagrimini LM (1993) Nucleotide Sequence of the Tobacco (*Nicotiana tabacum*) Anionic Peroxidase Gene. Plant Physiology **101:** 1117-1118

Diaz CL, Melchers LS, Hooykaas PJJ, Lugtenberg BJJ, Kijne JW (1989) Root lectin as a determinant of host-plant specificity in the *Rhizobium-legume* symbiosis. Nature **338:** 579-581

Dickey LF, Petracek ME, Nguyen TT, Hansen ER, Thompson WF (1998) Light Regulation of Fed-1 mRNA Requires an Element in the 5′ Untranslated Region and Correlates with Differential Polyribosome Association. The Plant Cell Online **10:** 475-484

Ding Y, Kalo P, Yendrek C, Sun J, Liang Y, Marsh JF, Harris JM, Oldroyd GED (2008) Abscisic Acid Coordinates Nod Factor and Cytokinin Signaling during the Regulation of Nodulation in Medicago truncatula. The Plant Cell Online **20:** 2681-2695

Donald R, Cashmore A (1990) Mutation of either G box or I box sequences profoundly affects expression from the *Arabidopsis rbcS-lA* promoter. The EMBO Journal **9:** 1717- 1726

Donaldson PA, Simmonds DH (2000) Susceptibility to *Agrobacterium tumefaciens* and cotyledonary node transformation in short-season soybean. Plant Cell Reports **19:** 478-484

Duan K, Yi K, Dang L, Huang H, Wu W, Wu P (2008) Characterization of a sub-family of Arabidopsis genes with the SPX domain reveals their diverse functions in plant tolerance to phosphorus starvation. The Plant Journal **54:** 965-975

Ebrahimie E, Naghavi MR, Hosseinzadeh A, Behamta MR, Mohammadi-Dehcheshmeh M, Sarrafi A, Spangenberg G (2007) Induction and comparison of different *in vitro* morphogenesis pathways using embryo of cumin (*Cuminum cyminum* L.) as a model material. Plant Cell Tiss Organ Cult **90:** 293–311

Elliott RC, Ross JJ, Smith JJ, Lester DR, Reid JB (2001) Feed-Forward Regulation of Gibberellin Deactivation in Pea. Journal of Plant Growth Regulation **20:** 87-94

Ellis C, Karafyllidis I, Wasternack C, Turner JG (2002) The Arabidopsis Mutant cev1 Links Cell Wall Signaling to Jasmonate and Ethylene Responses. The Plant Cell Online **14:** 1557-1566

Endre G, Kereszt A, Kevei Z, Mihacea S, Kalo P, Kiss GB (2002) A receptor kinase gene regulating symbiotic nodule development. Nature **417:** 962-965

Estrada-Navarrete G, Alvarado-Affantranger X, Olivares J-E, Díaz-Camino C, Santana O, Murillo E, Guillén G, Sánchez-Guevara N, Acosta J, Quinto C, Li D, Gresshoff PM, Sánchez F (2006) Agrobacterium rhizogenes Transformation of the Phaseolus spp.: A Tool for Functional Genomics. Molecular Plant-Microbe Interactions **19:** 1385-1393

Etzler ME, Kalsi G, Ewing NN, Roberts NJ, Day RB, Murphy JB (1999) A nod factor binding lectin with apyrase activity from legume roots. Proceedings of the National Academy of Sciences of the United States of America **96:** 5856-5861

Fang Y, Hirsch AM (1998) Studying Early Nodulin Gene *ENOD40* Expression and Induction by Nodulation Factor and Cytokinin in Transgenic Alfalfa. Plant Physiology **116:** 53-68

Ferguson BJ (2013) The development and regulation of soybean nodules. *In* A Comprehensive Survey of International Soybean Research - Genetics, Physiology, Agronomy and Nitrogen Relationships. InTech, pp 31–47

Ferguson BJ, Mathesius U (2003) Signaling Interactions During Nodule Development. Journal of Plant Growth Regulation **22:** 47-72

Ferguson BJ, Ross JJ, Reid JB (2005) Nodulation phenotypes of gibberellin and brassinosteroid mutants of pea. Plant Physiol **138:** 2396-2405

Fletcher WW, Alcorn JWS, Raymond JC (1959) Gibberellic Acid and Nodulation of Legumes. Nature **184:** 1576-1576

Fox R, Dimmic M (2006) A two-sample Bayesian t-test for microarray data. BMC Bioinformatics **7:** 126

Fu J, Liu H, Li Y, Yu H, Li X, Xiao J, Wang S (2011) Manipulating Broad-Spectrum Disease Resistance by Suppressing Pathogen-Induced Auxin Accumulation in Rice. Plant Physiology **155:** 589-602

Fujikake H, Yashima H, Tanabata S, Ishikawa S, Sato4 T, Toshikazu, Nishiwaki , Ohtake N, Sueyoshi K, Ishii S, Fujimaki S (2011) Effect of Nitrate on Nodulation and Nitrogen Fixation of Soybean. *In* HA El-Shemy, ed, Soybean Physiology and Biochemistry. InTech p488

Fujita K, Horiuchi H, Takato H, Kohno M, Suzuki S (2012) Auxin-responsive grape *Aux/IAA9* regulates transgenic *Arabidopsis* plant growth. Molecular Biology Reports **39:** 7823-7829

Gallou A, Declerck S, Cranenbrouck S (2012) Transcriptional regulation of defence genes and involvement of the WRKY transcription factor in arbuscular mycorrhizal potato root colonization. Functional & Integrative Genomics **12:** 183-198

Gao P, Bai X, Yang L, Lv D, Pan X, Li Y, Cai H, Ji W, Chen Q, Zhu Y (2011) osa-MIR393: a salinity- and alkaline stress-related microRNA gene. Molecular Biology Reports **38:** 237-242

Gentili F, Huss-Danell K (2002) Phosphorus modifies the effects of nitrogen on nodulation in split-root systems of Hippophaë rhamnoides. New Phytologist **153:** 53-61

Gentili F, Wall L, G., Huss-Danell K (2006) Effects of Phosphorus and Nitrogen on Nodulation are Seen Already at the Stage of Early Cortical Cell Divisions in Alnus incana. Annals of Botany **98:** 309-315

Gentleman R (2005) Bioinformatics and Computational Biology Solutions using R and Bioconductor.

Gilmartin PM, Memelink J, Hiratsuka K, Kay SA, Chua NH (1992) Characterization of a gene encoding a DNA binding protein with specificity for a light-responsive element. The Plant Cell Online **4:** 839-849

Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection roots. New Phytologist **84:** 489-500

Gobbato E, Marsh John F, Vernié T, Wang E, Maillet F, Kim J, Miller JB, Sun J, Bano SA, Ratet P, Mysore Kirankumar S, Dénarié J, Schultze M, Oldroyd Giles ED (2012) A GRAS-Type Transcription Factor with a Specific Function in Mycorrhizal Signaling. Current biology : CB **22:** 2236-2241

Godiard L, Lepage A, Moreau S, Laporte D, Verdenaud M, Timmers T, Gamas P (2011) *MtbHLH1*, a bHLH transcription factor involved in *Medicago truncatula* nodule vascular patterning and nodule to plant metabolic exchanges. New Phytologist **191:** 391-404

Goldsbrough AP, Albrecht H, Stratford R (1993) Salicylic acid-inducible binding of a tobacco nuclear protein to a 10 bp sequence which is highly conserved amongst stress-inducible genes. The Plant Journal **3:** 563-571

Gonzalez-Rizzo S, Crespi M, Frugier F (2006) The *Medicago truncatula CRE1* Cytokinin Receptor Regulates Lateral Root Development and Early Symbiotic Interaction with *Sinorhizobium meliloti*. Plant Cell **18:** 2680-2693

Gordon AJ, Ryle GJA, Mitchell DF, Powell DCE (1985) The Flux of 14C-Labelled Photosynthate through Soyabean Root Nodules during N_2 Fixation. Journal of Experimental Botany **36:** 756-769

Gough C, Jacquet C (2013) Nod factor perception protein carries weight in biotic interactions. Trends in Plant Science

Govindarajulu M, Kim S-Y, Libault M, Berg RH, Tanaka K, Stacey G, Taylor CG (2009) GS52 Ecto-Apyrase Plays a Critical Role during Soybean Nodulation. Plant Physiology **149:** 994-1004

Grant M, Brown I, Adams S, Knight M, Ainslie A, Mansfield J (2000) The RPM1 plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. The Plant Journal **23:** 441-450

Grec S, Vanham D, De Ribaucourt JC, Purnelle B, Boutry M (2003) Identification of regulatory sequence elements within the transcription promoter region of *NpABC1*, a gene encoding a plant ABC transporter induced by diterpenes. The Plant Journal **35:** 237-250

Green P, Kay S, A. , Chua N-H (1987) Sequence-specific interactions of a pea nuclear factor with lightresponsive elements upstream of the *rbcS-3A* gene. The EMBO Journal **6** 2543-2549

Grønlund M, Gustafsen C, Roussis A, Jensen D, Nielsen L, Marcker K, Jensen E (2003) The *Lotus japonicus ndx* gene family is involved in nodule function and maintenance. Plant Molecular Biology **52:** 303-316

Groth M, Takeda N, Perry J, Uchida H, Dräxl S, Brachmann A, Sato S, Tabata S, Kawaguchi M, Wang TL, Parniske M (2010) *NENA*, a *Lotus japonicus* Homolog of *Sec13*, Is Required for Rhizodermal Infection by Arbuscular Mycorrhiza Fungi and Rhizobia but Dispensable for Cortical Endosymbiotic Development. The Plant Cell Online **22:** 2509-2526

Gu M, Xu K, Chen A, Zhu Y, Tang G, Xu G (2010) Expression analysis suggests potential roles of microRNAs for phosphate and arbuscular mycorrhizal signaling in *Solanum lycopersicum*. Physiologia Plantarum **138:** 226-237

Habte M, Fox RL, Huang RS (1987) Determining vesicular arbuscular mycorrhizal effectiveness by monitoring P status of subleaflets of an indicator plant. Communications in Soil Science and Plant Analysis **18:** 1403-1420

Hansen J, Jørgensen J-E, Stougaard J, Marcker K (1989) Hairy roots — a short cut to transgenic root nodules. Plant Cell Reports **8:** 12-15

Hanson WC (1950) The photometric determination of phosphorus in fertilizers using the phosphovanado-molybdate complex. Journal of the Science of Food and Agriculture **1:** 172-173

Harrison MJ (2005) Signaling in the arbuscular mycorrhizal symbiosis. Annual Review of Microbiology **59:** 19-42

Harrison MJ, Dewbre GR, Liu J (2002) A Phosphate Transporter from Medicago truncatula Involved in the Acquisition of Phosphate Released by Arbuscular Mycorrhizal Fungi. The Plant Cell Online **14:** 2413-2429

Hartman GL, Gardner ME, Hymowitz T, Naidoo GC (2000) Evaluation of perennial Glycine species for Resistance to soybean fungal pathogens that cause sclerotinia stem rot and sudden death syndrome Crop Sci. **40:** 545–549

Hayashi S, Reid DE, Lorenc MT, Stiller J, Edwards D, Gresshoff PM, Ferguson BJ (2012) Transient Nod factor-dependent gene expression in the nodulation-competent zone of soybean (*Glycine max* [L.] Merr.) roots. Plant Biotechnology Journal **10:** 995-1010

Hayashi T, Banba M, Shimoda Y, Kouchi H, Hayashi M, Imaizumi-Anraku H (2010) A dominant function of *CCaMK* in intracellular accommodation of bacterial and fungal endosymbionts. The Plant Journal **63:** 141-154

Heckmann AB, Lombardo F, Miwa H, Perry JA, Bunnewell S, Parniske M, Wang TL, Downie JA (2006) *Lotus japonicus* Nodulation Requires Two GRAS Domain Regulators, One of Which Is Functionally Conserved in a Non-Legume. Plant Physiol. **142:** 1739-1750

Hedden P (1999) Recent advances in gibberellin biosynthesis. Journal of Experimental Botany **50:** 553-563

Hedden P, Phillips AL (2000) Gibberellin metabolism: new insights revealed by the genes. Trends in Plant Science **5:** 523-530

Hedden P, Thomas SG (2012) Gibberellin biosynthesis and its regulation. J Biochemical Journal **444:** 11-25

Helliwell CA, Chandler PM, Poole A, Dennis ES, Peacock WJ (2001) The *CYP88A* cytochrome P450, ent-kaurenoic acid oxidase, catalyzes three steps of the gibberellin biosynthesis pathway. Proceedings of the National Academy of Sciences **98:** 2065-2070

Henzi MX, Christey MC, McNeil DL (2000) Factors that influence *Agrobacterium rhizogenes*-mediated transformation of broccoli (*Brassica oleracea* L. var. *italica*). Plant Cell Reports **19:** 994-999

Hirsch S, Kim J, Muñoz A, Heckmann AB, Downie JA, Oldroyd GED (2009) GRAS Proteins Form a DNA Binding Complex to Induce Gene Expression during Nodulation Signaling in *Medicago truncatula*. The Plant Cell Online **21:** 545-557

Hong G-J, Xue X-Y, Mao Y-B, Wang L-J, Chen X-Y (2012) *Arabidopsis* MYC2 Interacts with DELLA Proteins in Regulating Sesquiterpene Synthase Gene Expression. The Plant Cell Online **24:** 2635-2648

Horváth B, Yeun LH, Domonkos Á, Halász G, Gobbato E, Ayaydin F, Miró K, Hirsch S, Sun J, Tadege M, Ratet P, Mysore KS, Ané J-M, Oldroyd GED, Kaló P (2011) *Medicago truncatula IPD3* Is a Member of the Common Symbiotic Signaling Pathway Required for Rhizobial and Mycorrhizal Symbioses. Molecular Plant-Microbe Interactions **24:** 1345-1358

Hsieh L-C, Lin S-I, Shih AC-C, Chen J-W, Lin W-Y, Tseng C-Y, Li W-H, Chiou T-J (2009) Uncovering Small RNA-Mediated Responses to Phosphate Deficiency in Arabidopsis by Deep Sequencing. Plant Physiology **151:** 2120-2132

Imaizumi-Anraku H, Takeda N, Charpentier M, Perry J, Miwa H, Umehara Y, Kouchi H, Murakami Y, Mulder L, Vickers K, Pike J, Allan Downie J, Wang T, Sato S, Asamizu E, Tabata S, Yoshikawa M, Murooka Y, Wu G-J, Kawaguchi M, Kawasaki S, Parniske M, Hayashi M (2005) Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots. Nature **433:** 527-531

Imsande J (1986) Inhibition of Nodule Development in Soybean by Nitrate or Reduced Nitrogen. Journal of Experimental Botany **37:** 348-355

Israel DW (1987) Investigation of the Role of Phosphorus in Symbiotic Dinitrogen Fixation. Plant Physiology **84:** 835-840

Ito H, Gray WM (2006) A Gain-of-Function Mutation in the *Arabidopsis* Pleiotropic Drug Resistance Transporter *PDR9* Confers Resistance to Auxinic Herbicides. Plant Physiology **142:** 63-74

Itzhaki H, Maxson JM, Woodson WR (1994) An ethylene-responsive enhancer element is involved in the senescence-related expression of the carnation glutathione-S-transferase (*GST1*) gene. Proceedings of the National Academy of Sciences **91:** 8925-8929

Jang S-W, Hamayun M, Sohn E-Y, Shin D-H, Kim K-U, Lee B-H, Lee I-J (2008) Effect of elevated nitrogen levels on endogenous gibberellin and jasmonic acid contents of three rice (*Oryza sativa* L.) cultivars. Journal of Plant Nutrition and Soil Science **171:** 181-186

Jensen JS, Marcker KA, Otten L, Schell J (1986) Nodule-specific expression of a chimaeric soybean leghaemoglobin gene in transgenic *Lotus corniculatus*. Nature **321:** 669-674

Jitacksorn S, Sadowsky MJ (2008) Nodulation Gene Regulation and Quorum Sensing Control Density-Dependent Suppression and Restriction of Nodulation in the *Bradyrhizobium japonicum*-Soybean Symbiosis. Appl. Environ. Microbiol. **74:** 3749-3756

Jones-Rhoades MW, Bartel DP (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Molecular cell **14:** 787-799

Kaiser BN, Finnegan PM, Tyerman SD, Whitehead LF, Bergersen FJ, Day DA, Udvardi MK (1998) Characterization of an Ammonium Transport Protein from the Peribacteroid Membrane of Soybean Nodules. Science **281:** 1202-1206

Kalendar R, Lee D, Schulman AH (2011) Java web tools for PCR, in silico PCR, and oligonucleotide assembly and analysis. Genomics **98:** 137-144

Kalo P, Gleason C, Edwards A, Marsh J, Mitra RM, Hirsch S, Jakab J, Sims S, Long SR, Rogers J, Kiss GB, Downie JA, Oldroyd GED (2005) Nodulation Signaling in Legumes Requires *NSP2*, a Member of the GRAS Family of Transcriptional Regulators. Science **308:** 1786-1789

Kanamori N, Madsen LH, Radutoiu S, Frantescu M, Quistgaard EMH, Miwa H, Downie JA, James EK, Felle HH, Haaning LL, Jensen TH, Sato S, Nakamura Y, Tabata S, Sandal N, Stougaard J (2006) From The Cover: A nucleoporin is required for induction of Ca^{2+} spiking in legume nodule development and essential for rhizobial and fungal symbiosis. PNAS **103:** 359-364

Karimi M, Inzé D, Depicker A (2002) GATEWAY™ vectors for *Agrobacterium*-mediated plant transformation. Trends in Plant Science **7:** 193-195

Kawaguchi M, Imaizumi-Anraku H, Fukai S, Syono K (1996) Unusual Branching in the Seedlings of Lotus japonicus—Gibberellins Reveal the Nitrogen-sensitive Cell Divisions within the Pericycle on Roots. Plant and Cell Physiology **37:** 461-470

