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Molecular genetic and biochemical analysis of gibberellic acid involvement in the stages of soybean (*Glycine max* L.) nodule development

Bethany Potten

BBiotech (Hon)

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Abstract

This thesis aimed to characterise four genes identified to be involved in early nodulation in *Glycine max* (soybean). The first gene studied was a *GmTIR-NBS-LRR* gene, a Toll/Interleukin 1 receptor - Nucleotide Binding Site - Leucine-Rich Repeat (TIR-NBS-LRR) suspected of being involved in host specificity in regards to rhizobia. Over-expression, silencing and histochemical promoter studies of this gene saw no phenotypic changes. This is due to the previously unknown existence of a transcript variant (TV) of the gene as well as the likely existence of alternative promoter(s) (AP). Future characterisation work will focus on understanding and identifying the TVs and possible APs of *GmTIR-NBS-LRR*.

The remaining three early nodulation genes are involved in GA biosynthesis, a plant hormone whose role in nodulation is still unclear. The genes *GmGA20ox a*, *GmGA2ox* and *GmGA3ox 1a* were analysed through silencing and histochemical promoter studies, over-expression and phytogenic analysis and histochemical promoter studies, respectably. Additionally, measurement of endogenous GAs in *G. max* roots which had yet to be reported was carried out. No phenotypic changes were observed following either silencing or over-expression for any of the GA biosynthesis genes. The histochemical promoter studies of *GmGA20ox a* highlighted likely role in facilitating infection thread formation and early nodule development in the cortical cells. *GmGA3ox 1a* appeared to be more general, but still NF dependent in its expression, through its widespread presence in the phloem during early nodulation.

Successful measurement of endogenous GA has shown that independent of nodulation, GA_3 is the more abundant bioactive GA in soybean compared to GA_1. This differs from *Pisum sativum* (pea) roots where GA_3 was not detected and GA_1 is most abundant.

The recent availability of *G. max* mutants of many of these genes through Soybase (http://soybase.org), coupled with the new method for measuring endogenous GA in *G. max* roots, opens up many pathways for further effective characterisation of these nodulation specific genes and thus, a better understanding of nodulation as a whole.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Contributor	Statement of contribution
Bethany Potten nee: van Hameren (Candidate)	Wrote the paper (50%)
Satomi Hayashi	Wrote the Paper (25%) Edited paper (20%)
Peter M Gresshoff	Edited paper (10%)
Brett J Ferguson	Wrote the paper (25%) Edited paper (70%)

Contributions by others to the thesis

Chapter 2, sections: 2.2, 2.4

Ms Dongxue Li (CILR, University of Queensland) created the control vectors; p35S::GUS and no promoter::GUS used for the histochemical β-glucuronidase (GUS) promoter study. As well as the modified pCAMBIA1305.1 vector used in this study to create new pGmTIR-NBS-LRR::GUS and pGmGA3ox::GUS vectors.

Dr Satomi Hayashi (CILR, University of Queensland) created the original pGmTIR-NBS-LRR::GUS and pGmGA3ox 1a::GUS vectors used to construct the new pGmTIR-NBS-LRR::GUS and pGmGA3ox::GUS vectors created and used in this study.

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Dr Satomi Hayashi (CILR, University of Queensland) created the *GmTIR-NBS-LRR* RNAi vector and *GmTIR-NBS-LRR* over-expression vector used in this chapter. As well as the original pGmTIR-NBS-LRR::GUS vector which was used to construct the new pGmTIR-NBS-LRR::GUS vector used in this study.

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Dr Satomi Hayashi (CILR, University of Queensland) created the *GmGA20ox a* RNAi vector and pGmGA20ox a::GUS vector used in this chapter. As well as the original pGmGA3ox 1a::GUS which was used to construct the new pGmGA3ox 1a::GUS vector used in this study.

Ms Dongxue Li (CILR, University of Queensland) created the modified pCAMBIA1305.1 vector used in this study to create new pGmGA3ox 1a::GUS. As well as the control vectors; p35S::GUS and no promoter::GUS.

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Early nodulation genes, endogenous hormone, gibberellin, gibberellin biosynthesis, legumes, nodulation, rhizobia, soybean.

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List of Abbreviations

35S – Cauliflower mosaic virus 35S promoter

2ODDs – 2-Oxoglutarate-Dependent Dioxygenases

AON – Autoregulation of Nodulation

CCC – Chlormequat Chloride

CPS – *ent*-Copalyl Diphosphate Synthase

dpi – Days Post-Inoculation

ENOD – Early Nodulin

EPS – Exopolysaccharide

ERF – Ets2 Repressor Factor

FLOT – Flotillin

GA – Gibberellic Acid

GAMTs – Gibberellin Methyltransferases

GA2ox – GA 2-oxidase

GA3ox – GA 3-oxidase

GA20ox – GA 20-oxidase

GC/MS-SIM – Gas Chromatography/Mass Spectrometry using Selected Ion Monitoring

GGDP – Geranylgeranyl Diphosphate

GUS – β-glucuronidase

HPLC – High Performance Liquid Chromatography

KAPP – Kinase-Associated Protein Phosphatase

KAO – *ent*-Kaurenoic Acid Oxidase

KO – *ent*-Kaurene

KS – *ent*-Kaurene Synthase

LPS – Lipopolysaccharide

LRR – Leucine-Rich Repeat

MS – Mass Spectrometry

NCR – Nodule Specific Cysteine-Rich

NF – Nod Factor

NFR – Nod Factor Receptor

P450s – Cytochrome P450 Monooxygenases

REM – Remorin

RNAi – RNA interference

qRT-PCR – Quantitative Reverse-Transcription PCR

SDI – Shoot-Derived Inhibitor

TLC – Thin Layer Chromatography

TNL – TIR-NBS-LRR

TIR-NBS-LRR – Toll/Interleukin 1 receptor - Nucleotide Binding Site - Leucine-Rich Repeat

UFD – Ubiquitin Fusion Degradation

UPLC – Ultra Performance Liquid Chromatography

Wpi – Weeks Post-Inoculation

WT – Wild Type

ZON – Zone Of Nodulation

Chapter 1 General Introduction: Nodulation and the Role of Hormones

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Please Note: Section 1.1 of chapter 1 is an exact replication of the review paper entitled "Advances in the identification of novel factors required in soybean nodulation, a process critical to sustainable agriculture and food security"

*Authors: **Bethany van Hameren*,** Satomi Hayashi, Peter M Gresshoff, Brett J Ferguson**

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Authors: Bethany van Hameren, Satomi Hayashi, Peter M Gresshoff, Brett J Ferguson*

Institutional Address: Australian Research Council Centre of Excellence for Integrative Legume Research, The University of Queensland, St. Lucia, Brisbane, Queensland 4072, Australia.

***Corresponding author:** Brett J Ferguson. Address as above, E-mail: b.ferguson1@uq.edu.au, Phone: +617-3346 9951, Fax: +617-3365 3556

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Advances in the Identification of Novel Factors Required in Soybean Nodulation, a Process Critical to Sustainable Agriculture and Food Security

Keywords: Legume; Nodule; Nitrogen-fixation; Rhizobia; Transcriptome; RNA-seq

1.1 Abstract

Nodulation is a process of organogenesis that results from a symbiotic relationship between legume plants and soil-dwelling, nitrogen-fixing bacteria, called rhizobia. The rhizobia are housed in newly formed structures on the host roots, called nodules. Within nodules, the rhizobia fix atmospheric N2 into useable forms of nitrogen for the plant. This process is highly important to agriculture, as nitrogen is critical for plant growth and development and is typically the main component of fertilizers. Although fertilizers are effective, they are expensive and often pollute, making biological alternatives, such as legume nodulation, attractive for use in agriculture. Nodulation is regulated by the auto regulation of nodulation (AON) pathway, which enables the host plant to balance its needs between nitrogen acquisition and energy expenditure. Current research is elucidating the nodule development and AON signalling networks. Recent technological advances, such as RNA-sequencing, are revolutionizing the discovery of genes that are critical to nodulation. The discovery of such genes not only enhances our knowledge of the nodulation signalling network, but may help to underpin future work to isolate superior legume crops via modern breeding and engineering practices. Here, recent advances using the cutting-edge technique of RNA sequencing to identify new nodulation genes in soybean are discussed.

1.2 Global Use of Nitrogen Fertiliser

Approximately half of the world's population is directly reliant upon nitrogen fertiliser use in agriculture for their food supply (Peoples *et al.*, 2009; Jensen *et al.*, 2012; Erisman *et al.*, 2008). Taking into account nitrogen fertiliser manufacture, transport and application, the fossil fuel consumed accounts for 50% of fossil fuel use in agriculture, and 5% of the global natural gas consumption annually (Crutzen *et al.*, 2007; Canfield *et al.*, 2010). With the rising cost of fossil fuels, the use of nitrogen fertiliser is becoming increasingly costly for farmers and is often too expensive in developing regions of the world (Ferguson *et al.*, 2013). Not only are nitrogen fertilisers expensive, they are inefficient, with 30-50% of nitrogen fertiliser typically lost to leaching. This run off can cause the eutrophication of waterways and other significant environmental problems (Vance, 2001). Nitrogen contaminated drinking water can also cause methemoglobinaemia, or "Blue-baby syndrome", a potentially fatal condition in infants (Murphy, 1991; Knobeloch *et al.*, 2000).

The global use of nitrogen fertiliser has been steadily increasing in most continents (Figure 1). Worryingly, this also means an increase in NOx gases, which are released when nitrogen fertiliser is broken down. These gases contribute to the formation of ground-level ozone, which causes yield reductions. Nitrous oxide (N2O) is also emitted by breakdown of nitrogen fertiliser (Vance, 2001; Sutton *et al.*, 2013) and is 292 times more active as a greenhouse gas than CO2 (Crutzen *et al.*, 2007). Agriculture was the main source of anthropogenic N2O emissions in 2005, making up 60% of the global total (Reay *et al.*, 2012). The majority of these emissions resulted from the application of nitrogen fertiliser (Reay *et al.*, 2012). By 2050 it is estimated that global nitrogen fertiliser use will increase by 50% in an attempt to boost food production and support a rising population (Sutton *et al.*, 2013). These numbers have experts calling for agricultural reform to diminish nitrogen fertiliser use.