Kereszt A, Li D, Indrasumunar A, Nguyen CDT, Nontachaiyapoom S, Kinkema M, Gresshoff PM (2007) *Agrobacterium rhizogenes*-mediated transformation of soybean to study root biology. Nat. Protocols **2:** 948-952

Kirsch C, Logemann E, Lippok B, Schmelzer E, Hahlbrock K (2001) A highly specific pathogen-responsive promoter element from the immediate-early activated *CMPG1* gene in Petroselinum crispum. The Plant Journal **26:** 217-227

Kobayashi K, Awai K, Takamiya K-i, Ohta H (2004) Arabidopsis Type B Monogalactosyldiacylglycerol Synthase Genes Are Expressed during Pollen Tube Growth and Induced by Phosphate Starvation. Plant Physiology **134:** 640-648

Kobayashi K, Nakamura Y, Ohta H (2009) Type A and type B monogalactosyldiacylglycerol synthases are spatially and functionally separated in the plastids of higher plants. Plant Physiology and Biochemistry **47:** 518-525

Kohl DH, Lin J-J, Shearer G, Schubert KR (1990) Activities of the pentose phosphate pathway and enzymes of proline metabolism in legume root nodules. Plant physiology **94:** 1258-1264

Kojima S, Bohner A, Gassert B, Yuan L, Wirén Nv (2007) *AtDUR3* represents the major transporter for high-affinity urea transport across the plasma membrane of nitrogen-deficient Arabidopsis roots. The Plant Journal **52:** 30-40

Konishi M, Yanagisawa S (2011) The Regulatory Region Controlling the Nitrate-Responsive Expression of a Nitrate Reductase Gene, *NIA1*, in *Arabidopsis*. Plant and Cell Physiology **52:** 824-836

Kouas S, Louche F, Debez A, Plassard C, Drevon JJ, Abdelly C (2009) Effect of phosphorus deficiency on acid phosphatase and phytase activities in common bean (*Phaseolus vulgaris* L.) under symbiotic nitrogen fixation. Symbiosis **3:** 141-149

Kretzschmar T, Kohlen W, Sasse J, Borghi L, Schlegel M, Bachelier JB, Reinhardt D, Bours R, Bouwmeester HJ, Martinoia E (2012) A petunia ABC protein controls strigolactone-dependent symbiotic signalling and branching. Nature **483:** 341-344

Krusell L, Madsen LH, Sato S, Aubert G, Genua A, Szczyglowski K, Duc G, Kaneko T, Tabata S, de Bruijn F, Pajuelo E, Sandal N, Stougaard J (2002) Shoot control of root development and nodulation is mediated by a receptor-like kinase. Nature **420:** 422

Kusaba S, Kano-Murakami Y, Matsuoka M, Tamaoki M, Sakamoto T, Yamaguchi I, Fukumoto M (1998) Alteration of hormone levels in transgenic tobacco plants overexpressing the rice homeobox gene *OSH1*. Plant Physiol **116:** 471-476

Lam E, Chua NH (1989) *ASF-2*: a factor that binds to the cauliflower mosaic virus 35S promoter and a conserved GATA motif in Cab promoters. The Plant Cell Online **1:** 1147-1156

Langlois-Meurinne M, Gachon CMM, Saindrenan P (2005) Pathogen-Responsive Expression of Glycosyltransferase Genes *UGT73B3* and *UGT73B5* Is Necessary for Resistance to *Pseudomonas syringae* pv *tomato* in *Arabidopsis*. Plant Physiology **139:** 1890-1901

Lee Y, Kende H (2002) Expression of α-Expansin and Expansin-Like Genes in Deepwater Rice. Plant Physiology **130:** 1396-1405

Lerouge P, Roche P, Faucher C, Maillet F, Truchet G, Prome JC, Denarie J (1990) Symbiotic host-specificity of Rhizobium meliloti is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature **344:** 781-784

Lescot M, Dehais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, Rouze P, Rombauts S (2002) PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res **30:** 325-327

Lescot M, Déhais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, Rouzé P, Rombauts S (2002) PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for *in silico* analysis of promoter sequences. Nucleic Acids Research **30:** 325-327

Li H, Dong Y, Yin H, Wang N, Yang J, Liu X, Wang Y, Wu J, Li X (2011) Characterization of the stress associated microRNAs in *Glycine max* by deep sequencing. BMC Plant Biology **11:** 170

Li H, Sun J, Xu Y, Jiang H, Wu X, Li C (2007) The bHLH-type transcription factor *AtAIB* positively regulates ABA response in Arabidopsis. Plant Molecular Biology **65:** 655-665

Li M, Xu W, Yang W, Kong Z, Xue Y (2007) Genome-Wide Gene Expression Profiling Reveals Conserved and Novel Molecular Functions of the Stigma in Rice. Plant Physiology **144:** 1797-1812

Libault M, Thibivilliers S, Bilgin DD, Radwan O, Benitez M, Clough SJ, Stacey G (2008) Identification of Four Soybean Reference Genes for Gene Expression Normalization. The Plant Genome **1:** 44-54

Libault M, Thibivilliers S, Bilgin DD, Radwan O, Benitez M, Clough SJ, Stacey G (2008) Identification of Four Soybean Reference Genes for Gene Expression Normalization. Plant Gen. **1:** 44-54

Lievens S, Goormachtig S, Den Herder J, Capoen W, Mathis R, Hedden P, Holsters M (2005) Gibberellins Are Involved in Nodulation of Sesbania rostrata. Plant Physiology **139:** 1366-1379

Lim CW, Lee YW, Hwang CH (2011) Soybean Nodule-Enhanced CLE Peptides in Roots Act as Signals in GmNARK-Mediated Nodulation Suppression. Plant and Cell Physiology **52:** 1613-1627

Lim WK, Wang K, Lefebvre C, Califano A (2007) Comparative analysis of microarray normalization procedures: effects on reverse engineering gene networks. Bioinformatics **23:** i282-i288

Limpens E, Franken C, Smit P, Willemse J, Bisseling T, Geurts R (2003) LysM Domain Receptor Kinases Regulating Rhizobial Nod Factor-Induced Infection. Science **302:** 630-633

Limpens E, Moling S, Hooiveld G, Pereira PA, Bisseling T, Becker JD, Küster H (2013) Cell- and Tissue-Specific Transcriptome Analyses of *Medicago truncatula* Root Nodules. PLoS ONE **8:** e64377

Lin P-C, Pomeranz MC, Jikumaru Y, Kang SG, Hah C, Fujioka S, Kamiya Y, Jang J-C (2011) The *Arabidopsis* tandem zinc finger protein *AtTZF1* affects ABA- and GA-mediated growth, stress and gene expression responses. The Plant Journal **65:** 253-268

Liu J-Q, Allan DL, Vance CP (2010) Systemic Signaling and Local Sensing of Phosphate in Common Bean: Cross-Talk between Photosynthate and MicroRNA399. Molecular Plant **3:** 428-437

Liu X, Zhang H, Zhao Y, Feng Z, Li Q, Yang H-Q, Luan S, Li J, He Z-H (2013) Auxin controls seed dormancy through stimulation of abscisic acid signaling by inducing ARF-mediated *ABI3* activation in *Arabidopsis*. Proceedings of the National Academy of Sciences **110:** 15485-15490

Livak KJ, Schmittgen TD (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods **25:** 402-408

Lohar DP, Schaff JE, Laskey JG, Kieber JJ, Bilyeu KD, Bird DM (2004) Cytokinins play opposite roles in lateral root formation, and nematode and Rhizobial symbioses. The Plant Journal **38:** 203-214

Lois R, Dietrich A, Hahlbrock K, Schulz W (1989) A phenylalanine ammonia-lyase gene from parsley: structure, regulation and identification of elicitor and light responsive cis-acting elements. The EMBO Journal **8:** 1641-1648

Loughlin PC (2007) Elucidation of a peibacteroid membrane-bound bHLH transcription factor required for legume nitrogen fixation. *In*, Ed November 2007, Adelaide, pp 4-20- 24-21

Lu B-R (2004) Conserving biodiversity of soybean gene pool in the biotechnology era. Plant Species Biology **19:** 115-125

Madsen EB, Madsen LH, Radutoiu S, Olbryt M, Rakwalska M, Szczyglowski K, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J (2003) A receptor kinase gene of the LysM type is involved in legumeperception of rhizobial signals. Nature **425:** 637-640

Maekawa T, Maekawa-Yoshikawa M, Takeda N, Imaizumi-Anraku H, Murooka Y, Hayashi M (2009) Gibberellin controls the nodulation signaling pathway in *Lotus japonicus*. The Plant Journal **58:** 183-194

Magome H, Nomura T, Hanada A, Takeda-Kamiya N, Ohnishi T, Shinma Y, Katsumata T, Kawaide H, Kamiya Y, Yamaguchi S (2013) *CYP714B1* and *CYP714B2* encode gibberellin 13-oxidases that reduce gibberellin activity in rice. Proceedings of the National Academy of Sciences **110:** 1947-1952

Maillet F, Poinsot V, Andre O, Puech-Pages V, Haouy A, Gueunier M, Cromer L, Giraudet D, Formey D, Niebel A, Martinez EA, Driguez H, Becard G, Denarie J (2011) Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature **469:** 58-63

Managbanag JR, Witten TM, Bonchev D, Fox LA, Tsuchiya M, Kennedy BK, Kaeberlein M (2008) Shortest-Path Network Analysis Is a Useful Approach toward Identifying Genetic Determinants of Longevity. PLoS ONE **3:** e3802

Manjunath A, Hue NV, Habte M (1989) Response ofLeucaena leucocephala to vesicular-arbuscular mycorrhizal colonization and rock phosphate fertilization in an Oxisol. Plant and Soil **114:** 127-133

Maoyin L, Ruth W, Xuemin W (2006) Quantitative profiling of Arabidopsis polar glycerolipids in response to phosphorus starvation. Roles of phospholipases D zeta1 and D zeta2 in phosphatidylcholine hydrolysis and digalactosyldiacylglycerol accumulation in phosphorus-starved plants. Plant Physiol **142:** 750-761

Marcker A, Lund M, Jensen EØ, Marcker KA (1984) Transcription of the soybean leghemoglobin genes during nodule development. EMBO J. **3:** 1691–1695

Marini A-M, Springael J-Y, Frommer WB, André B (2000) Cross-talk between ammonium transporters in yeast and interference by the soybean SAT1 protein. Molecular Microbiology **35:** 378-385

Marsh BAB (1971) Measurement of Length in Random Arrangements of Lines. Journal of Applied Ecology **8:** 265-267

Marsh JF, Rakocevic A, Mitra RM, Brocard L, Sun J, Eschstruth A, Long SR, Schultze M, Ratet P, Oldroyd GED (2007) *Medicago truncatula NIN* Is Essential for Rhizobial-Independent Nodule Organogenesis Induced by Autoactive Calcium/Calmodulin-Dependent Protein Kinase. Plant Physiol. **144:** 324-335

Martin D, Proebsting W, Parks TD, Dougherty W, Lange T, Lewis M, Gaskin P, Hedden P (1996) Feed-back regulation of gibberellin biosynthesis and gene expression in *Pisum sativum* L. Planta **200:** 159-166

Martínez-García M, Garcidueñas-Piña C, Guzmán P (1996) Gene isolation inArabidopsis thaliana by conditional overexpression of cDNAs toxic toSaccharomyces cerevisiae: identification of a novel early response zinc-finger gene. Molecular and General Genetics MGG **252:** 587-596

Mathesius U (2008) Auxin: at the root of nodule development? Functional Plant Biology **35:** 651-668

Mathesius U, Schlaman HRM, Spaink HP, Of Sautter C, Rolfe BG, Djordjevic MA (1998) Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoids and derivatives of chitin oligosaccharides. The Plant Journal **14:** 23-34

Mathur M, Satpathy M, Sachar RC (1992) Phytohormonal regulation of S-adenosylmethionine synthetase by gibberellic acid in wheat aleurones. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1137:** 338-348

Mathur M, Sharma N, Sachar RC (1993) Differential regulation of S-adenosylmethionine synthetase isozymes by gibberellic acid in dwarf pea epicotyls. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology **1162:** 283-290

Matsushima R, Fukao Y, Nishimura M, Hara-Nishimura I (2004) *NAI1* Gene Encodes a Basic-Helix-Loop-Helix-Type Putative Transcription Factor That Regulates the Formation of an Endoplasmic Reticulum-Derived Structure, the ER Body. Plant Cell **16:** 1536-1549

Matsushima R, Hayashi Y, Kondo M, Shimada T, Nishimura M, Hara-Nishimura I (2002) An Endoplasmic Reticulum-Derived Structure That Is Induced under Stress Conditions in *Arabidopsis*. Plant Physiol. **130:** 1807-1814

Mattanovich D, Rüker F, da Cämara Machado A, Laimer M, Regner F, Steinkeliner H, Himmler G, Katinger H (1989) Efficient transformation of *Agrobacterium* spp. by eletroporation. Nucleic Acids Research **17:** 6747

Matton D, Prescott G, Bertrand C, Camirand A, Brisson N (1993) Identification of cis-acting elements involved in the regulation of the pathogenesis-related gene *STH-2* in potato. Plant Molecular Biology **22:** 279-291

May A, Berger S, Hertel T, Köck M (2011) The *Arabidopsis thaliana* phosphate starvation responsive gene *AtPPsPase1* encodes a novel type of inorganic pyrophosphatase. Biochimica et Biophysica Acta (BBA) - General Subjects **1810:** 178-185

Mazurkiewicz D (2008) Functional Analysis of the (bHLH) Transcription Factor *GmSAT1* from the Peribacteroid Membrane of Glycine max

Mazurkiewicz D (2013) Characterisation of a novel family of eukaryotic ammonium transporter proteins. University of Adelaide

McClure PR, Israel DW (1979) Transport of Nitrogen in the Xylem of Soybean Plants. Plant Physiology **64:** 411-416

Messinese E, Mun J-H, Yeun LH, Jayaraman D, Rouge P, Barre A, Lougnon G, Schornack S, Bono J-J, Cook DR, Ane J-M (2007) A Novel Nuclear Protein Interacts With the Symbiotic *DMI3* Calcium- and Calmodulin-Dependent Protein Kinase of *Medicago truncatula*. Molecular Plant-Microbe Interactions **20:** 912-921

Meurer CA, Dinkins RD, Collins GB (1998) Factors affecting soybean cotyledonary node transformation. Plant Cell Reports **18:** 180-186

Middleton PH, Jakab J, Penmetsa RV, Starker CG, Doll J, Kaló P, Prabhu R, Marsh JF, Mitra RM, Kereszt A, Dudas B, VandenBosch K, Long SR, Cook DR, Kiss GB, Oldroyd GED (2007) An *ERF* Transcription Factor in *Medicago truncatula* That Is Essential for Nod Factor Signal Transduction. The Plant Cell Online **19:** 1221-1234

Mitra RM, Gleason CA, Edwards A, Hadfield J, Downie JA, Oldroyd GED, Long SR (2004) A Ca^{2+}/calmodulin-dependent protein kinase required for symbiotic nodule development: Gene identification by transcript-based cloning. Proceedings of the National Academy of Sciences of the United States of America **101:** 4701-4705

Montanini B, Levati E, Bolchi A, Kohler A, Morin E, Tisserant E, Martin F, Ottonello S (2011) Genome-wide search and functional identification of transcription factors in the mycorrhizal fungus Tuber melanosporum. New Phytologist **189:** 736-750

Morrone D, Chen X, Coates RM, Peters RJ (2010) Characterization of the kaurene oxidase *CYP701A3*, a multifunctional cytochrome P450 from gibberellin biosynthesis

Biochemical Journal **431:** 337-344

Mudd SH (1962) Activation of Methionine for Transmethylation: V. The mechanism of action of the methionine-activating enzyme. Journal of Biological Chemistry **237:** PC1372-PC1375

Mudge SR, Rae AL, Diatloff E, Smith FW (2002) Expression analysis suggests novel roles for members of the *Pht1* family of phosphate transporters in *Arabidopsis*. The Plant Journal **31:** 341-353

Murray JD (2011) Invasion by Invitation: Rhizobial Infection in Legumes. Molecular Plant-Microbe Interactions **24:** 631-639

Murray JD, Karas BJ, Sato S, Tabata S, Amyot L, Szczyglowski K (2007) A Cytokinin Perception Mutant Colonized by *Rhizobium* in the Absence of Nodule Organogenesis. Science **315:** 101-104

Naudin C, Corre-Hellou G, Voisin A-S, Oury V, Salon C, Crozat Y, Jeuffroy M-H (2011) Inhibition and recovery of symbiotic N_2 fixation by peas (*Pisum sativum* L.) in response to short-term nitrate exposure. Plant and Soil **346:** 275-287

Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, Voinnet O, Jones JDG (2006) A Plant miRNA Contributes to Antibacterial Resistance by Repressing Auxin Signaling. Science **312:** 436-439

Nikitin A, Egorov S, Daraselia N, Mazo I (2003) Pathway studio—the analysis and navigation of molecular networks. Bioinformatics **19:** 2155-2157

Nomura T, Magome H, Hanada A, Takeda-Kamiya N, Mander LN, Kamiya Y, Yamaguchi S (2013) Functional Analysis of Arabidopsis *CYP714A1* and *CYP714A2* Reveals That They are Distinct Gibberellin Modification Enzymes. Plant and Cell Physiology **54:** 1837-1851

Ohashi-Ito K, Bergmann DC (2007) Regulation of the *Arabidopsis* root vascular initial population by *LONESOME HIGHWAY*. Development **134:** 2959-2968

Ohashi-Ito K, Oguchi M, Kojima M, Sakakibara H, Fukuda H (2013) Auxin-associated initiation of vascular cell differentiation by *LONESOME HIGHWAY*. Development **140:** 765-769

Okazaki Y, Otsuki H, Narisawa T, Kobayashi M, Sawai S, Kamide Y, Kusano M, Aoki T, Hirai MY, Saito K (2013) A new class of plant lipid is essential for protection against phosphorus depletion. Nat Commun **4:** 1510