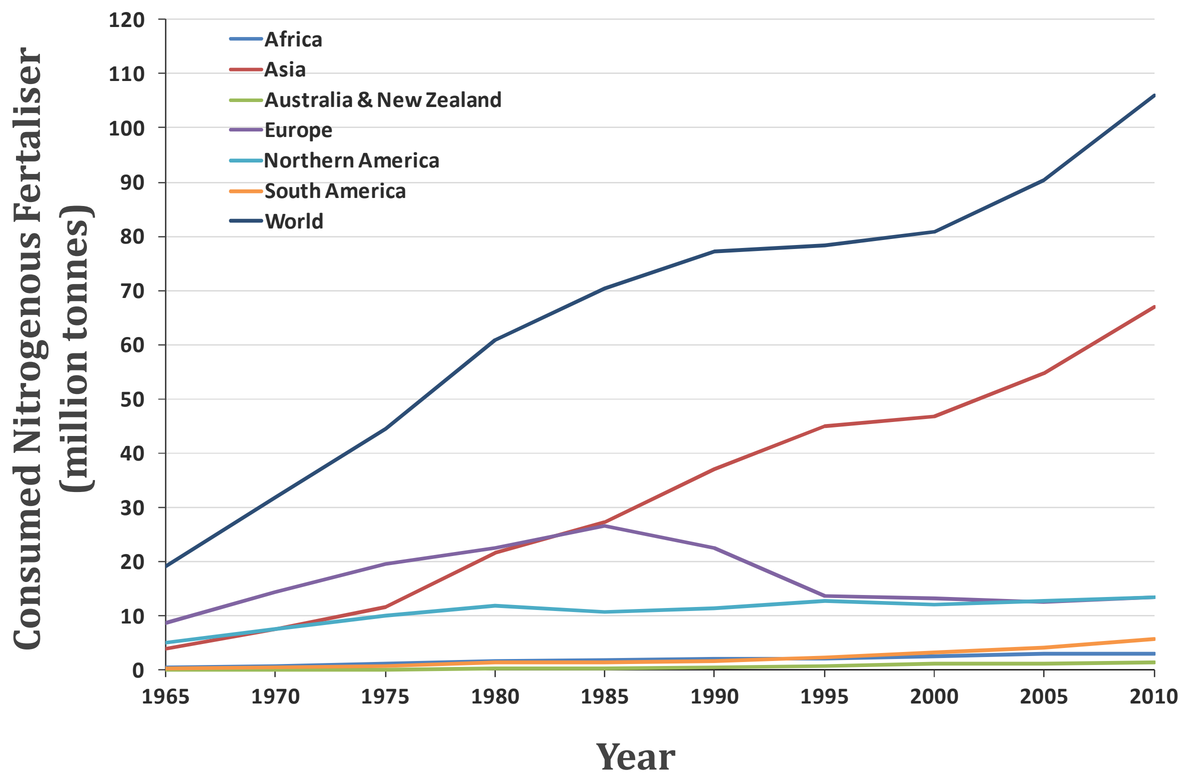

Figure 1. Global consumption of nitrogen fertiliser displayed per continent over a forty five year period, 1965-2010 (consumption in nutrients; tonnes; FAOSTAT, 2013). Data obtained from FAOSTAT.

1.3 Legume Crops as a Safe Alternative to Nitrogen Fertiliser

One safe alternative to the use of nitrogen-based fertilisers is to take advantage of biologically-fixed nitrogen. Legumes are able to form a relationship with specialised nitrogen-fixing soil bacteria, called rhizobia. The rhizobia convert atmospheric di-nitrogen into usable forms of nitrogen for the plant, whilst being housed in novel root organs, called nodules. The use of legumes as rotation crops is an important agricultural practice that many experts argue must be increased to help curb nitrogen fertiliser use (Jensen *et al.*, 2012; Sutton *et al.*, 2013; Ferguson *et al.*, 2013). Optimizing biological nitrogen fixation processes, such as nodulation, has the potential to increase crop yields and enhance soil fertility whilst simultaneously reducing farming costs and harmful environmental impacts (Hirel *et al.*, 2007; Peoples *et al.*, 2009; Canfield *et al.*, 2010; Ferguson *et al.*, 2013). However, it is only with an increase in our knowledge of nodulation processes and its genetic basis that we can fully reach this goal.

1.4 Nodule Organogenesis

The most common entry point for rhizobia invasion is the region of root where the root hairs are developing, called the Zone of Nodulation (ZON) (Bhuvaneswari *et al.*, 1981; Bhuvaneswari *et al.*, 1985; Calvert *et al.*, 1984; Hayashi *et al.*, 2012). Rhizobia attach to the root hair, triggering root hair deformation and curling (Yao *et al.*, 1969; Bhuvaneswari *et al.*, 1981; Bhuvaneswari *et al.*, 1981, 1985). This process involves the rearrangement of underlying microtubules which allow bacterial entry and the establishment of tubular structures called the infection threads (IT) (Gage, 2004; Ferguson *et al.*, 2010).

Occurring in parallel to rhizobia invasion are inner cellular changes which lead to nodule primordia formation (Ferguson *et al.*, 2010). The ITs full of rhizobia progress towards the nodule primordia. The convergence of the rhizobia in the ITs and the nodule primordia is essential for successful nodule formation. Once the rhizobia reach the developing nodule, they are released from the ITs into specialised structures called symbiosomes, in which they differentiate into bacteroids. Using their nitrogenase enzyme complex, the rhizobia bacteroids catalyses atmospheric N2 into ammonia, which can be used by the plant (Udvardi *et al.*, 1997).

There are two different morphological types of legume nodules: indeterminate and determinate. Indeterminate nodules are initiated by inner cortical cell divisions, followed by divisions in the endodermis and pericycle, and develop persistent meristems (Bond, 1948; Libbenga *et al.*, 1973; Newcomb, 1976; Newcomb *et al.*, 1979). Determinate nodules initially arise from cell division of the outer cortex and have transient meristems (Newcomb *et al.*, 1979; Turgeon *et al.*, 1982; Calvert *et al.*, 1984; Mathews *et al.*, 1989).

1.5 Signalling in Nodulation

Nodule formation is initiated via a highly-specific signal exchange between compatible rhizobia bacteria and legume plants (Ferguson *et al.*, 2003; Ferguson *et al.*, 2010; Hayashi *et al.*, 2013). Flavonoids are released into the soil by the plant, attracting compatible rhizobia species to the host plant. They also trigger the expression of rhizobia nodulation (*Nod*) genes, which leads to the production of novel Nod Factor (NF) signals that are recognized by the host plant (Dénarié *et al.*, 1996). Additional rhizobia-produced factors, such as exopolysaccharides (EPS) and lipopolysaccharides (LPS), are also known to be important for nodulation and in determining rhizobia-plant specificity.

Following perception of compatible partners, two main pathways are triggered within the plant: one involved in bacterial entry and infection and the other involved in cell divisions that lead to the formation of the nodule primordia (Ferguson *et al.*, 2010). Formation of a functional nodule requires synchronisation between these different signalling pathways. Many of the genes known to act in early nodulation are conserved between different legume species, but in many cases these orthologous genes have different names.

At the plant root periphery, LysM receptor kinases (*Lotus japonicus* Nod Factor Receptor 1 and 5; LjNFR1 and LjNFR5; soybean, *Glycine max* NFR1α/β and GmNFR5α/β; *Medicago truncatula* MtLYK3, MtLYK4 and MtNFP; and pea, *Pisum sativum* SYM2A and PsSYM10), perceive NF from compatible rhizobia (Limpens *et al.*, 2003; Madsen *et al.*, 2003; Radutoiu *et al.*, 2003; Arrighi *et al.*, 2006; Indrasumunar *et al.*, 2010; Broghammer *et al.*, 2012). These receptors are reported to associate with remorin proteins (MtSYMREM1), which may help in assembling a specialised NF receptor complex (Lefebvre *et al.*, 2010). Other factors, such as LjROP6, a Rho-like small GTPase, have also been found to interact with LjNFR5 (Ke *et al.*, 2012).

An additional receptor kinase (MsNORK/LjSYMRK/MtDMI2/ PsSYM19) also associates with SYMREM1 and appears to be involved in downstream signalling and possibly also the perception of NF (Lefebvre *et al.*, 2010). This receptor interacts with additional nodulation factors, including a coiled-coil protein (MtRPG; (Arrighi *et al.*, 2008), a transcription factor (LjSIP1; Zhu *et al.*, 2008) and a 3-hydroxy-3-methylglutaryl coenzyme reductase (MtHMGR1), which is involved in isoprenoid synthesis (Kevei *et al.*, 2007). However, the precise roles for these factors in nodulation are not yet known.

Perception of NF triggers Ca2+ fluxes, followed by the Ca2+ spiking, in root hair cells. The oscillation of Ca2+ in these cells is thought to initiate downstream signalling events (Felle *et al.*, 1999; Wais *et al.*, 2000; Walker *et al.*, 2000). Ca2+ spiking events require putative potassium ion-channels (MtDMI1, LjCASTOR and LjPOLLUX; Ané *et al.*, 2004; Imaizumi-Anraku *et al.*, 2005; Riely *et al.*, 2007), and two nucleoporins (LjNUP133 and LjNUP85; Kanamori *et al.*, 2006; Saito *et al.*, 2007). Acting downstream of the Ca2+ spiking, and likely perceiving the Ca2+ signal, is a calcium and calmodulin-dependent protein kinase (CCaMK; MtDMI3/PsSYM9; (Lévy *et al.*, 2004; Mitra *et al.*, 2004; Miwa *et al.*, 2006; Oldroyd *et al.*,

2004). Novel proteins shown to interact with CCaMK include LjCIP73, which belongs to the ubiquitin superfamily (Kang *et al.*, 2011), and MtIPD3/LjCYCLOPS, which regulates expression of *NSP1* (Smit *et al.*, 2005; Messinese *et al.*, 2007; Yano *et al.*, 2008). Following activation of CCaMK, a number of transcription factors, including Nodulation Signalling Pathways 1 (MtNSP1) and MtNSP2 (Kalo *et al.*, 2005; Smit *et al.*, 2005), Ets2 repressor factor (ERF), ERF required for nodulation (MtERN; Middleton *et al.*, 2007), and Nodule Inception (Lj/PsNIN; Schauser *et al.*, 1999; Borisov *et al.*, 2003) are activated. These transcription factors work in combination to activate the expression of the early nodulation (*ENOD*) genes in the epidermis (*e.g. MtENOD11*; Hirsch *et al.*, 2009).

CCaMK activation is also believed to trigger the increase in cytokinin level in these cells. Hormonal changes are detected by the cytokinin receptor, LjLHK1/MtCRE1, on the cortical cell membrane (Gonzalez-Rizzo *et al.*, 2006; Tirichine *et al.*, 2007). Activation of LjLHK1/MtCRE1 is thought to activate signalling within the cortical cells to initiate the cortical cell divisions required for the formation of the nodule (Gonzalez-Rizzo *et al.*, 2006; Tirichine *et al.*, 2007).

Other components that are essential for nodule development include SCAR/WAVE proteins that appear to have roles in root hair deformation and rhizobia infection (LjNAP1/MtRIT1 and LjPIR1; Yokota *et al.*, 2009; Miyahara *et al.*, 2010) and flotillin proteins that initiate the production of ITs for bacterial progression in the root (MtFLOT 2 and 4; Haney *et al.*, 2010). An ankyrin protein that may have a role in IT development (MtVAPYRIN) is also required (Murray *et al.*, 2011), in addition to a number of transcription factors (*e.g.* MtERF1 and EFD), U-box proteins (*e.g.* LjCERBERUS/MtLIN and MtPUB1) and early nodulin proteins of unknown function (*e.g.* ENOD11 and ENOD40 reviewed in Ferguson *et al.*, 2010). Further, a subunit of a signal peptidase complex (MtDNF1) that has a role in the processing of nodule specific cysteine-rich (NCR) proteins is an essential factor for rhizobia differentiation into nitrogen-fixing bacteroids in *M. truncatula* (Wang *et al.*, 2010).

1.6 Autoregulation of Nodulation

The formation and maintenance of nodules is an energy-intensive process. As such, the plant strictly regulates the number of nodules it forms, regardless of rhizobia availability, through the Autoregulation of Nodulation (AON). AON acts systemically, following nodule

development. Root-derived CLV3/ESR-related (CLE) peptide hormones are synthesised, called GmRIC1 and GmRIC2 in soybean, LjCLE-RS1 in *Lotus* and MtCLE12/13 in *Medicago* (Okamoto *et al.*, 2009; Mortier *et al.*, 2010; Lim *et al.*, 2011; Reid *et al.*, 2011a, 2013). These signals are predicted to travel to the shoot, presumably via the xylem (Delves *et al.*, 1986; Reid *et al.*, 2011a), where they are thought to be perceived by an LRR receptor kinase, GmNARK/LjHAR1/MtSUNN/PsSYM29 (Krusell *et al.*, 2002; Nishimura *et al.*, 2002; Searle *et al.*, 2003; Schnabel *et al.*, 2005). Mutants lacking a functional version of GmNARK/LjHAR1/MtSUNN/ PsSYM29 are unable to regulate their nodule numbers and exhibit a super- or hyper-nodulating phenotype (Figure 2). It is possible that this receptor acts in conjunction with other receptor components, such as Lj/PsCLAVATA2 and/or LjKLAVIER (Krusell *et al.*, 2002; Miyazawa *et al.*, 2010). Three additional factors, two Kinase-Associated Protein Phosphatases, GmKAPP1 and GmKAPP2 (Miyahara *et al.*, 2008) and a putative Ubiquitin Fusion Degradation protein, GmUFD1a (Reid *et al.*, 2012) have also been shown to possibly interact with GmNARK as part of the AON pathway.