Oldroyd GED (2013) Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. Nat Rev Micro **11:** 252-263

Oldroyd GED, Downie JA (2004) Calcium, kinases and nodulation signalling in legumes. Nat Rev Mol Cell Biol **5:** 566-576

Oldroyd GED, Downie JA (2008) Coordinating Nodule Morphogenesis with Rhizobial Infection in Legumes. Annual Review of Plant Biology **59:** 519-546

Oldroyd GED, Engstrom EM, Long SR (2001) Ethylene Inhibits the Nod Factor Signal Transduction Pathway of *Medicago truncatula*. The Plant Cell Online **13:** 1835-1849

Oldroyd GED, Murray JD, Poole PS, Downie JA (2011) The Rules of Engagement in the Legume-Rhizobial Symbiosis. Annual Review of Genetics **45:** 119-144

Oldroyd GED, Sharon RL (2003) Identification and Characterization of Nodulation-Signaling Pathway 2, a Gene of *Medicago truncatula* Involved in Nod Factor Signaling. Plant Physiology **131:** 1027-1032

Olhoft P, Bernal L, Grist L, Hill D, Mankin S, Shen Y, Kalogerakis M, Wiley H, Toren E, Song H-S, Hillebrand H, Jones T (2007) A novel *Agrobacterium rhizogenes*-mediated transformation method of soybean [*Glycine max* (L.) *Merrill*] using primary-node explants from seedlings. In Vitro Cellular & Developmental Biology - Plant **43:** 536-549

Olhoft P, Flagel L, Donovan C, Somers D (2003) Efficient soybean transformation using hygromycin B selection in the cotyledonary-node method. Planta **216:** 723-735

Op den Camp R, Streng A, De Mita S, Cao Q, Polone E, Liu W, Ammiraju JSS, Kudrna D, Wing R, Untergasser A, Bisseling T, Geurts R (2011) LysM-Type Mycorrhizal Receptor Recruited for Rhizobium Symbiosis in Nonlegume Parasponia. Science **331:** 909-912

Osnato M, Castillejo C, MatÃas-HernÃ¡ndez L, Pelaz S (2012) *TEMPRANILLO* genes link photoperiod and gibberellin pathways to control flowering in *Arabidopsis*. Nat Commun **3:** 808

Pacios-Bras C, Schlaman HM, Boot K, Admiraal P, Mateos Langerak J, Stougaard J, Spaink H (2003) Auxin distribution in *Lotus japonicus* during root nodule development. Plant Molecular Biology **52:** 1169-1180

Pan Y, Michael TP, Hudson ME, Kay SA, Chory J, Schuler MA (2009) Cytochrome P450 Monooxygenases as Reporters for Circadian-Regulated Pathways. Plant Physiology **150:** 858-878

Parniske M (2008) Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat Rev Micro **6:** 763-775

Parry G, Calderon-Villalobos LI, Prigge M, Peret B, Dharmasiri S, Itoh H, Lechner E, Gray WM, Bennett M, Estelle M (2009) Complex regulation of the *TIR1/AFB* family of auxin receptors. Proceedings of the National Academy of Sciences **106:** 22540-22545

Pastuglia M, Roby D, Dumas C, Cock JM (1997) Rapid induction by wounding and bacterial infection of an S gene family receptor-like kinase gene in *Brassica oleracea*. The Plant Cell Online **9:** 49-60

Penmetsa RV, Cook DR (1997) A Legume Ethylene-Insensitive Mutant Hyperinfected by Its Rhizobial Symbiont. Science **275:** 527-530

Penmetsa RV, Uribe P, Anderson J, Lichtenzveig J, Gish J-C, Nam YW, Engstrom E, Xu K, Sckisel G, Pereira M, Baek JM, Lopez-Meyer M, Long SR, Harrison MJ, Singh KB, Kiss GB, Cook DR (2008) The *Medicago truncatula* ortholog of *Arabidopsis EIN2*, sickle, is a negative regulator of symbiotic and pathogenic microbial associations. The Plant Journal **55:** 580-595

Phillips A, Huttly A (1994) Cloning of two gibberellin-regulated cDNAs from *Arabidopsis thaliana* by subtractive hybridization: expression of the tonoplast water channel, γ-TIP, is increased by GA3. Plant Molecular Biology **24:** 603-615

Pii Y, Crimi M, Cremonese G, Spena A, Pandolfini T (2007) Auxin and nitric oxide control indeterminate nodule formation. BMC Plant Biology **7:** 1-11

Plet J, Wasson A, Ariel F, Le Signor C, Baker D, Mathesius U, Crespi M, Frugier F (2011) MtCRE1-dependent cytokinin signaling integrates bacterial and plant cues to coordinate symbiotic nodule organogenesis in *Medicago truncatula*. The Plant Journal **65:** 622-633

Pueppke SG (1988) Nodulating associations among rhizobia and legumes of the genus Glycine subgenus Glycine. Plant and Soil **109:** 189-193

Quandt HJ, Puehler A, Broer I (1993) Transgenic root nodules of Vicia hirsuta: a fast and efficient system for the study of gene expression in indeterminate-type nodules. MPMI-Molecular Plant Microbe Interactions **6:** 699-706

Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y, Gronlund M, Sato S, Nakamura Y, Tabata S, Sandal N, Stougaard J (2003) Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. Nature **425:** 585-592

Rech EL, Golds TJ, Hammatt N, Mulligan BJ, Davey MR (1988) *Agrobacterium rhizogenes* mediated transformation of the wild soybeans *Glycine canescens* and *G. clandestina*: Production of transgenic plants of *G. canescens*. J. Exp. Bot. **39:** 1275-1285

Reid DE, Hayashi S, Lorenc M, Stiller J, Edwards D, Gresshoff PM, Ferguson BJ (2012) Identification of systemic responses in soybean nodulation by xylem sap feeding and complete transcriptome sequencing reveal a novel component of the autoregulation pathway. Plant Biotechnology Journal **10:** 680-689

Roberts NJ, Morieri G, Kalsi G, Rose A, Stiller J, Edwards A, Xie F, Gresshoff PM, Oldroyd GED, Downie JA, Etzler ME (2013) Rhizobial and Mycorrhizal Symbioses in *Lotus japonicus* Require Lectin Nucleotide Phosphohydrolase, Which Acts Upstream of Calcium Signaling. Plant Physiology **161:** 556-567

Rouster J, Leah R, Mundy J, Cameron-Mills V (1997) Identification of a methyl jasmonate-responsive region in the promoter of a lipoxygenase 1 gene expressed in barley grain. The Plant Journal **11:** 513-523

Rushton P, Torres J, Parniske M, Wernert P, Hahlbrock K, Somssich I (1996) Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley PR1 genes. The EMBO Journal **15** 5690-5700

Růžička K, Strader LC, Bailly A, Yang H, Blakeslee J, Łangowski Ł, Nejedlá E, Fujita H, Itoh H, Syōno K, Hejátko J, Gray WM, Martinoia E, Geisler M, Bartel B, Murphy AS, Friml J (2010) *Arabidopsis PIS1* encodes the ABCG37 transporter of auxinic compounds including the auxin precursor indole-3-butyric acid. Proceedings of the National Academy of Sciences **107:** 10749-10753

Ryu H, Cho H, Choi D, Hwang I (2012) Plant hormonal regulation of nitrogen-fixing nodule organogenesis. Molecules and Cells **34:** 117-126

Saito K, Yoshikawa M, Yano K, Miwa H, Uchida H, Asamizu E, Sato S, Tabata S, Imaizumi-Anraku H, Umehara Y, Kouchi H, Murooka Y, Szczyglowski K, Downie JA, Parniske M, Hayashi M, Kawaguchi M (2007) *NUCLEOPORIN85* Is Required for Calcium Spiking, Fungal and Bacterial Symbioses, and Seed Production in *Lotus japonicus*. The Plant Cell Online **19:** 610-624

Sakai T, Takahashi Y, Nagata T (1996) Analysis of the Promoter of the Auxin-Inducible Gene, parC, of Tobacco. Plant and Cell Physiology **37:** 906-913

Sakamoto T, Kamiya N, Ueguchi-Tanaka M, Iwahori S, Matsuoka M (2001) KNOX homeodomain protein directly suppresses the expression of a gibberellin biosynthetic gene in the tobacco shoot apical meristem. Genes Dev **15:** 581-590

Sakamoto T, Morinaka Y, Ishiyama K, Kobayashi M, Itoh H, Kayano T, Iwahori S, Matsuoka M, Tanaka H (2003) Genetic manipulation of gibberellin metabolism in transgenic rice. Nat Biotech **21:** 909-913

Samac DA, Tesfaye M, Dornbusch M, Saruul P, Temple SJ (2004) A comparison of constitutive promoters for expression of transgenes in alfalfa (Medicago sativa). Transgenic research **13:** 349-361

Sandal N, Krusell L, Radutoiu S, Olbryt M, Pedrosa A, Stracke S, Sato S, Kato T, Tabata S, Parniske M, Bachmair A, Ketelsen T, Stougaard J (2002) A Genetic Linkage Map of the Model Legume *Lotus japonicus* and Strategies for Fast Mapping of New Loci. Genetics **161:** 1673-1683

Sasabe M, Toyoda K, Shiraishi T, Inagaki Y, Ichinose Y (2002) cDNA cloning and characterization of tobacco ABC transporter: *NtPDR1* is a novel elicitor-responsive gene. FEBS Letters **518:** 164-168

Sasaki A, Ashikari M, Ueguchi-Tanaka M, Itoh H, Nishimura A, Swapan D, Ishiyama K, Saito T, Kobayashi M, Khush GS, Kitano H, Matsuoka M (2002) Green revolution: A mutant gibberellin-synthesis gene in rice. Nature **416:** 701-702

Sato T, Onoma N, Fujikake H, Ohtake N, Sueyoshi K, Ohyama T (2001) Changes in four leghemoglobin components in nodules of hypernodulating soybean (*Glycine max* [L] *Merr.*) mutant and its parent in the early nodule developmental stage. Plant and Soil **237:** 129-135

Schaarschmidt S, Gresshoff P, Hause B (2013) Analyzing the soybean transcriptome during autoregulation of mycorrhization identifies the transcription factors *GmNF-YA1a/b* as positive regulators of arbuscular mycorrhization. Genome Biology **14:** R62

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. Nat. Protocols **3:** 1101-1108

Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T, Nelson W, Hyten DL, Song Q, Thelen JJ, Cheng J, Xu D, Hellsten U, May GD, Yu Y, Sakurai T, Umezawa T, Bhattacharyya MK, Sandhu D, Valliyodan B, Lindquist E, Peto M, Grant D, Shu S, Goodstein D, Barry K, Futrell-Griggs M, Abernathy B, Du J, Tian Z, Zhu L, Gill N, Joshi T, Libault M, Sethuraman A, Zhang X-C, Shinozaki K, Nguyen HT, Wing RA, Cregan P, Specht J, Grimwood J, Rokhsar D, Stacey G, Shoemaker RC, Jackson SA (2010) Genome sequence of the palaeopolyploid soybean. Nature **463:** 178-183

Schneider G, Jensen E, Spray CR, Phinney BO (1992) Hydrolysis and reconjugation of gibberellin A20 glucosyl ester by seedlings of *Zea mays* L. Proc Natl Acad Sci U S A **89:** 8045-8048

Schubert KR (1981) Enzymes of purine biosynthesis and catabolism in Glycine max I. Comparison of activities with N2 fixation and composition of xylem exudate during nodule development. Plant physiology **68:** 1115-1122

Schultze M, Kondorosi A (1998) Regulation of symbiotic root nodule development. Annual Review of Genetics **32:** 33-57

Searle IR, Men AE, Laniya TS, Buzas DM, Iturbe-Ormaetxe I, Carroll BJ, Gresshoff PM (2003) Long-Distance Signaling in Nodulation Directed by a CLAVATA1-Like Receptor Kinase. Science **299:** 109-112

Secco D, Wang C, Arpat BA, Wang Z, Poirier Y, Tyerman SD, Wu P, Shou H, Whelan J (2012) The emerging importance of the SPX domain-containing proteins in phosphate homeostasis. New Phytologist **193:** 842-851

Sehnke PC, Laughner BJ, Linebarger CRL, Gurley WB, Ferl RJ (2005) Identification and characterization of *GIP1*, an *Arabidopsis thaliana* protein that enhances the DNA binding affinity and reduces the oligomeric state of G-box binding factors. Cell Res **15:** 567-575

Seo M, Hanada A, Kuwahara A, Endo A, Okamoto M, Yamauchi Y, North H, Marion-Poll A, Sun TP, Koshiba T, Kamiya Y, Yamaguchi S, Nambara E (2006) Regulation of hormone metabolism in *Arabidopsis* seeds: phytochrome regulation of abscisic acid metabolism and abscisic acid regulation of gibberellin metabolism. Plant J **48:** 354-366

Singh S, Parniske M (2012) Activation of calcium- and calmodulin-dependent protein kinase (*CCaMK*), the central regulator of plant root endosymbiosis. Current Opinion in Plant Biology **15:** 444-453

Sinharoy S, Saha S, Chaudhury SR, DasGupta M (2009) Transformed Hairy Roots of Arachis hypogea: A Tool for Studying Root Nodule Symbiosis in a Non–Infection Thread Legume of the Aeschynomeneae Tribe. Molecular Plant-Microbe Interactions **22:** 132-142

Smit P, Raedts J, Portyanko V, Debelle F, Gough C, Bisseling T, Geurts R (2005) NSP1 of the GRAS Protein Family Is Essential for Rhizobial Nod Factor-Induced Transcription. Science **308:** 1789-1791

Smith SE, Smith FA (2011) Roles of Arbuscular Mycorrhizas in Plant Nutrition and Growth: New Paradigms from Cellular to Ecosystem Scales. Annual Review of Plant Biology **62:** 227-250

Somers DA, Samac DA, Olhoft PM (2003) Recent advances in legume transformation. Plant physiology **131:** 892-899

Soyano T, Kouchi H, Hirota A, Hayashi M (2013) NODULE INCEPTION Directly Targets *NF-Y* Subunit Genes to Regulate Essential Processes of Root Nodule Development in *Lotus japonicus*. PLoS Genet **9:** e1003352

Stacey G, McAlvin CB, Kim S-Y, Olivares J, Soto MJ (2006) Effects of Endogenous Salicylic Acid on Nodulation in the Model Legumes *Lotus japonicus* and *Medicago truncatula*. Plant Physiology **141:** 1473-1481

Stenzel I, Ziethe K, Schurath J, Hertel SC, Bosse D, Köck M (2003) Differential expression of the *LePS2* phosphatase gene family in response to phosphate availability, pathogen infection and during development. Physiologia Plantarum **118:** 138-146

Stiller J, Martirani L, Tuppale S, Chian R-J, Chiurazzi M, Gresshoff PM (1997) High frequency transformation and regeneration of transgenic plants in the model legume Lotus japonicus. Journal of Experimental Botany **48:** 1357-1365

Stracke S, Kistner C, Yoshida S, Mulder L, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J, Szczyglowski K, Parniske M (2002) A plant receptor-like kinase required for both bacterial and fungal symbiosis. Nature **417:** 959(954)

Strader LC, Monroe-Augustus M, Rogers KC, Lin GL, Bartel B (2008) Arabidopsis iba response5 Suppressors Separate Responses to Various Hormones. Genetics **180:** 2019-2031

Stukkens Y, Bultreys A, Grec S, Trombik T, Vanham D, Boutry M (2005) *NpPDR1*, a Pleiotropic Drug Resistance-Type ATP-Binding Cassette Transporter from *Nicotiana plumbaginifolia*, Plays a Major Role in Plant Pathogen Defense. Plant Physiology **139:** 341-352

Su CH, Shih CH, Chang TH, Tsai HK (2010) Genome-wide analysis of the cis-regulatory modules of divergent gene pairs in yeast. Genomics **96:** 352-361

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP (2005) Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. Proceedings of the National Academy of Sciences of the United States of America **102:** 15545-15550

Subramanian S, Fu Y, Sunkar R, Barbazuk WB, Zhu J-K, Yu O (2008) Novel and nodulation-regulated microRNAs in soybean roots. BMC Genomics **9:** 160

Sugiyama A, Shitan N, Yazaki K (2007) Involvement of a Soybean ATP-Binding Cassette-Type Transporter in the Secretion of Genistein, a Signal Flavonoid in Legume-Rhizobium Symbiosis. Plant Physiology **144:** 2000-2008

Sugiyama A, Shitan N, Yazaki K (2008) Signaling from soybean roots to *Rhizobium*: An ATP-binding cassette-type transporter mediates genistein secretion. Plant Signaling & Behavior **3:** 38-40

Sun J, Cardoza V, Mitchell DM, Bright L, Oldroyd G, Harris JM (2006) Crosstalk between jasmonic acid, ethylene and Nod factor signaling allows integration of diverse inputs for regulation of nodulation. The Plant Journal **46:** 961-970

Sun T-p, Kamiya Y (1997) Regulation and cellular localization of ent-kaurene synthesis. Physiologia Plantarum **101:** 701-708

Sun TP, Kamiya Y (1994) The *Arabidopsis GA1* locus encodes the cyclase ent-kaurene synthetase A of gibberellin biosynthesis. Plant Cell **6:** 1509-1518

Suzaki T, Ito M, Kawaguchi M (2013) Induction of localized auxin response during spontaneous nodule development in *Lotus japonicus*. Plant Signaling & Behavior **8:** e23359

Suzaki T, Yano K, Ito M, Umehara Y, Suganuma N, Kawaguchi M (2012) Positive and negative regulation of cortical cell division during root nodule development in *Lotus japonicus* is accompanied by auxin response. Development **139:** 3997-4006

Suzuki A, Akune M, Kogiso M, Imagama Y, Osuki K-i, Uchiumi T, Higashi S, Han S-Y, Yoshida S, Asami T, Abe M (2004) Control of Nodule Number by the Phytohormone Abscisic Acid in the Roots of Two Leguminous Species. Plant and Cell Physiology **45:** 914-922