Once the root-derived CLE peptide signal has been perceived, a novel Shoot-Derived Inhibitor (SDI) is produced which travels to the roots, presumably via the phloem, where it inhibits further nodulation (Ferguson *et al.*, 2010; Reid *et al.*, 2011b). Although SDI has yet to be identified, it has been shown to be NF dependent, heat stable, small (<1KDa) and unlikely to be a protein or RNA (Lin *et al.*, 2010; Lin *et al.*, 2011).

Nodulation is not only regulated by the number of nodulation events, but also in response to environmental factors such as stress (*e.g.* ethylene), soil acidity and soil nitrate (*e.g.* Carroll *et al.*, 1985; Lorteau *et al.*, 2001; Ferguson *et al.*, 2005a; Ferguson *et al.*, 2005b; Ferguson *et al.*, 2011; Lin *et al.*, 2012; Ferguson *et al.*, 2013). This gives the host plant the ability to regulate nodule development in response to its surrounding environment, thus optimizing nodulation and nitrogen-fixation under a variety of growing conditions.

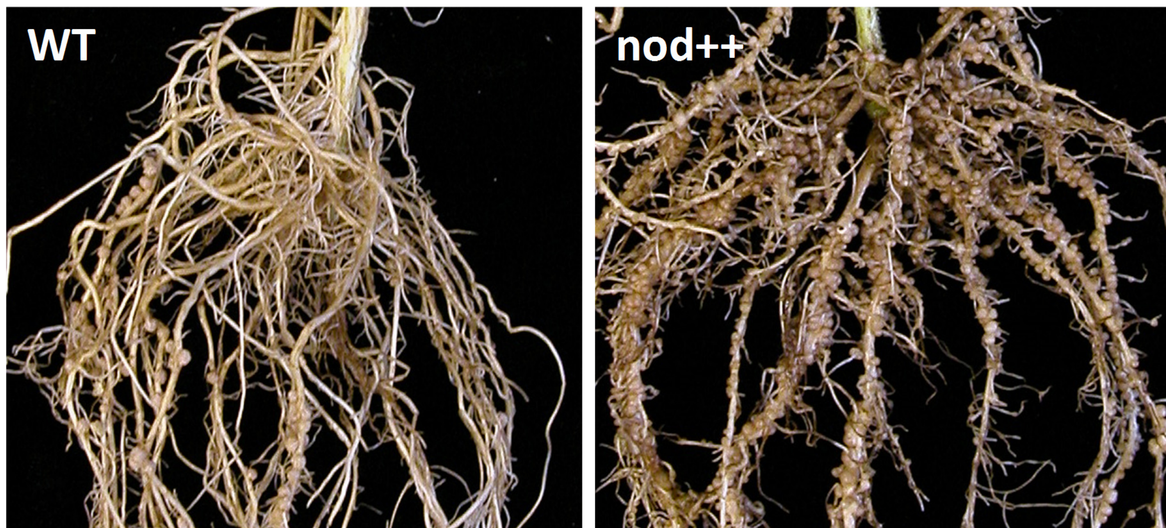

Figure 2. Root systems of wild-type (WT) and supernodulating mutant (nod++) soybean plants exhibiting mature nodule structures as a result of a symbiotic relationship with *Bradyrhizobium japonicum.*

1.7 The New Generation of Gene Discovery: RNA-seq

The identification of factors acting in the development and control of legume nodules has considerably increased our understanding of these processes. Moreover, it has provided novel targets for breeding and engineering programs dedicated to generating superior crop species. Recent technological advances have significantly increased the speed and efficiency with which new molecular components can be discovered. This includes new, high-throughput sequencing technology that has enabled the genomes of many legume species to be assembled in recent years, including soybean, *L. japonicus, M. truncatula*, chickpea and pigeon pea (Cannon *et al.*, 2009; Schmutz *et al.*, 2010; Young *et al.*, 2011; Varshney *et al.*, 2012; 2013). Similarly, next-generation RNA-sequencing (RNA-seq) technology enables the complete transcriptome of a given plant sample to be determined. This includes establishing the expression of both known and unknown genes in a sample. This cannot be achieved using other techniques, such as microarrays.

Soybean is one legume species that has recently been subjected to a number of RNA-seq studies seeking to identify new factors required for nodulation within its transcriptome. Indeed, soybean is often used as a model legume species (Ferguson *et al.*, 2009; Ferguson *et al.*, 2013), as it has had its complete genome sequenced (Schmutz *et al.*, 2010), with gene atlases and gene expression databases also being publically available (Libault *et al.*, 2010a; Libault *et al.*, 2010b; Severin *et al.*, 2010). It is also amenable to a number of molecular approaches, including

Agrobacterium rhizogenes-mediated transformation (*e.g.*, Kereszt *et al.*, 2007; Lin *et al.*, 2011), that are essential for follow-up research aimed at confirming and functionally characterising the role of candidate genes in nodulation. In addition, a number of mutant and TILLING populations are also available, which can considerably assist genetic studies (Carroll *et al.*, 1985; Bolon *et al.*, 2011; Cooper *et al.*, 2008; Batley J *et al.*, 2013). Physiologically, soybeans are also excellent for scientific purposes, being fast growing, high yielding, amenable to grafting and of appropriate size for most field and laboratory studies (Ferguson, 2013).

Soybean research is highly applicable to other legume crops including pea, lentil, chickpea, bean, peanut, lucerne, clover and faba bean (Rispail *et al.*, 2010). Soybean is also an important crop in its own right, with production of ~250 million tonnes globally in 2011, accounting for 50% of the world's oilseed production. It generates 200 kg N ha-1 in aboveground biomass each growing season with 58-68% of its nitrogen content resulting from symbiotic nitrogen fixation (Salvagiotti *et al.*, 2008; Peoples *et al.*, 2009; Jensen *et al.*, 2012). As a rotation crop, or "green manure", soybean can be ploughed back into the soil whether their seed has been harvested or not. This provides farmers with some flexibility and helps to replenish the soil nitrogen content, as in addition to the aboveground biomass, the roots and nodules contain 30-60% of the overall plant nitrogen content (Mahieu *et al.*, 2007; McNeill *et al.*, 2008).

Three separate studies have reported using RNA-seq to identify differentially-expressed genes in the transcriptome of rhizobia-inoculated soybean roots (Libault *et al.*, 2010a; Hayashi *et al.*, 2012; Barros de Carvalho *et al.*, 2013). The genes identified represent candidates required for nodule development. An additional study used RNA-seq to determine the transcriptome of soybean leaves to identify differentially-expressed gene candidates acting in AON (Reid *et al.*, 2012).

The work of both Libault *et al.* (2010a) and Hayashi *et al.* (2012) focused on the early stages of nodulation, with samples harvested 48 hours post inoculation Libault *et al.* (2010a) focused on root hairs and stripped roots, enabling a tissue-specific analysis to be conducted. In contrast, Hayashi *et al.* (2012) focused on the ZON of the tap root, enabling nodulation-specific transcripts to be concentrated by removing transcripts found throughout the remaining portion of the root system that are not specifically nodulation related. These studies both identified a number of new nodulation gene candidates. One such candidate, *GmNMNa*, has already been

followed up and confirmed to be involved in the regulation of rhizobia infection (Libault *et al.*, 2011).

Barros de Carvalho *et al.* (2013) also investigated the transcriptome of soybean root tissue; however, these authors focused their study on whole root systems harvested 10 days after rhizobia inoculation. The expression data from these samples showcase genes involved later in the nodulation pathway, including those involved in nodule maturation and growth.

To identify novel components functioning in the AON pathway, Reid *et al.* (2012) used RNA-seq to determine the transcriptome of soybean leaves. Leaf tissue was collected from soybean shoots fed with xylem sap taken from soybean plants that were either nodulating or non-nodulating. This led to the identification of the putative ubiquitin fusion degradation protein, *GmUFD1a*, whose product may interact with GmNARK in the regulation of legume nodulation.

1.8 Conclusion

Collectively, the four abovementioned datasets have led to the identification of numerous new gene candidates potentially having roles in the development and regulation of soybean nodules. The confirmation and subsequent functional characterization of these genes aids in the understanding of the signalling mechanisms involved in legume nodulation. Moreover, the identification of critical nodulation genes could one day help to benefit the isolation of superior cultivars for use in agriculture and help to reduce the over-application of nitrogen fertilisers in agriculture.

1.9 Acknowledgements

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End of "Advances in the identification of novel factors required in soybean nodulation, a process critical to sustainable agriculture and food security"

1.10 RNA-seq identifies a nodulation specific TIR-NBS-LRR

A high throughput RNA-seq study has revealed several early nodulation genes which are up-regulated in *G. max* roots in the ZON within 48 hours of inoculation with WT *B. japonicum* and not expressed after inoculation with the NF deficient mutant *nodC*$^-$ (Hayashi *et al.*, 2012). These genes include one encoding a Toll/Interleukin 1 receptor - Nucleotide Binding Site - Leucine-Rich Repeat (TIR-NBS-LRR; TNL): *GmTIR-NBS-LRR* (Glyma12g03040; Hayashi *et al.*, 2012).

TNLs are well-known to be involved in plant defense, but in the nodulation process at least two soybean TNL genes; *Rj2* and *Rfg1,* are known to enforce symbiosis specificity. *Rj2* restricts nodulation specific *B. japonicum* strains and *Rfg1* does so with *Sinorhizobium fredii* strains (Yang *et al.*, 2010). It is possible that *GmTIR-NBS-LRR* plays a similar role.

Here we investigated the role of *GmTIR-NBS-LRR* through promoter studies utilising the histochemical GUS staining method, gene silencing and over-expression techniques. All three studies are facilitated by hairy root transformation using *Agrobacterium rhizogenes* strain K599.

1.11 The role of plant hormones in nodulation

The roles of plant hormones in nodulation are often complex and intertwining. Cytokinin is known to be involved in nodulation and is vital to it. Its roles in nodulation are diverse and include; regulating cell division in the cortex, regulating auxin levels and an involvement in nodule maturation (reviewed in Ferguson *et al.* (2014)). Interestingly, cytokinin has recently been reported to also have a negative role in nodulation, acting in AON pathway to control nodule numbers (Sasaki *et al.*, 2014). Auxin, gibberellins (GAs) and brassinosteroids also all appear to have a role in nodulation: however, the exact nature of their roles is often unclear (Ferguson and Mathesius, 2003; Ferguson *et al.*, 2005a; Ferguson and Mathesius, 2014; Bensmihen, 2015). Conversely, abscisic acid, jasmonic acid, ethylene and salicylic acid are plant hormones which are suspected to be inhibitory to nodule formation (Oldroyd *et al.*, 2001; Suzuki *et al.*, 2004; Stacey *et al.*, 2006; Biswas, 2008; Ferguson and Mathesius, 2014). New techniques in gene identification such as RNA-seq, as well as improved methods for measuring endogenous levels of hormones, open up new avenues with which to explore how these important plant signals are utilised in nodulation.

1.12 The three GAs biosynthesis pathways; plants, fungi and bacteria

Bioactive GAs are involved in many plant development processes, including stem elongation, leaf expansion, flower and seed development and seed germination. While only five GAs are considered to be bioactive, namely GA_1, GA_3, GA_4, GA_5 and GA_7, hundreds of GAs have been identified in plants. These non-bioactive GAs are precursors or deactivated metabolites of the bioactive GAs. GAs are also found in fungi and bacteria, though their biosynthesis pathway differs from each other and that found in plants. GA_1 is the bioactive GA which has been found in the greatest variety of plant species, followed by GA_4 which is thought to be the major bioactive GA in *Arabidopsis thaliana,* whilst GA_3 and GA_7 seem to be less common (Yamaguchi, 2008; Davies, 2010).