Takanashi K, Sugiyama A, Yazaki K (2011) Involvement of auxin distribution in root nodule development of *Lotus japonicus*. Planta **234:** 73-81

Takeda N, Maekawa T, Hayashi M (2012) Nuclear-Localized and Deregulated Calcium- and Calmodulin-Dependent Protein Kinase Activates Rhizobial and Mycorrhizal Responses in *Lotus japonicus*. The Plant Cell Online **24:** 810-822

Tanaka-Ueguchi M, Itoh H, Oyama N, Koshioka M, Matsuoka M (1998) Over-expression of a tobacco homeobox gene, *NTH15*, decreases the expression of a gibberellin biosynthetic gene encoding GA 20-oxidase. Plant J **15:** 391-400

Tanaka K, Nguyen CT, Libault M, Cheng J, Stacey G (2011) Enzymatic Activity of the Soybean Ecto-Apyrase *GS52* Is Essential for Stimulation of Nodulation. Plant Physiology **155:** 1988-1998

Thomas SG, Phillips AL, Hedden P (1999) Molecular cloning and functional expression of gibberellin 2- oxidases, multifunctional enzymes involved in gibberellin deactivation. Proc Natl Acad Sci U S A **96:** 4698-4703

Tirichine L, Imaizumi-Anraku H, Yoshida S, Murakami Y, Madsen LH, Miwa H, Nakagawa T, Sandal N, Albrektsen AS, Kawaguchi M, Downie A, Sato S, Tabata S, Kouchi H, Parniske M, Kawasaki S, Stougaard J (2006) Deregulation of a Ca^{2+}/calmodulin-dependent kinase leads to spontaneous nodule development. Nature **441:** 1153-1156

Torres MA, Jones JDG, Dangl JL (2006) Reactive Oxygen Species Signaling in Response to Pathogens. Plant Physiology **141:** 373-378

Toyomasu T, Kawaide H, Sekimoto H, von Numers C, Phillips AL, Hedden P, Kamiya Y (1997) Cloning and characterization of a cDNA encoding gibberellin 20-oxidase from rice (*Oryza sativa*) seedlings. Physiologia Plantarum **99:** 111-118

Trindade I, Capitão C, Dalmay T, Fevereiro M, Santos Dd (2010) miR398 and miR408 are up-regulated in response to water deficit in *Medicago truncatula*. Planta **231:** 705-716

Tully R, van Berkum P, Lovins K, Keister D (1998) Identification and sequencing of a cytochrome P450 gene cluster from *Bradyrhizobium japonicum*. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression **1398:** 243-255

Turner M, Nizampatnam NR, Baron M, Coppin S, Damodaran S, Adhikari S, Arunachalam S, Yu O, Subramanian S (2013) Ectopic expression of miR160 results in auxin hypersensitivity, cytokinin hyposensitivity, and inhibition of symbiotic nodule development in soybean. Plant Physiology

Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K (1993) An *Arabidopsis* myb homolog is induced by dehydration stress and its gene product binds to the conserved *MYB* recognition sequence. The Plant Cell Online **5:** 1529-1539

Van de Velde W, Mergeay J, Holsters M, Goormachtig S (2003) Agrobacterium rhizogenes-mediated transformation of Sesbania rostrata. Plant Science **165:** 1281-1288

Varbanova M, Porter K, Lu F, Ralph J, Hammerschmidt R, Jones AD, Day B (2011) Molecular and Biochemical Basis for Stress-Induced Accumulation of Free and Bound p-Coumaraldehyde in Cucumber. Plant Physiology **157:** 1056-1066

Venable JH, Coggeshall R (1965) A simplified lead citrate stain for use in electron microscopy. The Journal of Cell Biology **25:** 407-408

Vernié T, Moreau S, de Billy F, Plet J, Combier J-P, Rogers C, Oldroyd G, Frugier F, Niebel A, Gamas P (2008) *EFD* Is an *ERF* Transcription Factor Involved in the Control of Nodule Number and Differentiation in *Medicago truncatula*. The Plant Cell Online **20:** 2696-2713

Vidal EA, Araus V, Lu C, Parry G, Green PJ, Coruzzi GM, Gutiérrez RA (2010) Nitrate-responsive miR393/*AFB3* regulatory module controls root system architecture in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences **107:** 4477-4482

Vidal EA, Moyano TC, Riveras E, Contreras-López O, Gutiérrez RA (2013) Systems approaches map regulatory networks downstream of the auxin receptor *AFB3* in the nitrate response of *Arabidopsis thaliana* roots. Proceedings of the National Academy of Sciences **110:** 12840-12845

Vierheilig H, Coughlan AP, Wyss U, Piché Y (1998) Ink and Vinegar, a Simple Staining Technique for Arbuscular-Mycorrhizal Fungi. Applied and Environmental Microbiology **64:** 5004-5007

Wais RJ, Galera C, Oldroyd G, Catoira R, Penmetsa RV, Cook D, Gough C, Denarie J, Long SR (2000) Genetic analysis of calcium spiking responses in nodulation mutants of *Medicago truncatula*. Proceedings of the National Academy of Sciences of the United States of America **97:** 13407-13412

Wang R, Xing X, Wang Y, Tran A, Crawford NM (2009) A Genetic Screen for Nitrate Regulatory Mutants Captures the Nitrate Transporter Gene *NRT1.1*. Plant Physiology **151:** 472-478

Wang Y, Zhang C, Hao Q, Sha A, Zhou R, Zhou X, Yuan L (2013) Elucidation of miRNAs-Mediated Responses to Low Nitrogen Stress by Deep Sequencing of Two Soybean Genotypes. PLoS ONE **8:** e67423

Wasson AP, Pellerone FI, Mathesius U (2006) Silencing the Flavonoid Pathway in *Medicago truncatula* Inhibits Root Nodule Formation and Prevents Auxin Transport Regulation by Rhizobia. Plant Cell **18:** 1617-1629

Wieczorek K, Hofmann J, Blöchl A, Szakasits D, Bohlmann H, Grundler FMW (2008) Arabidopsis endo-1,4-β-glucanases are involved in the formation of root syncytia induced by Heterodera schachtii. The Plant Journal **53:** 336-351

Williams ME, Foster R, Chua NH (1992) Sequences flanking the hexameric G-box core CACGTG affect the specificity of protein binding. The Plant Cell Online **4:** 485-496

Windels D, Vazquez F (2011) miR393: Integrator of environmental cues in auxin signaling? Plant Signaling & Behavior **6:** 1672-1675

Xu F, Liu Q, Chen L, Kuang J, Walk T, Wang J, Liao H (2013) Genome-wide identification of soybean microRNAs and their targets reveals their organ-specificity and responses to phosphate starvation. BMC Genomics **14:** 66

Yamaguchi-Shinozaki K, Mundy J, Chua N-H (1990) Four tightly linked rab genes are differentially expressed in rice. Plant Molecular Biology **14:** 29-39

Yamaguchi S (2008) Gibberellin metabolism and its regulation. Annu Rev Plant Biol **59:** 225-251

Yamaguchi S, Kamiya Y (2000) Gibberellin Biosynthesis: Its Regulation by Endogenous and Environmental Signals. Plant and Cell Physiology **41:** 251-257

Yano K, Yoshida S, Muller J, Singh S, Banba M, Vickers K, Markmann K, White C, Schuller B, Sato S, Asamizu E, Tabata S, Murooka Y, Perry J, Wang TL, Kawaguchi M, Imaizumi-Anraku H, Hayashi M, Parniske M (2008) CYCLOPS, a mediator of symbiotic intracellular accommodation. Proceedings of the National Academy of Sciences **105:** 20540-20545

Yokota K, Soyano T, Kouchi H, Hayashi M (2010) Function of GRAS Proteins in Root Nodule Symbiosis is Retained in Homologs of a Non-Legume, Rice. Plant and Cell Physiology **51:** 1436-1442

Zanetti ME, Blanco FA, Beker MP, Battaglia M, Aguilar OM (2010) A C Subunit of the Plant Nuclear Factor *NF-Y* Required for Rhizobial Infection and Nodule Development Affects Partner Selection in the Common Bean–*Rhizobium etli Symbiosis*. The Plant Cell Online **22:** 4142-4157

Zarcinas BA, Cartwright B (1983) Analysis of soil and plant material by inductively coupled plasma-optical emission spectrometry. CSIRO, Melbourne

Zarcinas BA, Cartwright B, Spouncer LR (1987) Nitric acid digestion and multi-element analysis of plant material by inductively coupled plasma spectrometry. Communications in Soil Science and Plant Analysis **18:** 131-146

Zhang W, Wang C, Qin C, Wood T, Olafsdottir G, Welti R, Wang X (2003) The Oleate-Stimulated Phospholipase D, *PLDδ*, and Phosphatidic Acid Decrease H_2O_2-Induced Cell Death in *Arabidopsis*. The Plant Cell Online **15:** 2285-2295

Zhang Y, Ni Z, Yao Y, Nie X, Sun Q (2007) Gibberellins and heterosis of plant height in wheat (*Triticum aestivum* L.). BMC Genetics **8:** 40

Zhang Y, Zhang B, Yan D, Dong W, Yang W, Li Q, Zeng L, Wang J, Wang L, Hicks LM, He Z (2011) Two *Arabidopsis* cytochrome P450 monooxygenases, *CYP714A1* and *CYP714A2*, function redundantly in plant development through gibberellin deactivation. The Plant Journal **67:** 342-353

Zhao M, Ding H, Zhu J-K, Zhang F, Li W-X (2011) Involvement of miR169 in the nitrogen-starvation responses in Arabidopsis. New Phytologist **190:** 906-915

Zhao X, Yu X, Foo E, Symons GM, Lopez J, Bendehakkalu KT, Xiang J, Weller JL, Liu X, Reid JB, Lin C (2007) A Study of Gibberellin Homeostasis and Cryptochrome-Mediated Blue Light Inhibition of Hypocotyl Elongation. Plant Physiology **145:** 106-118

Zhu X, Lei G, Jiang T, Liu Y, Li G, Zheng S (2012) Cell wall polysaccharides are involved in P-deficiency-induced Cd exclusion in *Arabidopsis thaliana*. Planta **236:** 989-997

9. Appendixes

Appendix 1 List of down-regulated genes in *sat1* nodules

Appendix 2 List of up-regulated genes in *sat1* nodules

Appendix 3 List of down-regulated genes in *sat1* roots

Appendix 4 List of up-regulated genes in *sat1* roots

Appendix 1

Down-regulated genes in *sat1* nodules. The data is ordered based on the mean fold-change differences between *sat1* and empty vector control nodules. A negative fold-change value indicates a down regulation of the gene. Those genes identified as down regulated were found to be statistically significant using a Bayesian t-test ($p < 0.05$, n=3-4).

Gene ID	*Gene Name*	*Description*	*Best Arabidopsis Match*	*Fold change*	*P-value*
Glyma01g00980	NRPC2	nuclear RNA polymerase C2	AT5G45140	-7.03	1.9E-05
Glyma18g38670	ELI3-2	elicitor-activated gene 3-2	AT4G37990	-5.34	1.6E-12
Glyma05g21680	BRU6	Auxin-responsive GH3 family protein	AT2G14960	-4.75	2.2E-04
Glyma13g31690	scpl31	serine carboxypeptidase-like 31	AT1G11080	-4.75	7.2E-08
Glyma19g32860		Unknown		-4.66	1.6E-04
Glyma13g01140	TCH4	Xyloglucan endotransglucosylase/hydrolase family protein	AT5G57560	-4.62	3.4E-06
Glyma13g10790	ZIP1	zinc transporter 1 precursor	AT3G12750	-4.11	6.9E-04
Glyma03g29440	SIP2	seed imbibition 2	AT3G57520	-3.66	1.8E-04
Glyma15g06680		basic helix-loop-helix (bHLH) DNA-binding superfamily	AT4G37850	-3.56	5.8E-07
Glyma15g02110		Unknown		-3.47	2.9E-04
Glyma11g05510	GH3.1	Auxin-responsive GH3 family protein	AT2G14960	-3.46	1.3E-06
Glyma10g44170	HPT1	homogentisate phytyltransferase 1	AT2G18950	-3.43	2.4E-05
Glyma03g24490		Unknown		-3.35	8.4E-06
Glyma17g06560		Unknown	AT5G57123	-3.25	1.9E-06
Glyma17g10050		Gibberellin-regulated family protein	AT5G14920	-3.21	2.3E-06
Glyma17g18040	BRU6	Auxin-responsive GH3 family protein	AT4G37390	-3.2	1.3E-03
Glyma13g21410		Unknown		-3.09	1.0E-03
Glyma19g01120		Oxidoreductase, zinc-binding dehydrogenase family	AT1G23740	-3.04	2.3E-05
Glyma10g02210	SAG21	senescence-associated gene 21	AT4G02380	-3.04	5.8E-03
Glyma19g01150		Oxidoreductase, zinc-binding dehydrogenase family	AT1G23740	-3	1.2E-03
Glyma20g30980	GI	gigantea protein (GI)	AT1G22770	-2.99	3.3E-04
Glyma03g08020		Unknown		-2.98	7.2E-03
Glyma11g31530		RNA-binding KH domain-containing protein	AT3G08620	-2.95	1.8E-08
Glyma06g44120		Unknown		-2.88	2.0E-04
Glyma16g16800		Unknown		-2.88	3.3E-05
Glyma04g40640	PRR5	pseudo-response regulator 5	AT5G24470	-2.86	1.4E-07
Glyma16g28570		disease resistance family protein / LRR family protein	AT2G34930	-2.82	2.3E-04
Glyma01g42640	AT-	winged-helix DNA-binding transcription factor family	AT4G11660	-2.8	5.7E-04
Glyma19g01200	FDH	formate dehydrogenase	AT5G14780	-2.73	7.0E-04
Glyma10g20390		Unknown		-2.73	3.8E-03
Glyma17g14680	BETA-	beta vacuolar processing enzyme	AT1G62710	-2.72	8.0E-05
Glyma01g14740		Translation elongation factor EFG/EF2 protein	AT2G45030	-2.65	1.6E-04
Glyma04g04270		HXXXD-type acyl-transferase family protein	AT5G07850	-2.62	4.2E-03
Glyma17g03860		Unknown		-2.62	1.1E-02
Glyma05g18360		Unknown		-2.61	8.9E-03
Glyma08g14070		beta-1,4-N-acetylglucosaminyltransferase family protein	AT1G12990	-2.58	3.7E-05
Glyma16g25080		disease resistance protein (TIR-NBS-LRR class), putative	AT5G17680	-2.56	7.7E-03
Glyma17g05920	NF-YA8	nuclear factor Y, subunit A8	AT1G17590	-2.53	4.0E-03
Glyma14g27020		Unknown		-2.5	1.9E-07
Glyma08g26570		Unknown		-2.5	3.7E-04
Glyma03g11610		NAD(P)-linked oxidoreductase superfamily protein	AT2G37790	-2.47	6.0E-05
Glyma05g09130	GLCAK	glucuronokinase G	AT3G01640	-2.46	2.9E-03
Glyma19g01980		ABC transporter family protein	AT3G28345	-2.45	4.5E-04
Glyma01g28790		Unknown		-2.44	3.2E-04
Glyma09g29240		Unknown		-2.44	4.2E-04
Glyma10g14830		Unknown		-2.44	1.5E-04
Glyma15g33040		Unknown		-2.44	4.9E-04
Glyma16g32100		Unknown		-2.44	4.2E-04
Glyma14g35810		sequence-specific DNA binding transcription	AT5G64340	-2.44	2.4E-03
Glyma14g39560		HSP20-like chaperones superfamily protein	AT5G37670	-2.43	1.8E-03
Glyma06g35580		Tyrosine transaminase family protein	AT5G53970	-2.42	4.2E-03
Glyma05g15860		Unknown		-2.4	1.7E-05