Three enzyme classes are used throughout the GA biosynthesis pathway, terpene synthases (TPSs), cytochrome P450 monooxygenases (P450s) and 2-oxoglutarate-dependent dioxygenases (2ODDs). The pathway begins with geranylgeranyl diphosphate (GGDP) that is converted into *ent*-kaurene by two TPSs, *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS). Here, fungi and plants differ in their biosynthesis pathway as fungi possess a bifunctional CPS/KS, rather than separate CPS and KS (Rojas *et al.*, 2001; Figure 1-1). In plants, the *ent*-kaurene is metabolised by a P450, *ent*-kaurene oxidase (KO), producing *ent*-kaurenonic acid converted by P450 *ent*-kaurenoic acid oxidase (KAO) into GA_{12} (Nelson *et al.*, 2004). GA_{12} is a precursor to both GA_4 and GA_1; it is converted by three 2ODDs, GA 20-oxidase (GA20ox), GA 3-oxidase (GA3ox) and GA 13-oxidase (GA13ox). GA20ox converts GA_{12} through several oxidations to GA_{15}, GA_{24} and finally GA_9 where GA3ox converts GA_9 to the bioactive GA_4. In the case of GA_{12} becoming GA_1, GA13ox first converts GA_{12} to GA_{53}, from there GA20ox converts GA_{53} through GA_{44}, GA_{19} and GA_{20} before GA3ox converts GA_{20} into bioactive GA_1. GA3ox also converts GA_{20} into GA_5 and from there, into either GA_6 or bioactive GA_3 (Spray *et al.*, 1996; Itoh *et al.*, 2001; Appleford *et al.*, 2006; Figure 1-1).

In fungi, P450s, not 2ODDs, act at as GA3oxs, GA20oxs and GA13oxs, rather than 2ODDS, as in plants. GA_{12} is converted to GA_{14} by GA3ox, then into GA_4 by GA20ox (Hedden *et al.*, 2001; Tudzynski *et al.*, 2002). Recently, GA_4-desaturase was shown to be a 2ODDs; it is responsible for deactivation of GA_4 into GA_7 (Bhattacharya *et al.*, 2012). GA_7 is then converted into GA_3 by a GA13ox, another P450 (Hedden *et al.*, 2001; Tudzynski *et al.*, 2003). GA_1 is produced by conversion of GA_4 by GA13ox (Figure 1-1; Hedden *et al.*, 2001; Tudzynski *et al.*, 2003).

In plants, deactivation of both bioactive and non-bioactive GAs is primarily carried out by another 2ODD class, GA 2-oxidases (GA2ox). GA2oxs are separated into three classes. Class I and Class II are separated by phylogenetic relationships (Lee *et al.*, 2005) and both use C_{19}-GAs as substrates, while Class III uses only C_{20}-GAs (Lee *et al.*, 2005; Schomburg *et al.*, 2003). GA2oxs are responsible for the conversion of GA_{12} to GA_{110}, GA_9 to GA_{51}, GA_4 to GA_{34}, GA_{53} to GA_{97}, GA_{30} to GA_{29} and GA_1 to GA_8 (Thomas *et al.*, 1999). A P450 has also been shown to deactivate GA_4, GA_9 and GA_{12} in rice, by epoxidising the 16, 17-double bond of non 13-hydrosylated GAs (Zhu *et al.*, 2006). 16, 17-dihydrodiols occur in many plant species, thus it is suggested that 16α,17-epoxidation is a general GA deactivation mechanism (Zhu *et al.*, 2006; Yamaguchi, 2008). Gibberellin methyltransferases (GAMTs) have been found in *Arabidopsis* to deactivate both bioactive and non-bioactive GAs, though it is not yet known how they act in the pathway (Varbanova *et al.*, 2007).

The multiple 2ODDs involved in GA biosynthesis in plants are encoded by a multigene family. The individual genes are differentially regulated depending on different environmental and developmental cues (Yamaguchi, 2008). GA3ox genes *AtGA3ox1* and *AtGA3ox2* are not required for reproductive development, but do play both distinct and overlapping roles in vegetative development (Mitchum, Yamaguchi, Hanada et al. 2006; Mitchum *et al.*, 2006). As the major biosynthesis factors in the pathway, the 2ODDs also act a key regulator. It is the expression of the 2ODDs, rather than earlier pathway components, which affects the level of bioactive GA present in the system (Croker *et al.*, 1999; Huang *et al.*, 1998; Fleet *et al.*, 2003). Plants required that GA biosynthesis is tightly regulated as the optimum level of GA present in a given tissue is vital to normal growth and development (Bhattacharya *et al.*, 2012). In contrast, as fungi mostly use GA to manipulate plant behaviour, their biosynthetic pathway does not seem to possess such tight regulation (Bhattacharya *et al.*, 2012).

Rhizobia have been shown to produce and secrete bioactive and non-bioactive GAs into their surrounds when in liquid culture (Atzorn *et al.*, 1988; Marcassa, 2014). Multiple strains of *Rhizobium phaseoli,* including nodulation mutants, produce predominantly GA_1, GA_4 and GA_9 and in some strains GA_{20} in liquid culture (Atzorn *et al.*, 1988). *B. japonicum* strains E109, USDA110 and SEMIA5080 produce GA_3 in liquid culture, but all at differing levels to each other, with E109 producing the greatest amount of GA_3 and SEMIA5080 the least (Boiero *et al.*, 2007).