Gene ID	*Gene Name*	*Description*	*Best Arabidopsis Match*	*Fold change*	*P-value*
Glyma06g05280	BCAT-2	branched-chain amino acid transaminase 2	AT1G10070	-2.4	6.2E-03
Glyma12g05080		Unknown	AT5G66580	-2.39	1.5E-06
Glyma04g02220		ACT-like protein tyrosine kinase family protein	AT4G38470	-2.39	4.5E-03
Glyma06g40740		Disease resistance protein (TIR-NBS-LRR class) family	AT4G12010	-2.38	8.6E-04
Glyma06g07160		Unknown	AT3G11760	-2.37	2.6E-05
Glyma01g42030	PME1	pectin methylesterase inhibitor 1	AT4G12390	-2.34	1.3E-02
Glyma17g26660		Unknown		-2.33	9.0E-06
Glyma18g05710		Leucine-rich repeat protein kinase family protein	AT1G06840	-2.31	5.4E-05
Glyma18g44390		Unknown		-2.3	2.1E-03
Glyma11g05030	HHP4	heptahelical protein 4	AT4G37680	-2.29	2.2E-04
Glyma13g25270	TPS04	terpene synthase 04	AT1G61120	-2.28	3.4E-02
Glyma08g04150		Unknown		-2.28	1.8E-03
Glyma20g11250		Unknown		-2.25	1.9E-04
Glyma14g38800	ATM3	ABC transporter of the mitochondrion 3	AT5G58270	-2.24	3.4E-03
Glyma04g11230		tetratricopeptide repeat (TPR)-containing protein	AT5G65160	-2.24	1.5E-06
Glyma18g46610		Acyl-CoA thioesterase family protein	AT1G01710	-2.23	1.0E-02
Glyma06g10700	EXO	Phosphate-responsive 1 family protein	AT4G08950	-2.23	2.1E-02
Glyma06g13280	GLT1	NADH-dependent glutamate synthase 1	AT5G53460	-2.22	7.2E-03
Glyma12g07860	PRR7	pseudo-response regulator 7	AT5G02810	-2.22	1.7E-03
Glyma08g27810		Unknown		-2.21	3.7E-04
Glyma13g32240		Unknown		-2.21	1.1E-05
Glyma02g04770	SERAT3;2	serine acetyltransferase 3;2	AT4G35640	-2.2	4.2E-02
Glyma09g04750	ATL2	TOXICOS EN LEVADURA 2	AT3G16720	-2.2	3.1E-02
Glyma17g04360	PDR9	pleiotropic drug resistance 9	AT3G53480	-2.2	6.2E-04
Glyma11g04220		Unknown		-2.19	1.0E-02
Glyma17g17840		Unknown		-2.19	2.6E-03
Glyma06g45180	ULT1	Developmental regulator, ULTRAPETALA	AT4G28190	-2.17	4.0E-03
Glyma17g11590		Leucine-rich repeat family protein	AT1G15740	-2.17	5.3E-03
Glyma16g24940		disease resistance protein (TIR-NBS-LRR class), putative	AT5G36930	-2.15	7.9E-04
Glyma15g14000	FAH1	fatty acid hydroxylase 1	AT2G34770	-2.15	6.9E-04
Glyma03g39100		Adenine nucleotide alpha hydrolases-like superfamily	AT3G62550	-2.13	2.9E-04
Glyma07g08500	RPS2	NB-ARC domain-containing disease resistance protein	AT4G26090	-2.12	1.9E-02
Glyma01g23460		Unknown		-2.11	6.9E-05
Glyma12g17020		Unknown		-2.11	2.6E-05
Glyma01g05550	SBH2	sphingoid base hydroxylase 2	AT1G69640	-2.11	6.8E-03
Glyma13g01130	XTR6	xyloglucan endotransglycosylase 6	AT5G57560	-2.11	3.5E-02
Glyma17g11370		RING U-box superfamily protein	AT2G15580	-2.11	5.7E-03
Glyma08g07580	WBC11	white-brown complex homolog protein 11	AT1G17840	-2.11	1.5E-02
Glyma04g04750	LBD39	LOB domain-containing protein 39	AT5G67420	-2.11	1.0E-02
Glyma12g30240	FRU	FER-like regulator of iron uptake	AT2G28160	-2.1	2.4E-02
Glyma07g31200	scpl31	serine carboxypeptidase-like 31	AT1G11080	-2.1	8.0E-03
Glyma03g11580		NAD(P)-linked oxidoreductase superfamily protein	AT2G37790	-2.09	8.7E-04
Glyma06g45020		Unknown		-2.09	1.7E-02
Glyma20g24510		F1F0-ATPase inhibitor protein, putative	AT5G04750	-2.08	2.0E-02
Glyma19g35270	PDR12	pleiotropic drug resistance 12	AT1G15520	-2.08	2.7E-07
Glyma06g30920		Unknown		-2.08	4.1E-03
Glyma15g19380		Unknown		-2.07	5.2E-03
Glyma18g44250	PYD4	PYRIMIDINE 4	AT3G08860	-2.06	4.5E-02
Glyma11g07270		Unknown		-2.06	3.2E-04
Glyma20g17440		uricase / urate oxidase	AT2G26230	-2.04	1.3E-05
Glyma18g42430		Unknown		-2.03	1.5E-02
Glyma18g42290		RING U-box superfamily protein	AT5G01160	-2.03	1.9E-03
Glyma15g07600	scpl31	serine carboxypeptidase-like 31	AT1G11080	-2.01	1.6E-03
Glyma18g14100		Unknown		-2	3.2E-03
Glyma09g30390	NUDT4	nudix hydrolase homolog 4	AT1G18300	-2	3.0E-02
Glyma06g05140		Unknown		-1.99	2.9E-02
Glyma12g12260		Unknown		-1.98	3.0E-02
Glyma19g38490		alpha/beta-Hydrolases superfamily protein	AT3G11620	-1.98	1.0E-04
Glyma06g45030		Unknown		-1.98	2.1E-02
Glyma02g12710		Unknown		-1.98	5.8E-03

Gene ID	*Gene Name*	*Description*	*Best Arabidopsis Match*	*Fold change*	*P-value*
Glyma18g17150		Unknown		-1.98	1.6E-02
Glyma10g43850	TT5	Chalcone-flavanone isomerase family protein	AT3G55120	-1.97	9.1E-03
Glyma11g22950		Unknown		-1.97	7.7E-03
Glyma11g12510		Unknown		-1.97	9.8E-05
Glyma14g38130		Unknown	AT2G40435	-1.96	8.5E-04
Glyma03g05270		Unknown		-1.96	1.6E-02
Glyma07g10140		DNAse I-like superfamily protein	AT1G73875	-1.96	3.7E-02
Glyma14g24140	ALDH3H1	aldehyde dehydrogenase 3H1	AT1G44170	-1.95	2.6E-02
Glyma20g28230	MSS1	Major facilitator superfamily protein	AT5G26340	-1.95	6.6E-04
Glyma18g52250		NAD(P)-linked oxidoreductase superfamily protein	AT1G59950	-1.95	2.6E-02
Glyma03g28650		Calcium-binding EF-hand family protein	AT2G41410	-1.95	3.8E-03
Glyma12g36510		NB-ARC domain-containing disease resistance protein	AT4G27220	-1.95	3.1E-02
Glyma11g36240		Unknown		-1.94	3.9E-04
Glyma16g32540	AGL6	AGAMOUS-like 6	AT2G45650	-1.94	8.8E-03
Glyma15g08940		splicing factor, putative	AT5G64270	-1.93	1.3E-02
Glyma09g07240	GI	gigantea protein (GI)	AT1G22770	-1.93	6.7E-04

Appendix 2

Up-regulated genes in *sat1* nodules. The data is ordered based on the mean fold-change differences between *sat1* and empty vector control nodules. A positive fold-change value indicates an up-regulated gene. Those genes identified as up-regulated were found to be statistically significant using a Bayesian t-test ($p < 0.05$, n=3-4).

Gene ID	*Gene Name*	*Description*	*Best Arabidopsis Match*	*Fold change*	*P-value*
Glyma19g31480		Gibberellin-regulated family protein	AT1G74670	5.74	4.7E-05
Glyma02g38920		Histone superfamily protein	AT3G53730	4.83	2.1E-04
Glyma16g28840		Unknown		4.78	5.5E-05
Glyma17g14890		Bifunctional inhibitor lipid-transfer protein	AT1G62510	4.57	1.2E-04
Glyma20g31700	OMT1	O-methyltransferase 1	AT5G54160	3.87	2.3E-03
Glyma17g14850		Bifunctional inhibitor lipid-transfer protein	AT1G62510	3.76	1.6E-03
Glyma13g12190		Unknown		3.65	4.7E-03
Glyma07g04400	GER3	germin 3	AT5G20630	3.48	7.1E-04
Glyma03g34310	GAMMA-	gamma tonoplast intrinsic protein	AT2G36830	3.44	2.4E-05
Glyma04g39860		Peroxidase superfamily protein	AT5G05340	3.41	1.3E-03
Glyma01g37540	BGAL3	beta-galactosidase 3	AT4G36360	3.29	2.3E-03
Glyma17g14270	SDD1	Subtilase family protein	AT1G04110	3.19	4.1E-03
Glyma11g35030	PIP1;4	plasma membrane intrinsic protein 1;4	AT4G00430	3.17	2.1E-05
Glyma06g15030		Peroxidase superfamily protein	AT5G05340	3.11	4.4E-05
Glyma02g47770		Unknown		3.02	2.5E-06
Glyma13g37020		Unknown		3	1.7E-02
Glyma19g43010		Unknown		2.98	1.3E-04
Glyma14g09510		N-terminal nucleophile aminohydrolases (Ntn hydrolases)	AT3G16150	2.96	1.1E-02
Glyma18g03470	CEL2	cellulase 2	AT1G02800	2.87	2.9E-02
Glyma04g17600		Eukaryotic aspartyl protease family protein	AT5G07030	2.87	5.2E-03
Glyma10g40060	AGL62	AGAMOUS-like 62	AT5G60440	2.83	5.3E-03
Glyma10g01600		Cytochrome c oxidase subunit Vc family protein	AT2G47380	2.77	6.7E-05
Glyma18g50780	RGP1	reversibly glycosylated polypeptide 1	AT3G02230	2.77	7.2E-04
Glyma19g44060	ARA12	Subtilase family protein	AT5G67360	2.72	2.6E-03
Glyma05g04380		Bifunctional inhibitor lipid-transfer protein	AT1G62510	2.71	2.2E-03
Glyma14g38110		O-methyltransferase family protein	AT4G35160	2.7	1.6E-03
Glyma18g51250	bZIP42	basic leucine-zipper 42	AT3G30530	2.69	4.1E-03
Glyma18g06220		Peroxidase superfamily protein	AT5G05340	2.65	3.0E-02
Glyma17g03850		Unknown		2.64	3.8E-03
Glyma20g23520		GRAM domain-containing protein ABA-responsive	AT5G08350	2.59	1.8E-06
Glyma0973s00200		Unknown		2.59	5.2E-03
Glyma04g42120	ARPN	plantacyanin	AT2G02850	2.55	1.1E-02
Glyma07g37050	LPD1	lipoamide dehydrogenase 1	AT3G16950	2.54	5.1E-04
Glyma02g47880	FLA2	FASCICLIN-like arabinogalactan 2	AT4G12730	2.53	3.5E-03
Glyma20g39220		basic helix-loop-helix (bHLH) DNA-binding superfamily	AT1G05805	2.46	8.2E-07
Glyma01g16140	EXPB2	expansin B2	AT1G65680	2.45	4.6E-03
Glyma07g08710		TCP family transcription factor	AT2G45680	2.44	2.1E-04
Glyma08g23600		Eukaryotic aspartyl protease family protein	AT1G79720	2.43	2.9E-03
Glyma13g21290		Unknown		2.42	4.1E-03
Glyma12g08990	CSLA02	cellulose synthase-like A02	AT5G22740	2.42	1.3E-04
Glyma18g02610	SAG20	senescence associated gene 20	AT3G10985	2.41	1.1E-03
Glyma03g12150	GIP1	AtGCP3 interacting protein 1	AT4G09550	2.41	1.5E-02
Glyma10g34460	CYP76C4	cytochrome P450, family 76, subfamily C, polypeptide 4	AT2G45580	2.4	6.3E-04
Glyma16g26630	XTH5	xyloglucan endotransglucosylase hydrolase 5	AT5G13870	2.39	4.9E-05
Glyma10g30560		winged-helix DNA-binding transcription factor family	AT1G06760	2.39	6.6E-03
Glyma13g12160		Unknown		2.38	8.8E-03
Glyma15g22220	VPS54	VPS54	AT4G19490	2.34	4.1E-02
Glyma08g15280		Phosphoglycerate mutase family protein	AT1G22170	2.33	4.9E-02
Glyma04g33920	BNQ3	BANQUO 3	AT3G47710	2.33	1.2E-03
Glyma09g09780		Unknown		2.33	4.5E-02
Glyma20g27280	TUA4	tubulin alpha-4 chain	AT1G04820	2.31	7.1E-03
Glyma05g02890		Pectin lyase-like superfamily protein	AT4G24780	2.3	2.7E-02

Gene ID	*Gene Name*	*Description*	*Best Arabidopsis Match*	*Fold change*	*P-value*
Glyma04g20330	EBF1	EIN3-binding F box protein 1	AT2G25490	2.29	6.1E-03
Glyma15g06240		Unknown	AT5G09520	2.29	5.3E-03
Glyma18g05160	OXS3	oxidative stress 3	AT5G56550	2.29	4.7E-02
Glyma15g38440		Unknown		2.28	3.3E-03
Glyma08g19290		UDP-Glycosyltransferase superfamily protein	AT2G22590	2.28	7.5E-03
Glyma18g08180	UKL4	uridine kinase-like 4	AT4G26510	2.26	8.8E-03
Glyma13g12040		Unknown		2.24	3.9E-03
Glyma19g36200		Unknown	AT4G36920	2.24	3.1E-03
Glyma17g04330	MTO3	S-adenosylmethionine synthetase family protein	AT3G17390	2.22	8.0E-04
Glyma04g15590		nodulin MtN21 EamA-like transporter family protein	AT5G07050	2.21	4.0E-02
Glyma08g09670	GAD	glutamate decarboxylase	AT5G17330	2.2	1.2E-04
Glyma06g19400	CA2	carbonic anhydrase 2	AT5G14740	2.19	1.1E-04
Glyma08g16420	AAC2	ADP ATP carrier 2	AT3G08580	2.19	2.0E-03
Glyma20g24670		Pectin lyase-like superfamily protein	AT5G04310	2.18	7.1E-03
Glyma16g02970		Unknown		2.13	1.3E-03
Glyma04g11400	SAH7	Pollen Ole e 1 allergen and extensin family protein	AT4G08685	2.13	1.1E-02
Glyma17g14860		Bifunctional inhibitor lipid-transfer protein	AT1G62510	2.13	8.8E-04
Glyma16g01650	PME3	pectin methylesterase 3	AT3G14310	2.13	2.6E-02
Glyma13g01590	COX6B	cytochrome C oxidase 6B	AT1G22450	2.12	1.2E-02
Glyma08g03150		Mitochondrial outer membrane translocase complex,	AT5G41685	2.08	1.2E-02
Glyma06g42890	MBF1B	multiprotein bridging factor 1B	AT2G42680	2.08	1.2E-02
Glyma17g17970	AGP18	arabinogalactan protein 18	AT4G37450	2.08	4.9E-03
Glyma05g04390		Bifunctional inhibitor lipid-transfer protein	AT1G62510	2.08	3.3E-03
Glyma06g02650	TUB1	tubulin beta-1 chain	AT1G75780	2.08	4.3E-03
Glyma19g37000	GAMMA-	gamma tonoplast intrinsic protein	AT2G36830	2.07	1.0E-03
Glyma04g40530	RCI3	Peroxidase superfamily protein	AT1G05260	2.07	2.5E-02
Glyma05g03920		Protein of unknown function (DUF1442)	AT2G45360	2.06	1.8E-03
Glyma11g36220	SULTR2;1	slufate transporter 2;1	AT5G10180	2.06	4.4E-02
Glyma04g03760		Unknown		2.06	4.1E-04
Glyma07g33870	LEJ2	Cystathionine beta-synthase (CBS) family protein	AT4G36910	2.05	2.5E-02
Glyma10g32370	MPI7	CAMV movement protein interacting protein 7	AT1G04260	2.05	8.1E-03
Glyma14g40170	CAD9	cinnamyl alcohol dehydrogenase 9	AT4G39330	2.05	6.3E-03
Glyma14g38530		Unknown		2.03	2.6E-02
Glyma17g36650	GATL2	galacturonosyltransferase-like 2	AT1G19300	2.03	7.6E-03
Glyma10g03920		pfkB-like carbohydrate kinase family protein	AT1G06730	2.02	1.7E-03
Glyma18g51630		Unknown		2.02	2.3E-02
Glyma06g42850		Peroxidase superfamily protein	AT5G05340	2.01	2.4E-02
Glyma07g35620	EXPA15	expansin A15	AT1G26770	2.01	3.6E-02
Glyma16g04950	XTH5	xyloglucan endotransglucosylase hydrolase 5	AT5G13870	2.01	2.8E-03
Glyma02g38240	bHLH093	beta HLH protein 93	AT5G65640	2.01	7.7E-03
Glyma08g28220	bZIP42	basic leucine-zipper 42	AT3G30530	2.01	6.1E-03
Glyma15g20350		RING U-box superfamily prote	AT3G53690	2	3.6E-02

Appendix 3

Down-regulated genes in *sat1* roots. The data is ordered based on the mean fold-change differences between *sat1* and empty vector control roots. A negative fold-change value indicates a down regulation of the gene. Those genes identified as down regulated were found to be statistically significant using a Bayesian t-test ($p < 0.05$, n=3-4).

Gene ID	*Gene Name*	*Description*	*Best Arabidopsis Match*	*Fold change*	*P-value*
Glyma0466s00200		SGNH hydrolase-type esterase superfamily protein	AT3G11210	-18.59	3.5E-10
Glyma08g20810	pvHAD1	Pyridoxal phosphate phosphatase-related protein	AT1G17710	-10.02	2.4E-13
Glyma16g04950	XTH5	xyloglucan endotransglucosylase/hydrolase	AT5G13870	-7.74	5.0E-10
Glyma10g32020		O-methyltransferase family protein	AT4G35160	-7.62	4.9E-07
Glyma08g46520	CYP93D1	cytochrome P450, family 93, subfamily D, polypeptide 1	AT5G06900	-6.9	1.6E-09
Glyma02g30010	CYP93D1	cytochrome P450, family 93, subfamily D, polypeptide 1	AT5G06900	-6.89	1.7E-12
Glyma19g32990	ACT7	actin 7	AT5G09810	-6.49	1.2E-11
Glyma08g28580	TBL19	TRICHOME BIREFRINGENCE-LIKE 19	AT5G15900	-6.46	2.0E-10
Glyma06g48260	CSLG1	cellulose synthase like G1	AT4G24010	-6.31	4.9E-09
Glyma08g01680	HMA5	heavy metal atpase 5	AT1G63440	-6.15	1.6E-09
Glyma10g22070	CYP71B34	cytochrome P450, family 71, subfamily B, polypeptide 34	AT3G26300	-6.13	7.5E-10
Glyma06g02630		Pathogenesis-related thaumatin superfamily protein	AT1G20030	-5.75	1.2E-09
Glyma08g04090				-5.55	1.7E-11
Glyma11g20090	NRT2.4	nitrate transporter 2.4	AT5G60770	-5.4	2.8E-08
Glyma08g24720	MLP43	MLP-like protein 43	AT1G70890	-5.25	8.0E-08
Glyma06g08350				-5.24	8.3E-04
Glyma19g33950	GA1	Terpenoid cyclases/Protein prenyltransferases superfamily	AT4G02780	-5.2	8.8E-08
Glyma03g04880		Peroxidase superfamily protein	AT5G05340	-4.84	1.7E-06
Glyma01g42440	NRS/ER	nucleotide-rhamnose synthase/epimerase-reductase	AT1G63000	-4.8	6.1E-08
Glyma08g19290		UDP-Glycosyltransferase superfamily protein	AT2G22590	-4.63	2.7E-11
Glyma06g14450	ABCB19	ATP binding cassette subfamily B19	AT3G28860	-4.63	3.0E-07
Glyma20g00760		Exostosin family protein	AT1G68470	-4.59	6.0E-08
Glyma18g16710	TRX2	thioredoxin 2	AT5G39950	-4.49	1.8E-11
Glyma18g08130		unknown		-4.42	1.5E-05
Glyma15g36200		nodulin MtN21 /EamA-like transporter family protein	AT5G07050	-4.41	1.4E-08
Glyma01g41990	ATBETAF	Glycosyl hydrolases family 32 protein	AT1G12240	-4.38	1.8E-07
Glyma11g03000	EXPA7	expansin A7	AT1G12560	-4.34	2.4E-09
Glyma13g32310		Quinone reductase family protein	AT4G27270	-4.34	3.9E-13
Glyma01g44270	4CL3	4-coumarate:CoA ligase 3	AT1G65060	-4.27	1.0E-06
Glyma18g12660	RHM1	rhamnose biosynthesis 1	AT1G78570	-4.22	5.7E-09
Glyma17g03860		unknown		-4.21	9.5E-07
Glyma18g35220		2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase	AT5G59540	-4.2	1.3E-03
Glyma03g34760	CYP76G1	cytochrome P450, family 76, subfamily G, polypeptide 1	AT3G52970	-4.19	1.2E-07
Glyma05g04470		Bifunctional inhibitor/lipid-transfer protein	AT2G45180	-4.16	2.0E-05
Glyma10g43850	TT5	Chalcone-flavanone isomerase family protein	AT3G55120	-4.16	4.2E-07
Glyma11g02950	MVA1	hydroxymethylglutaryl-CoA synthase	AT4G11820	-4.13	5.4E-12
Glyma08g25950	CYP72A15	cytochrome P450, family 72, subfamily A, polypeptide 15	AT3G14690	-4.12	6.8E-10
Glyma10g22000	CYP71B34	cytochrome P450, family 71, subfamily B, polypeptide 34	AT3G26300	-4.06	7.7E-06
Glyma20g28350		Copper amine oxidase family protein	AT1G31690	-3.95	1.1E-07
Glyma01g07120	UPS2	ureide permease 2	AT2G03530	-3.9	2.5E-04
Glyma13g23150	MGD2	monogalactosyldiacylglycerol synthase 2	AT5G20410	-3.9	2.4E-07
Glyma09g36360	PAP10	purple acid phosphatase 10	AT2G27190	-3.88	2.0E-07
Glyma08g47160		Pectin lyase-like superfamily protein	AT4G13710	-3.84	1.3E-05
Glyma16g26970	AGP14	arabinogalactan protein 14		-3.76	1.5E-07
Glyma06g09000	CCD8	carotenoid cleavage dioxygenase 8	AT4G32810	-3.74	5.2E-07
Glyma16g28540	RLP33	receptor like protein 33	AT3G05660	-3.69	2.7E-05
Glyma17g00880		Adenine nucleotide alpha hydrolases-like superfamily protein	AT5G47740	-3.67	1.4E-07
Glyma19g04280	DMR6	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase	AT5G24530	-3.63	5.3E-06
Glyma03g31110	GA1	Terpenoid cyclases/Protein prenyltransferases superfamily	AT4G02780	-3.63	6.4E-06

Gene ID	*Gene Name*	*Description*	*Best Arabidopsis Match*	*Fold change*	*P-value*
Glyma10g12130		2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase	AT1G52790	-3.63	1.4E-08
Glyma16g29300		disease resistance family protein	AT2G34930	-3.61	1.7E-04
Glyma01g05540	ORC6	origin recognition complex protein 6	AT1G26840	-3.6	4.4E-07
Glyma12g30240	FRU	FER-like regulator of iron uptake	AT2G28160	-3.58	1.1E-03
Glyma14g37440	ASN1	glutamine-dependent asparagine synthase 1	AT3G47340	-3.58	7.1E-07
Glyma18g06220		Peroxidase superfamily protein	AT5G05340	-3.56	5.5E-07
Glyma04g18650		unknown		-3.53	1.3E-04
Glyma02g09200		unknown		-3.51	1.9E-06
Glyma09g28310		Kunitz family trypsin and protease inhibitor protein	AT1G17860	-3.51	1.3E-04
Glyma18g48120				-3.47	5.5E-04
Glyma03g27840		Major facilitator superfamily protein	AT1G68570	-3.45	2.9E-04
Glyma16g03280		unknown		-3.45	2.3E-05
Glyma06g37390		unknown		-3.43	1.3E-06
Glyma20g27280	TUA4	tubulin alpha-4 chain	AT1G04820	-3.42	5.2E-05
Glyma18g06060	EXPA8	expansin A8	AT2G40610	-3.42	6.7E-07
Glyma17g36130	PAH2	phosphatidic acid phosphohydrolase 2	AT5G42870	-3.41	5.3E-04
Glyma13g38270	CYCP2;1	cyclin p2;1	AT3G21870	-3.33	1.7E-03
Glyma15g16490		2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase	AT3G21420	-3.3	3.6E-06
Glyma05g36930	GH9C2	glycosyl hydrolase 9C2	AT1G64390	-3.29	3.5E-07
Glyma14g07810	CPuORF5	conserved peptide upstream open reading frame 5		-3.29	7.7E-08
Glyma16g28570		disease resistance family protein / LRR family protein	AT2G34930	-3.26	3.0E-06
Glyma05g32570		unknown		-3.23	1.5E-03
Glyma12g32160	RCI3	Peroxidase superfamily protein	AT1G05260	-3.23	3.0E-04
Glyma04g08520		Sulfite exporter TauE/SafE family protein	AT2G25737	-3.22	3.3E-07
Glyma03g05510		Disease resistance-responsive (dirigent-like protein) family	AT5G49040	-3.22	3.0E-03
Glyma18g08120				-3.2	7.1E-07
Glyma06g22430	IRX6	COBRA-like extracellular glycosyl-phosphatidyl inositol-	AT5G15630	-3.17	2.7E-05
Glyma17g14860		Bifunctional inhibitor/lipid-transfer protein	AT1G62510	-3.14	2.5E-03
Glyma19g35730		DNAse I-like superfamily protein	AT2G37440	-3.12	1.8E-05
Glyma12g08520	XTH32	xyloglucan endotransglucosylase/hydrolase	AT2G36870	-3.1	3.9E-04
Glyma17g14190	AGL14	AGAMOUS-like 14	AT4G11880	-3.09	2.1E-05
Glyma15g11930	EFE	ethylene-forming enzyme	AT1G05010	-3.08	8.7E-05
Glyma06g10750		RNA-binding (RRM/RBD/RNP motifs) family protein	AT1G78260	-3.01	4.4E-05
Glyma05g25510		unknown		-3	5.2E-06
Glyma05g14220		unknown		-2.99	4.1E-07
Glyma17g17970	AGP18	arabinogalactan protein 18	AT4G37450	-2.98	1.1E-03
Glyma10g22080	CYP71B34	cytochrome P450, family 71, subfamily B, polypeptide 34	AT3G26300	-2.97	8.9E-03
Glyma16g07830		2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase	AT1G52800	-2.94	9.5E-06
Glyma03g03580				-2.94	2.5E-05
Glyma13g39650	FRU	FER-like regulator of iron uptake	AT2G28160	-2.93	8.5E-05
Glyma03g14200	SQD2	sulfoquinovosyldiacylglycerol 2	AT5G01220	-2.93	1.3E-05
Glyma08g20820		Pyridoxal phosphate phosphatase-related protein	AT1G17710	-2.92	6.7E-05
Glyma06g20200	HA11	H(+)-ATPase 11	AT5G62670	-2.91	3.6E-06
Glyma18g12150		unknown		-2.89	2.6E-04
Glyma08g38740				-2.89	2.4E-04
Glyma06g08630		Sulfite exporter TauE/SafE family protein	AT2G25737	-2.88	3.8E-06
Glyma03g05580		Disease resistance-responsive (dirigent-like protein) family	AT1G58170	-2.87	1.6E-04
Glyma19g38130	SNRNP-G	probable small nuclear ribonucleoprotein G	AT2G23930	-2.87	4.8E-05
Glyma05g04270	SKU5	Cupredoxin superfamily protein	AT4G12420	-2.86	2.7E-04
Glyma10g06710	ACA7	alpha carbonic anhydrase 7	AT1G08080	-2.84	2.7E-05
Glyma05g33790	XPL1	S-adenosyl-L-methionine-dependent methyltransferases	AT3G18000	-2.83	2.1E-06
Glyma09g16400		unknown		-2.82	1.9E-06
Glyma06g42080	THI1	thiazole biosynthetic enzyme, chloroplast (ARA6) (THI1)	AT5G54770	-2.82	3.8E-05
Glyma07g20860	LACS2	long-chain acyl-CoA synthetase 2	AT1G49430	-2.82	8.9E-05
Glyma13g34560	PPC3	phosphoenolpyruvate carboxylase 3	AT3G14940	-2.8	5.5E-08
Glyma16g04750	APY2	apyrase 2	AT5G18280	-2.8	1.7E-02
Glyma10g12700	CYP71B34	cytochrome P450, family 71, subfamily B, polypeptide 34	AT3G26300	-2.79	4.6E-04
Glyma10g22060	CYP71B34	cytochrome P450, family 71, subfamily B, polypeptide 34	AT3G26300	-2.79	5.1E-04
Glyma10g12710	CYP71B34	cytochrome P450, family 71, subfamily B, polypeptide 34	AT3G26300	-2.79	4.1E-05

Gene ID	*Gene Name*	*Description*	*Best Arabidopsis Match*	*Fold change*	*P-value*
Glyma11g36410		O-methyltransferase family protein	AT4G35160	-2.79	7.1E-07
Glyma14g39910		Prolyl oligopeptidase family protein	AT1G76140	-2.78	3.9E-04
Glyma10g03370	EDA4	Bifunctional inhibitor/lipid-transfer protein	AT2G48140	-2.78	9.1E-06
Glyma02g38580		Protein of unknown function, DUF599	AT5G24600	-2.78	2.6E-04
Glyma15g40060	CID9	CTC-interacting domain 9	AT3G14450	-2.77	7.0E-05
Glyma02g40940			AT5G16550	-2.77	4.6E-04
Glyma06g12840		nodulin MtN21 /EamA-like transporter family protein	AT1G70260	-2.77	3.9E-06
Glyma08g02590		unknown		-2.76	1.2E-03
Glyma01g03190		MATE efflux family protein	AT3G26590	-2.76	7.4E-06
Glyma19g34100	EDA4	Bifunctional inhibitor/lipid-transfer protein	AT2G48140	-2.75	5.9E-06
Glyma06g07260	SPX2	SPX domain gene 2	AT2G26660	-2.73	3.7E-06
Glyma20g35960	ENODL8	early nodulin-like protein 8	AT1G64640	-2.73	7.0E-06
Glyma03g22860	NPC3	non-specific phospholipase C3	AT3G03530	-2.73	4.9E-06
Glyma20g38200	PLDP1	phospholipase D P1	AT3G16785	-2.71	7.9E-05
Glyma01g01690		Mono-di-acylglycerol lipase, N-terminal;Lipase, class 3	AT4G16070	-2.7	2.2E-05
Glyma11g29680		unknown		-2.7	2.7E-03
Glyma19g36620	PAL1	PHE ammonia lyase 1	AT2G37040	-2.7	1.2E-06
Glyma12g03050	GH9C1	glycosyl hydrolase 9C1	AT1G48930	-2.69	1.1E-04
Glyma14g36390	IAA9	indole-3-acetic acid inducible 9	AT5G65670	-2.68	3.4E-04
Glyma09g01680		Bifunctional inhibitor/lipid-transfer protein	AT2G45180	-2.68	8.5E-04
Glyma09g01810	PGLP2	2-phosphoglycolate phosphatase 2	AT5G47760	-2.66	6.8E-04
Glyma02g08920	CESA6	cellulose synthase 6	AT4G39350	-2.66	1.3E-05
Glyma14g09620		Gibberellin-regulated family protein	AT5G59845	-2.65	1.9E-03
Glyma01g39350		alpha/beta-Hydrolases superfamily protein	AT5G42930	-2.62	2.1E-05
Glyma19g29180	ATMS1	Cobalamin-independent synthase family protein	AT5G17920	-2.61	6.1E-05
Glyma10g35480	ARF16	auxin response factor 16	AT4G30080	-2.6	3.8E-08
Glyma04g03200	bZIP44	basic leucine-zipper 44	AT1G75390	-2.6	3.4E-04
Glyma12g07420	FLA11	FASCICLIN-like arabinogalactan-protein 11	AT5G03170	-2.59	1.1E-03
Glyma13g42770		Pyridoxal phosphate phosphatase-related protein	AT1G73010	-2.58	2.0E-06
Glyma03g14050		unknown		-2.58	2.4E-05
Glyma18g00350	PSY	PHYTOENE SYNTHASE	AT5G17230	-2.58	7.3E-06
Glyma03g36620	RCI3	Peroxidase superfamily protein	AT1G05260	-2.56	8.8E-04
Glyma07g06720				-2.56	1.8E-07
Glyma04g08910	CCD8	carotenoid cleavage dioxygenase 8	AT4G32810	-2.55	9.9E-05
Glyma18g51260		6-phosphogluconate dehydrogenase family protein	AT3G02360	-2.52	1.5E-09
Glyma08g43610		unknown		-2.51	1.7E-04
Glyma15g16710		Peroxidase superfamily protein	AT1G30870	-2.5	5.1E-05
Glyma10g02090		Pollen Ole e 1 allergen and extensin family protein	AT2G47540	-2.49	1.0E-07
Glyma01g02820			AT4G38060	-2.48	8.7E-07
Glyma11g03810		2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase	AT4G16765	-2.48	2.8E-04
Glyma01g40320		VQ motif-containing protein	AT2G22880	-2.48	1.4E-08
Glyma13g36930	FLA6	FASCICLIN-like arabinogalactan 6	AT5G44130	-2.48	1.8E-04
Glyma10g25800		disease resistance family protein / LRR family protein	AT2G34930	-2.47	1.1E-06
Glyma14g07800	bZIP44	basic leucine-zipper 44	AT1G75390	-2.47	2.3E-10
Glyma05g15230		AMP-dependent synthetase and ligase family protein	AT5G63380	-2.46	3.0E-06
Glyma14g09510		N-terminal nucleophile aminohydrolases (Ntn hydrolases)	AT3G16150	-2.46	3.6E-04
Glyma13g43970		Protein of unknown function, DUF642	AT5G11420	-2.46	9.3E-05
Glyma18g20480		unknown		-2.45	1.4E-05
Glyma16g26020		GDSL-like Lipase/Acylhydrolase superfamily protein	AT2G23540	-2.45	2.6E-06
Glyma07g05230	SRF6	STRUBBELIG-receptor family 6	AT1G53730	-2.45	7.1E-07
Glyma02g38920		Histone superfamily protein	AT3G53730	-2.44	1.5E-03
Glyma16g01780		RNA-binding (RRM/RBD/RNP motifs) family protein	AT3G54770	-2.44	1.1E-04
Glyma15g41100		unknown		-2.43	4.2E-04
Glyma18g17580		Purple acid phosphatases superfamily protein	AT1G13750	-2.42	4.5E-05
Glyma08g12020		O-Glycosyl hydrolases family 17 protein	AT3G13560	-2.42	1.9E-03
Glyma08g21420		HAD superfamily, subfamily IIIB acid phosphatase	AT4G25150	-2.42	3.6E-06
Glyma20g33740		Disease resistance protein (CC-NBS-LRR class) family	AT1G53350	-2.41	6.0E-04
Glyma02g47880	FLA2	FASCICLIN-like arabinogalactan 2	AT4G12730	-2.41	6.6E-04
Glyma04g11550	ACC1	acetyl-CoA carboxylase 1	AT1G36160	-2.4	1.8E-07
Glyma10g30110		HXXXD-type acyl-transferase family protein	AT5G17540	-2.4	4.0E-06

Gene ID	*Gene Name*	*Description*	*Best Arabidopsis Match*	*Fold change*	*P-value*
Glyma17g12340	SPX2	SPX domain gene 2	AT2G26660	-2.39	1.8E-03
Glyma10g34970	PPC4	phosphoenolpyruvate carboxylase 4	AT1G68750	-2.39	1.9E-05
Glyma13g38310	RCI3	Peroxidase superfamily protein	AT1G05260	-2.39	4.6E-05
Glyma07g02000	TINY2	Integrase-type DNA-binding superfamily protein	AT5G11590	-2.37	1.3E-06
Glyma04g34370	HA11	H(+)-ATPase 11	AT5G62670	-2.37	3.1E-05
Glyma06g45550	MYB14	myb domain protein 14	AT2G31180	-2.37	9.1E-04
Glyma11g29920		Peroxidase superfamily protein	AT5G05340	-2.37	8.4E-04
Glyma02g01970		Pollen Ole e 1 allergen and extensin family protein	AT2G47540	-2.36	3.3E-04
Glyma20g36320		RmlC-like cupins superfamily protein	AT3G05950	-2.36	6.4E-05
Glyma19g13540		2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase	AT1G52790	-2.36	1.2E-04
Glyma19g45130	SRF6	STRUBBELIG-receptor family 6	AT1G53730	-2.35	4.4E-05
Glyma06g43050	PPC1	phosphoenolpyruvate carboxylase 1	AT1G53310	-2.35	4.3E-05
Glyma02g09220		unknown		-2.35	6.0E-03
Glyma11g28150		unknown		-2.35	3.0E-04
Glyma12g05770	BGLU15	beta glucosidase 15	AT2G44450	-2.34	4.8E-05
Glyma06g11790		nodulin MtN21 /EamA-like transporter family protein	AT4G08300	-2.34	3.6E-04
Glyma12g07430	FLA11	FASCICLIN-like arabinogalactan-protein 11	AT5G60490	-2.33	6.0E-06
Glyma05g18360		unknown		-2.33	2.5E-02
Glyma08g21650	TINY2	Integrase-type DNA-binding superfamily protein	AT5G11590	-2.33	4.5E-06
Glyma05g35290		unknown		-2.33	4.5E-04
Glyma12g19510		unknown		-2.32	1.4E-06
Glyma02g41210		SGNH hydrolase-type esterase superfamily protein	AT5G37690	-2.32	1.6E-05
Glyma03g31950	PHT1;7	phosphate transporter 1;7	AT3G54700	-2.32	2.5E-07
Glyma12g03680		Protein kinase protein with adenine nucleotide alpha	AT2G16750	-2.32	1.4E-05
Glyma14g24130		unknown		-2.32	9.3E-06
Glyma18g47810		Protein phosphatase 2C family protein	AT3G02750	-2.31	3.0E-06
Glyma07g00920	LOX1	lipoxygenase 1	AT1G55020	-2.31	7.2E-03
Glyma04g37320		Major facilitator superfamily protein	AT5G14120	-2.31	4.0E-05
Glyma03g27830		Major facilitator superfamily protein	AT1G68570	-2.31	4.7E-11
Glyma03g20170		vacuolar iron transporter (VIT) family protein	AT5G24290	-2.3	1.2E-06
Glyma10g35470	GATA9	GATA transcription factor 9	AT3G54810	-2.29	1.7E-04
Glyma19g22460		AMP-dependent synthetase and ligase family protein	AT5G63380	-2.29	2.9E-04
Glyma15g06260		hydroxyproline-rich glycoprotein family protein		-2.28	9.4E-04
Glyma02g15630		NAD(P)-binding Rossmann-fold superfamily protein	AT3G59710	-2.28	3.6E-05
Glyma09g23600	UGT88A1	UDP-glucosyl transferase 88A1	AT3G16520	-2.28	2.5E-07
Glyma17g31230		unknown		-2.28	1.8E-03
Glyma19g32620				-2.28	2.7E-04
Glyma06g12270	AAP3	amino acid permease 3	AT1G77380	-2.27	1.4E-02
Glyma09g05300		Domain of unknown function (DUF23)	AT5G44670	-2.27	1.5E-04
Glyma08g26670	CYP716A1	cytochrome P450, family 716, subfamily A, polypeptide 1	AT5G36110	-2.27	1.2E-07
Glyma13g44140		Pectin lyase-like superfamily protein	AT5G17200	-2.27	1.5E-04
Glyma13g28450		Major facilitator superfamily protein	AT5G18840	-2.27	7.9E-04
Glyma18g49240	AT5MAT	HXXXD-type acyl-transferase family protein	AT5G39050	-2.26	2.1E-04
Glyma04g07220	CESA1	cellulose synthase 1	AT4G32410	-2.25	5.7E-04
Glyma11g13970		Leucine-rich repeat (LRR) family protein	AT3G20820	-2.25	1.3E-08
Glyma10g35870				-2.24	1.6E-03
Glyma18g12210	SHT	spermidine hydroxycinnamoyl transferase	AT2G19070	-2.24	1.6E-04
Glyma06g02770		lysine decarboxylase family protein	AT2G37210	-2.24	2.8E-04
Glyma02g16080	IAA7	indole-3-acetic acid 7	AT3G23050	-2.24	1.3E-04
Glyma20g38560	TT5	Chalcone-flavanone isomerase family protein	AT3G55120	-2.24	1.1E-02
Glyma18g48170	BIR1	BAK1-interacting receptor-like kinase 1	AT5G48380	-2.24	1.1E-04
Glyma15g01370		Protein of unknown function, DUF642	AT5G11420	-2.23	5.4E-08
Glyma06g02650	TUB1	tubulin beta-1 chain	AT1G75780	-2.23	1.6E-05
Glyma16g19800		unknown		-2.23	3.9E-10
Glyma14g34330		Protein of unknown function (DUF581)	AT1G78020	-2.23	6.4E-05
Glyma10g06630		ACT-like superfamily protein	AT2G36840	-2.22	2.2E-03
Glyma14g25410		unknown		-2.22	9.5E-04
Glyma15g21160		unknown		-2.22	7.1E-04
Glyma07g38740	AILP1	Aluminium induced protein with YGL and LRDR motifs	AT5G19140	-2.21	3.9E-03
Glyma12g32280	bHLH115	basic helix-loop-helix (bHLH) DNA-binding superfamily	AT1G51070	-2.2	1.9E-04

Gene ID	*Gene Name*	*Description*	*Best Arabidopsis Match*	*Fold change*	*P-value*
Glyma15g30110	MLP43	MLP-like protein 43	AT1G70890	-2.2	3.8E-03
Glyma08g22880	AVP1	Inorganic H pyrophosphatase family protein	AT1G15690	-2.2	5.8E-03
Glyma15g00330		Ubiquitin-specific protease family C19-related protein	AT1G78880	-2.2	4.2E-02
Glyma13g02050		Protein of unknown function (DUF581)	AT1G78020	-2.19	1.6E-04
Glyma15g41940		DNA glycosylase superfamily protein	AT3G12710	-2.19	7.6E-05
Glyma03g25380	AP4.3A	protein kinase family protein	AT2G32800	-2.19	7.8E-05
Glyma14g23280		nodulin MtN21 /EamA-like transporter family protein	AT4G08290	-2.19	5.6E-04
Glyma05g21820	AGP18	arabinogalactan protein 18	AT4G37450	-2.19	5.5E-03
Glyma06g45910	RCI3	Peroxidase superfamily protein	AT1G05260	-2.19	2.4E-04
Glyma05g15220		AMP-dependent synthetase and ligase family protein	AT5G63380	-2.18	7.1E-06
Glyma02g25950		Polyketide cyclase/dehydrase and lipid transport superfamily	AT5G28010	-2.18	3.2E-05
Glyma16g27900		Peroxidase superfamily protein	AT1G71695	-2.18	8.7E-03
Glyma08g29090	POM1	Chitinase family protein	AT1G05850	-2.17	1.8E-02
Glyma14g38670		Leucine-rich repeat protein kinase family protein	AT1G06840	-2.17	3.0E-03
Glyma11g00710	MSS1	Major facilitator superfamily protein	AT5G26340	-2.17	2.1E-06
Glyma10g42680	UGT73B5	UDP-glucosyl transferase 73B5	AT2G15480	-2.17	1.8E-07
Glyma11g20720	FLA11	FASCICLIN-like arabinogalactan-protein 11	AT5G60490	-2.17	3.5E-04
Glyma11g36210	SULTR2;1	slufate transporter 2;1	AT5G10180	-2.16	6.7E-06
Glyma13g32930		Cox19-like CHCH family protein	AT5G64400	-2.16	6.7E-03
Glyma06g09500	TUA2	tubulin alpha-2 chain	AT1G50010	-2.16	1.4E-02
Glyma19g42040	SWEET17	Nodulin MtN3 family protein	AT4G15920	-2.16	4.6E-04
Glyma17g35530		Gibberellin-regulated family protein	AT5G59845	-2.16	2.4E-03
Glyma03g08570		unknown		-2.15	1.9E-04
Glyma20g24400		GHMP kinase family protein	AT3G54250	-2.15	5.6E-04
Glyma10g39660		Protein of unknown function (DUF3049)	AT5G19260	-2.15	2.0E-04
Glyma14g17260	FPF1	flowering promoting factor 1	AT5G24860	-2.15	1.7E-03
Glyma05g21930		alpha/beta-Hydrolases superfamily protein	AT4G37470	-2.14	1.6E-04
Glyma03g28850	BG1	beta-1,3-glucanase 1	AT3G57270	-2.14	7.6E-04
Glyma04g00570	ADF5	actin depolymerizing factor 5	AT2G16700	-2.14	1.9E-02
Glyma11g25650		Eukaryotic aspartyl protease family protein	AT5G07030	-2.13	1.8E-05
Glyma03g40680	ATB2	NAD(P)-linked oxidoreductase superfamily protein	AT1G60710	-2.13	3.4E-05
Glyma05g00420	RBOH F	respiratory burst oxidase protein F	AT1G64060	-2.12	3.8E-03
Glyma10g00460		Protein of unknown function (DUF506)	AT3G22970	-2.12	7.5E-04
Glyma13g03850		unknown		-2.12	6.3E-07
Glyma08g06060	KUP6	K+ uptake permease 6	AT1G70300	-2.12	8.0E-07
Glyma17g14730	SKU5	Cupredoxin superfamily protein	AT4G12420	-2.11	2.5E-05
Glyma06g17590		HXXXD-type acyl-transferase family protein	AT5G41040	-2.11	1.7E-04
Glyma08g14700	SULTR2;1	slufate transporter 2;1	AT5G10180	-2.11	5.5E-04
Glyma18g51880		Disease resistance-responsive (dirigent-like protein) family	AT2G39430	-2.11	1.6E-06
Glyma17g38120	sks5	SKU5 similar 5	AT1G76160	-2.11	3.6E-05
Glyma14g04670		unknown		-2.1	1.5E-02
Glyma03g22960	MLO1	Seven transmembrane MLO family protein	AT4G02600	-2.09	1.8E-03
Glyma14g10480	RAX2	Duplicated homeodomain-like superfamily protein	AT5G65790	-2.09	5.9E-05
Glyma06g12850		nodulin MtN21 /EamA-like transporter family protein	AT1G70260	-2.09	5.5E-05
Glyma10g24080	EXPB2	expansin B2	AT1G65680	-2.08	9.1E-03
Glyma12g34570	RD22	BURP domain-containing protein	AT5G25610	-2.08	9.0E-06
Glyma12g02610	sks5	SKU5 similar 5	AT1G76160	-2.08	2.1E-07
Glyma15g15370	BXL1	beta-xylosidase 1	AT5G49360	-2.08	5.9E-06
Glyma09g09390	APA1	aspartic proteinase A1	AT1G11910	-2.08	8.2E-07
Glyma19g35270	PDR12	pleiotropic drug resistance 12	AT1G15520	-2.07	1.0E-02
Glyma13g03870		unknown		-2.07	2.5E-03
Glyma02g34510		unknown		-2.07	2.3E-05
Glyma10g05170		DNAse I-like superfamily protein	AT2G37440	-2.07	1.5E-02
Glyma06g40070		unknown		-2.07	3.4E-03
Glyma15g13080	GH9A1	glycosyl hydrolase 9A1	AT5G49720	-2.06	5.6E-05
Glyma20g16110		unknown		-2.06	8.1E-05
Glyma12g33530	FLA9	FASCICLIN-like arabinoogalactan 9	AT5G44130	-2.06	3.1E-04
Glyma08g14160			AT5G65300	-2.06	1.8E-03
Glyma11g04970		VQ motif-containing protein	AT2G22880	-2.05	1.2E-04
Glyma06g07440		Protein of unknown function, DUF642	AT5G11420	-2.05	8.8E-04

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Gene ID	*Gene Name*	*Description*	*Best Arabidopsis Match*	*Fold change*	*P-value*
Glyma06g02290		Pyridoxal-dependent decarboxylase family protein	AT5G11880	-2.05	2.7E-04
Glyma07g06520	RNS2	ribonuclease 2	AT2G39780	-2.04	6.4E-06
Glyma02g45640	PAR2	phy rapidly regulated 2	AT2G42870	-2.04	1.1E-02
Glyma09g02610		Peroxidase superfamily protein	AT5G06730	-2.04	6.8E-03
Glyma18g48580	AIR3	Subtilisin-like serine endopeptidase family protein	AT2G04160	-2.03	3.3E-02
Glyma20g33420		unknown		-2.03	8.2E-03
Glyma02g02680	NRT1.7	nitrate transporter 1.7	AT1G69870	-2.03	2.0E-04
Glyma20g38590		Peroxidase superfamily protein	AT5G05340	-2.03	2.3E-05
Glyma19g13520		2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase	AT1G52800	-2.03	2.3E-03
Glyma04g14790	DUR3	solute:sodium symporters;urea transmembrane transporters	AT5G45380	-2.02	3.4E-03
Glyma09g07100	BGAL12	beta-galactosidase 12	AT4G26140	-2.02	3.6E-03
Glyma07g03120		Plant protein of unknown function (DUF247)	AT3G02650	-2.02	1.5E-03
Glyma17g14850		Bifunctional inhibitor/lipid-transfer protein	AT1G62510	-2.02	2.5E-04
Glyma18g20510	NRT2.5	nitrate transporter2.5	AT1G12940	-2.02	5.8E-03
Glyma19g03120	RAP2.11	related to AP2 11	AT5G19790	-2.01	4.5E-04
Glyma06g07460		Protein of unknown function, DUF642	AT5G11420	-2.01	2.3E-04
Glyma03g36000	NHL1	NDR1/HIN1-like 1	AT3G11660	-2.01	3.8E-03
Glyma16g03270		unknown		-2.01	2.4E-03
Glyma20g32130		unknown		-2	2.1E-02
Glyma13g39850	NRT2.4	nitrate transporter 2.4	AT5G60770	-2	3.5E-02

Appendix 4

Up-regulated genes in *sat1* roots. The data is ordered based on the mean fold-change differences between *sat1* and empty vector control roots. A positive fold-change value indicates an up-regulated gene. Those genes identified as up-regulated were found to be statistically significant using a Bayesian t-test ($p < 0.05$, n=3-4).

Gene ID	*Gene Name*	*Description*	*Best Arabidopsis Match*	*Fold change*	*P-value*
Glyma11g34650			AT3G55646	20.38	2.2E-15
Glyma12g12610		Unknown		13.19	2.1E-12
Glyma10g31210		RmlC-like cupins superfamily protein	AT3G05950	12.65	3.6E-12
Glyma12g12600	RMA1	RING membrane-anchor 1	AT4G03510	11.94	9.6E-13
Glyma06g05280	BCAT-2	branched-chain amino acid transaminase 2	AT1G10070	10.9	3.3E-16
Glyma08g37180		hydroxyproline-rich glycoprotein family protein	AT5G65660	10.41	5.0E-05
Glyma17g05920	NF-YA8	nuclear factor Y, subunit A8	AT1G17590	8.36	9.1E-09
Glyma07g39580	TDT	tonoplast dicarboxylate transporter	AT5G47560	8.11	2.7E-13
Glyma01g42030	PME1	pectin methylesterase inhibitor 1	AT4G12390	7.38	1.2E-10
Glyma15g04910			AT2G28570	6.57	3.9E-11
Glyma07g04810		Dormancy /auxin associated family protein	AT1G54070	5.6	1.7E-12
Glyma17g37020			AT1G19530	5.13	7.9E-07
Glyma11g05280	LBD38	LOB domain-containing protein 38	AT5G67420	4.93	7.0E-06
Glyma20g36300		RmlC-like cupins superfamily protein	AT3G05950	4.78	2.0E-09
Glyma13g40340				4.78	1.1E-06
Glyma12g04770		BTB, POZ domain-containing protein	AT3G50780	4.74	3.9E-09
Glyma18g00830		Protein of unknown function (DUF 3339)	AT3G48660	4.59	7.6E-07
Glyma03g40730	BZO2H3	bZIP transcription factor family protein	AT5G28770	4.36	2.1E-09
Glyma13g16770	NF-YA3	nuclear factor Y, subunit A3	AT1G72830	4.35	1.6E-06
Glyma13g01970		Phosphoglycerate mutase family protein	AT1G22170	4.27	1.4E-02
Glyma10g28900		Adenine nucleotide alpha hydrolases-like superfamily	AT3G62550	4.22	1.0E-09
Glyma01g00930	OXS3	oxidative stress 3	AT5G56550	4.17	1.8E-08
Glyma12g01520				4.16	9.0E-09
Glyma11g27480	ASN1		AT3G47340	4.15	8.0E-04
Glyma06g10430		Unknown		4.08	3.1E-06
Glyma08g23540	J8	Chaperone DnaJ-domain superfamily protein	AT1G80920	4.04	7.4E-06
Glyma11g06960	GBF1	G-box binding factor 1	AT4G36730	3.96	2.8E-06
Glyma05g00750			AT3G25400	3.95	1.1E-05
Glyma12g29470		Protein of unknown function, DUF584	AT5G60680	3.91	3.7E-04
Glyma15g42080	emb2742	CTP synthase family protein	AT3G12670	3.88	3.7E-10
Glyma06g12190	NRAMP6	NRAMP metal ion transporter 6	AT1G15960	3.73	4.5E-03
Glyma04g05280	ELF3	hydroxyproline-rich glycoprotein family protein	AT2G25930	3.69	7.0E-09
Glyma10g03340		Protein of unknown function (DUF581)	AT3G22550	3.65	2.7E-07
Glyma13g29620		Protein of unknown function (DUF 3339)		3.65	1.5E-06
Glyma20g37350		Unknown		3.59	1.8E-04
Glyma09g35670	ASE1		AT2G16570	3.53	3.5E-07
Glyma17g06560			AT5G57123	3.49	7.9E-09
Glyma07g08410		Unknown		3.49	2.7E-05
Glyma07g15070	OXS3	oxidative stress 3	AT5G56550	3.48	7.4E-08
Glyma09g41340	SIP1	SOS3-interacting protein 1	AT5G58380	3.44	1.4E-06
Glyma14g40110		RING U-box superfamily protein	AT5G42200	3.43	1.4E-11
Glyma07g33050	MEE14	maternal effect embryo arrest 14	AT2G15890	3.41	6.9E-06
Glyma10g06050		Protein of unknown function, DUF584	AT5G03230	3.37	1.3E-07
Glyma05g24000		Unknown		3.31	6.6E-05
Glyma05g31390	ACX2	acyl-CoA oxidase 2	AT5G65110	3.26	1.4E-07
Glyma06g04490	DEAR3	DREB and EAR motif protein 3	AT5G67190	3.25	2.6E-06
Glyma19g39920	SAG21	senescence-associated gene 21	AT4G02380	3.22	9.0E-07
Glyma18g05160	OXS3	oxidative stress 3	AT5G56550	3.22	1.2E-05
Glyma10g02580		Thioredoxin superfamily protein	AT3G62930	3.22	1.9E-05
Glyma17g07690		nodulin MtN21 EamA-like transporter family protein	AT4G28040	3.19	7.0E-07
Glyma07g33180		Peroxidase superfamily protein	AT1G49570	3.16	7.6E-05
Glyma10g40320			AT3G19615	3.16	7.4E-06

Gene ID	*Gene Name*	*Description*	*Best Arabidopsis Match*	*Fold change*	*P-value*
Glyma18g51280	GDU2		AT4G25760	3.16	1.3E-03
Glyma06g10630		VQ motif-containing protein	AT1G78310	3.16	5.6E-07
Glyma14g07990			AT1G19530	3.16	5.3E-07
Glyma01g38500		Protein of unknown function (DUF607)	AT2G23790	3.16	1.7E-03
Glyma11g12480	CCR2	cold, circadian rhythm, and rna binding 2	AT2G21660	3.14	1.5E-09
Glyma09g37270		Transmembrane amino acid transporter family protein	AT3G28960	3.14	1.5E-06
Glyma02g06550	GBF1	G-box binding factor 1	AT4G36730	3.13	3.9E-08
Glyma01g41930	NRT1.1	nitrate transporter 1.1	AT1G12110	3.11	4.8E-03
Glyma13g02510	NIA1	nitrate reductase 1	AT1G77760	3.1	3.0E-03
Glyma20g26910	LBD38	LOB domain-containing protein 38	AT5G67420	3.09	1.1E-04
Glyma10g02210	SAG21	senescence-associated gene 21	AT4G02380	3.05	9.8E-03
Glyma05g02880	BT2	BTB and TAZ domain protein 2	AT3G48360	3.05	4.4E-05
Glyma07g10880				3.04	1.3E-03
Glyma07g02990		NAD(P)-binding Rossmann-fold superfamily protein	AT2G33590	3.04	1.3E-04
Glyma01g00980	NRPC2	nuclear RNA polymerase C2	AT5G45140	3.02	5.6E-09
Glyma01g36630		ACT-like protein tyrosine kinase family protein	AT4G35780	3.01	9.4E-05
Glyma13g27840		Eukaryotic aspartyl protease family protein	AT1G03220	3	3.2E-04
Glyma13g16590		Peroxidase superfamily protein	AT5G19890	3	5.4E-06
Glyma13g31870		Unknown		2.99	4.4E-02
Glyma13g12190		Unknown		2.99	1.5E-04
Glyma06g43620	BIN2	Protein kinase superfamily protein	AT4G18710	2.97	4.9E-03
Glyma04g08050			AT5G24890	2.96	8.2E-11
Glyma07g36980		Unknown		2.95	4.6E-04
Glyma09g32230			AT1G67910	2.91	2.2E-07
Glyma02g14860	MCCB	3-methylcrotonyl-CoA carboxylase	AT4G34030	2.9	4.9E-05
Glyma13g31060	DYL1	dormancy-associated protein-like 1	AT1G28330	2.9	5.6E-05
Glyma11g07090		Major facilitator superfamily protein	AT2G18480	2.89	5.4E-04
Glyma07g04050	NF-YA3	nuclear factor Y, subunit A3	AT1G72830	2.88	6.5E-06
Glyma20g01580		Leucine-rich repeat (LRR) family protein	AT1G49750	2.88	3.2E-06
Glyma14g39820		BTB, POZ domain-containing protein	AT2G30600	2.88	5.7E-03
Glyma17g34240		Mitochondrial substrate carrier family protein	AT5G26200	2.88	9.5E-08
Glyma15g08300	DYL1	dormancy-associated protein-like 1	AT1G28330	2.86	2.1E-05
Glyma16g01390		Dormancy /auxin associated family protein	AT1G54070	2.86	3.1E-10
Glyma07g15060				2.82	3.0E-05
Glyma15g36520		Unknown		2.82	4.8E-10
Glyma03g07460	FAC1	AMP deaminase, putative myoadenylate deaminase,	AT2G38280	2.81	9.5E-03
Glyma12g04040	ASE1		AT2G16570	2.79	3.1E-05
Glyma01g00950				2.77	2.9E-06
Glyma09g35090		Leucine-rich repeat protein kinase family protein	AT5G20480	2.77	2.1E-06
Glyma11g14570		Octicosapeptide	AT3G26510	2.76	2.7E-05
Glyma03g30200	HAT14	homeobox from Arabidopsis thaliana	AT5G06710	2.76	6.9E-09
Glyma03g29940		myb-like HTH transcriptional regulator family protein	AT5G06800	2.76	7.1E-03
Glyma15g36320		Unknown		2.75	2.3E-11
Glyma03g36870		Thioredoxin superfamily protein	AT3G62930	2.74	1.0E-02
Glyma03g36810		B-box type zinc finger protein with CCT domain	AT2G47890	2.73	1.8E-04
Glyma06g45850	RMA1	RING membrane-anchor 1	AT4G03510	2.72	5.8E-05
Glyma13g07900	EXL2	EXORDIUM like 2	AT5G64260	2.71	3.2E-04
Glyma08g36820	PCK1	phosphoenolpyruvate carboxykinase 1	AT4G37870	2.7	4.1E-04
Glyma11g06180		Peroxidase superfamily protein	AT1G49570	2.67	1.7E-04
Glyma18g07090		RING U-box superfamily protein	AT3G47160	2.66	3.2E-07
Glyma11g19860		CHY-type CTCHY-type	AT5G22920	2.65	5.7E-06
Glyma17g07530	TPS11	trehalose phosphatase synthase 11	AT2G18700	2.65	1.3E-02
Glyma19g00600		Galactose mutarotase-like superfamily protein	AT3G01590	2.65	5.2E-09
Glyma09g35830		Unknown		2.65	4.9E-05
Glyma12g01120	RTFL12	ROTUNDIFOLIA like 12		2.63	6.8E-05
Glyma11g08730	SEN1	Rhodanese Cell cycle control phosphatase	AT4G35770	2.62	3.4E-02
Glyma10g02910				2.61	1.5E-07
Glyma08g17490		Aldolase-type TIM barrel family protein	AT1G16350	2.59	5.4E-05
Glyma14g11160			AT5G21940	2.55	9.4E-06
Glyma14g29580		VQ motif-containing protein	AT1G21326	2.54	8.9E-03

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Gene ID	*Gene Name*	*Description*	*Best Arabidopsis Match*	*Fold change*	*P-value*
Glyma13g37450		Integrase-type DNA-binding superfamily protein	AT2G20880	2.53	3.8E-04
Glyma03g34270	ACA7	alpha carbonic anhydrase 7	AT1G08080	2.52	7.2E-04
Glyma06g08100			AT5G24890	2.51	3.3E-04
Glyma11g36900			AT1G27290	2.5	1.3E-07
Glyma07g02180		AMP-dependent synthetase and ligase family protein	AT3G16170	2.49	1.3E-03
Glyma19g32850		myb-like HTH transcriptional regulator family protein	AT5G06800	2.49	4.8E-07
Glyma11g03430	NRT1.1	nitrate transporter 1.1	AT1G12110	2.48	1.2E-02
Glyma15g01060		Tetratricopeptide repeat (TPR)-like superfamily	AT1G80130	2.47	8.6E-05
Glyma08g00950		Unknown		2.46	3.9E-04
Glyma17g07720				2.46	3.6E-04
Glyma05g04060		MATE efflux family protein	AT4G22790	2.46	4.5E-05
Glyma12g33020		Integrase-type DNA-binding superfamily protein	AT2G20880	2.45	3.0E-04
Glyma08g04730	PDLP6	plasmodesmata-located protein 6	AT2G01660	2.44	2.4E-02
Glyma06g13280	GLT1	NADH-dependent glutamate synthase 1	AT5G53460	2.44	2.6E-03
Glyma07g04330	GER3	germin 3	AT5G20630	2.44	1.9E-04
Glyma08g28820	Rap2.6L	related to AP2 6l	AT5G13330	2.44	1.3E-03
Glyma03g34670		Exostosin family protein	AT5G03795	2.43	1.9E-04
Glyma17g14680	BETA-VPE	beta vacuolar processing enzyme	AT1G62710	2.43	1.9E-05
Glyma17g03850		Unknown		2.41	1.6E-03
Glyma10g33190			AT4G14450	2.4	2.2E-04
Glyma13g27110			AT2G16385	2.39	2.3E-03
Glyma13g18160		C2H2 and C2HC zinc fingers superfamily protein	AT5G43540	2.39	4.2E-05
Glyma11g27720	ASN1		AT3G47340	2.39	5.9E-04
Glyma19g43420	BZO2H3	bZIP transcription factor family protein	AT5G28770	2.38	5.1E-03
Glyma05g36100	MIOX4	myo-inositol oxygenase 4	AT4G26260	2.38	1.6E-05
Glyma20g24510		F1F0-ATPase inhibitor protein, putative	AT5G04750	2.38	6.4E-09
Glyma09g36210	RTFL12	ROTUNDIFOLIA like 12		2.38	4.8E-04
Glyma13g00380	WRKY11	WRKY DNA-binding protein 11	AT2G24570	2.36	1.4E-03
Glyma16g02200	EMB2301	NAC (No Apical Meristem) domain transcriptional	AT2G46770	2.35	7.5E-05
Glyma07g39420	EFE	ethylene-forming enzyme	AT1G05010	2.35	1.4E-03
Glyma09g31690			AT1G13360	2.35	1.0E-03
Glyma08g03850			AT4G29110	2.35	4.3E-04
Glyma07g09710	SULTR3;5	sulfate transporter 3;5	AT5G19600	2.34	4.1E-05
Glyma05g09130			AT3G01640	2.34	1.8E-03
Glyma10g30560		winged-helix DNA-binding transcription factor	AT1G06760	2.33	1.5E-03
Glyma20g38930	HPT1	homogentisate phytyltransferase 1	AT2G18950	2.33	1.5E-04
Glyma07g16760	SIS		AT5G02020	2.32	1.0E-04
Glyma09g24680		Chaperone DnaJ-domain superfamily protein	AT4G36040	2.31	7.8E-04
Glyma05g03920		Protein of unknown function (DUF1442)	AT2G45360	2.31	1.4E-04
Glyma15g02250		Homeodomain-like superfamily protein	AT3G16350	2.31	1.6E-06
Glyma11g36910		Protein of unknown function (DUF 3339)	AT3G48660	2.28	1.2E-04
		Unknown		2.28	8.3E-04
Glyma10g39480		Unknown		2.28	2.8E-03
Glyma12g00400		Unknown		2.28	1.8E-03
Glyma05g27970	CYP78A5	cytochrome P450, family 78, subfamily A,	AT1G13710	2.26	1.7E-03
Glyma08g16810			AT1G05575	2.26	6.0E-05
Glyma10g29190		Dormancy /auxin associated family protein	AT1G56220	2.25	1.9E-04
Glyma02g03820	TPS9	trehalose-phosphatase	AT1G23870	2.24	1.7E-06
Glyma12g32980		Protein of unknown function (DUF789)	AT4G03420	2.24	1.2E-07
Glyma03g37060		Heavy metal transport/detoxification superfamily	AT3G06130	2.23	4.3E-04
Glyma13g02960		nodulin MtN21 /EamA-like transporter family protein	AT4G08300	2.23	5.6E-05
Glyma11g08000	HIS1-3	histone H1-3	AT2G18050	2.23	1.1E-03
Glyma13g25520		Unknown		2.22	2.4E-02
Glyma05g22120	LBD39	LOB domain-containing protein 39	AT5G67420	2.21	9.3E-03
Glyma08g10890		Galactose oxidase/kelch repeat superfamily protein	AT1G67480	2.2	5.1E-03
Glyma06g42890	MBF1B	multiprotein bridging factor 1B	AT2G42680	2.2	1.9E-05
Glyma15g15140				2.2	5.8E-03
Glyma13g35300		Dynein light chain type 1 family protein	AT1G23220	2.19	3.7E-04
Glyma04g05580	EIF4A1	eukaryotic translation initiation factor 4A1	AT3G13920	2.19	1.7E-02
Glyma04g39860		Peroxidase superfamily protein	AT5G05340	2.19	1.3E-02

Gene ID	*Gene Name*	*Description*	*Best Arabidopsis Match*	*Fold change*	*P-value*
Glyma07g03580			AT5G20100	2.18	5.7E-06
8g20800		Copper amine oxidase family protein	AT2G42490	2.18	4.1E-03
Glyma08g22900		Leucine-rich repeat family protein	AT1G15740	2.18	1.4E-03
Glyma06g46740		nodulin MtN21 /EamA-like transporter family protein	AT5G07050	2.18	2.0E-06
Glyma17g04920		lysine-ketoglutarate reductase/saccharopine	AT4G33150	2.17	2.9E-03
Glyma02g16390	MCCA	methylcrotonyl-CoA carboxylase alpha chain,	AT1G03090	2.16	9.2E-04
Glyma13g23310		serine-rich protein-related	AT5G25280	2.16	5.5E-03
Glyma04g41550		Unknown		2.16	2.1E-04
Glyma14g07420		Unknown		2.15	9.2E-03
Glyma17g11500		serine-rich protein-related	AT5G11090	2.15	1.1E-03
Glyma17g37580	MP	Transcriptional factor B3 family protein /auxin-	AT1G19850	2.15	2.2E-05
Glyma13g01870	ANNAT1	annexin 1	AT1G35720	2.14	8.0E-03
Glyma13g11590	COL9	CONSTANS-like 9	AT3G07650	2.14	2.4E-05
Glyma18g32730		Unknown		2.14	4.6E-03
Glyma13g29170		Unknown		2.14	1.5E-04
Glyma18g03130	ELF4	Protein of unknown function (DUF1313)	AT2G40080	2.14	1.6E-03
Glyma04g11290	RAP2.4	related to AP2 4	AT1G78080	2.13	1.0E-06
Glyma20g38470		alpha/beta-Hydrolases superfamily protein	AT2G39420	2.13	1.5E-06
Glyma02g09480	ATTPS6	UDP-Glycosyltransferase / trehalose-phosphatase	AT1G68020	2.12	8.5E-04
Glyma03g37740		RING/FYVE/PHD zinc finger superfamily protein	AT4G02075	2.12	1.1E-03
Glyma06g15560		Unknown		2.12	7.8E-04
Glyma18g18590	TPS10	trehalose phosphate synthase	AT1G23870	2.12	1.5E-07
Glyma12g34420	SCL5	scarecrow-like 5	AT5G48150	2.12	9.5E-07
Glyma07g35310			AT2G03440	2.12	1.4E-06
Glyma01g09460				2.11	7.5E-03
Glyma01g40010	LBD38	LOB domain-containing protein 38	AT5G67420	2.11	6.4E-05
Glyma07g26910	bHLH121	basic helix-loop-helix (bHLH) DNA-binding	AT3G19860	2.11	2.0E-04
Glyma11g35800	SAG20	senescence associated gene 20	AT3G10985	2.11	1.1E-03
Glyma08g45510		Kunitz family trypsin and protease inhibitor protein	AT1G17860	2.1	1.7E-04
Glyma01g32450	WNK4	with no lysine (K) kinase 4	AT5G58350	2.1	2.0E-04
Glyma07g34230	HB17	homeobox-leucine zipper protein 17	AT2G01430	2.09	1.7E-04
Glyma17g17110				2.08	5.1E-04
Glyma05g35120			AT5G16110	2.08	9.4E-03
Glyma16g26430		Unknown		2.08	5.9E-03
Glyma07g08420	PP2-A13	phloem protein 2-A13	AT3G61060	2.07	6.7E-04
Glyma20g22530		Carbohydrate-binding X8 domain superfamily protein	AT1G09460	2.07	9.3E-04
Glyma16g07540		Protein of unknown function (DUF567)	AT3G11740	2.06	2.1E-03
Glyma11g38150			AT5G65030	2.06	1.2E-07
Glyma15g18490		Protein of unknown function (DUF761)	AT2G26110	2.06	1.1E-04
Glyma13g44720	CIPK5	CBL-interacting protein kinase 5	AT5G10930	2.06	1.4E-03
Glyma11g08720		ACT-like protein tyrosine kinase family protein	AT4G35780	2.06	1.9E-05
Glyma08g10010	CYP77A5P	cytochrome P450, family 77, subfamily A,	AT3G18270	2.05	1.4E-02
Glyma13g32050		PapD-like superfamily protein	AT4G21450	2.05	1.3E-04
Glyma14g36690	LSU3	response to low sulfur 3	AT3G49570	2.05	6.8E-05
Glyma15g15120		Unknown		2.05	2.9E-05
Glyma02g44410			AT5G35732	2.04	2.7E-03
Glyma02g40000		Peroxidase superfamily protein	AT5G05340	2.04	3.4E-03
Glyma04g02440	ATSBT5.2	Subtilisin-like serine endopeptidase family protein	AT1G20160	2.03	1.6E-03
Glyma08g39210		Unknown		2.03	2.8E-04
Glyma08g44700	GT72B1	UDP-Glycosyltransferase / trehalose-phosphatase	AT4G01070	2.03	3.4E-05
Glyma03g05060		Unknown		2.02	6.1E-05
Glyma15g09750	ARF8	auxin response factor 8	AT1G30330	2.02	3.9E-02
Glyma01g37450	GATA5	GATA transcription factor 5	AT5G66320	2.02	7.4E-05
Glyma20g30290	WRKY27	WRKY DNA-binding protein 27	AT5G52830	2.01	3.1E-03
Glyma01g37090		Chaperone DnaJ-domain superfamily protein	AT4G36040	2.01	1.9E-05
Glyma03g33850	EIN2	NRAMP metal ion transporter family protein	AT5G03280	2.01	8.5E-04
Glyma17g05960			AT5G35460	2	3.0E-05
Glyma02g46070	CPK9	calmodulin-domain protein kinase 9	AT3G20410	2	1.5E-02
Glyma08g45520		Kunitz family trypsin and protease inhibitor protein	AT1G17860	2	3.7E-05