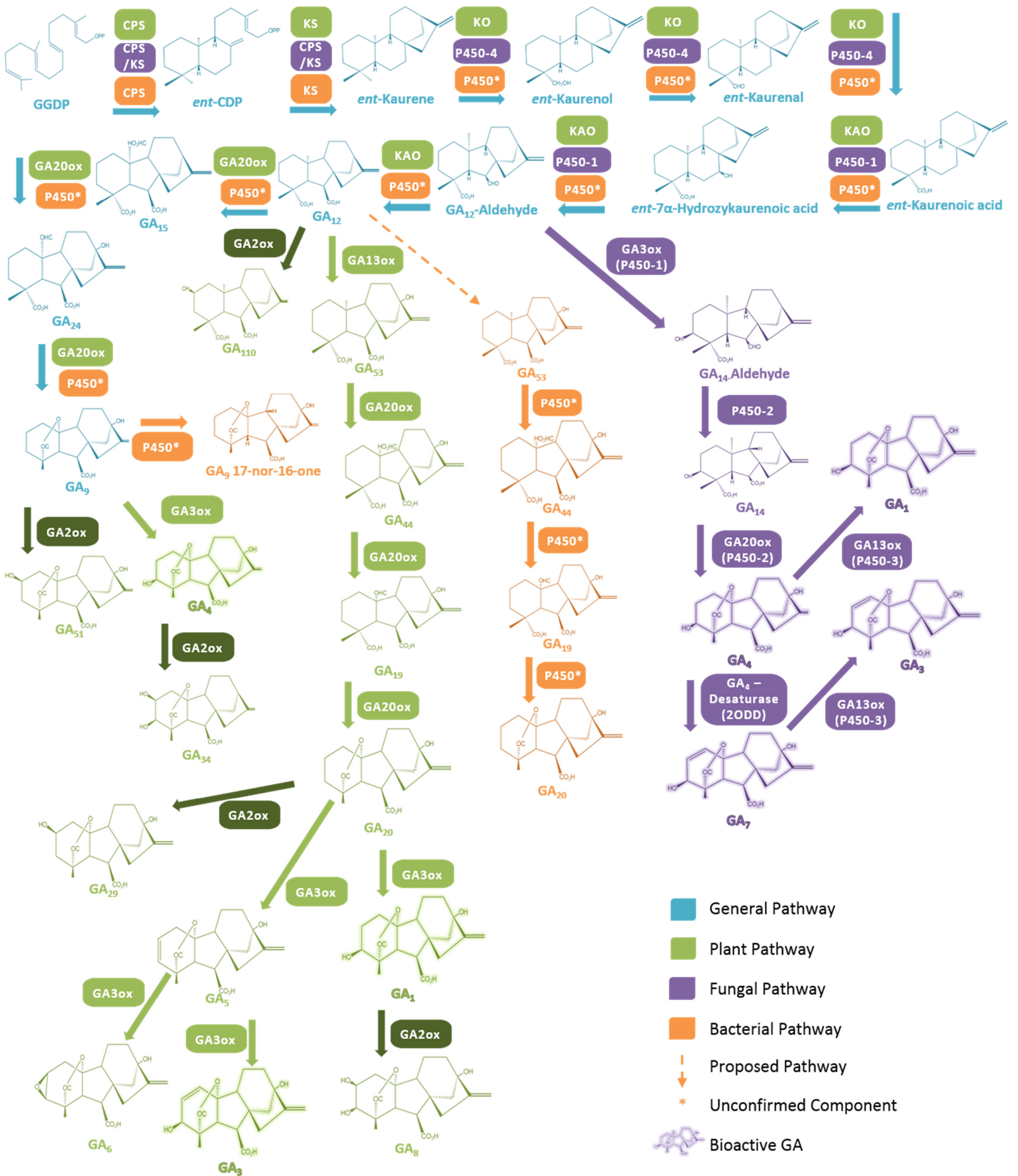


Figure 1-1 - Gibberellin biosynthesis pathways of plants (green), fungi (purple) and bacteria (orange) and shared pathway components (blue). Unconfirmed components are highlighted by * and proposed pathway steps are highlighted by a dashed arrow. Bioactive GAs are highlighted by a glow around the chemical structure.

While the GA biosynthesis pathway is mostly known for plants and fungi, it remains unclear in bacteria. The most studied microorganism in GA biosynthesis is *B. japonicum,* which has been shown to possess a diterpenoid biosynthetic operon that contains genes proposed to be involved in GA biosynthesis (Tully *et al.*, 1993; Morrone *et al.*, 2009; Hershey *et al.*, 2014). It is known that *B. japonicum* contains separate CPS and KS genes, similar to the system found in plants, rather than a bifunctional CPS/KS found in fungi (Morrone *et al.*, 2009). Similar to other rhizobacteria, the operon in *B. japonicum* contains only three P450s, differing from the four found in *Gibberella fujikuroi* but producing the same GA_3 (Morrone *et al.*, 2009). The operon also contains a short chain alcohol dehydrogenase, which indicates that, like fungal GA biosynthesis, it is possible that few or even no 2ODDs are involved in GA biosynthesis in *B. japonicum* (Morrone *et al.*, 2009). Given that *B. japonicum* shares some similarities in GA biosynthesis to both plants and fungi, it is suggested that the bacterial GA biosynthesis pathway has its own composition, thus resulting in three differing biosynthesis pathways.

Recent work by Méndez *et al.* (2014) has enabled the proposal of the GA biosynthesis pathway present in *B. japonicum* bacteroids. Given that this work did not demonstrate a production or even presence of GA_3, as was found in the liquid culture of *B. japonicum* by Boiero *et al.* (2007), it is likely that the biosynthesis pathway in bacteroids differs to that of the free-living bacterium (Méndez *et al.*, 2014). This is possibly due to the low O_2 environment of the nodule interior which induces specific GA biosynthesis enzymes (Méndez *et al.*, 2014). It has been suggested that bacteroids do not produce bioactive GAs directly, instead they secret GA_9 into the plant tissue, possibly to then be converted via the plant biosynthesis pathway into bioactive GAs (Méndez *et al.*, 2014).

The *B. japonicum* bacteroid biosynthesis pathway to GA_9 differs only from plants in that GA_9 then also converted to GA_9 17-nor-16-one (Figure 1-1; Méndez *et al.*, 2014). The exact components which act in the bacteroid biosynthesis pathway to convert GAs from one to another are not known; this presents another possible difference in the bacteroid pathway (Méndez *et al.*, 2014). However, it is currently assumed given what is known about the presumed GA biosynthesis operon and the fungal biosynthesis pathway, that P450s take the role of GA20ox and GA3ox (Méndez *et al.*, 2014). It is not yet known if the bacteroid pathway to GA_{53} is similar to that in plants, disregarding the P450/ODD difference. However, given the similarities in the GA_9 pathway, it is proposed to be similar (Figure 1-1). If the plant pathway to GA_{53} is similar to that of bacteroids, then the pathway to GA_{20} is also similar. Both bacteroids

and plants process through GA_{53} to GA_{44} then GA_{19} to GA_{20}; however, it has not been shown that bacteroids continue from GA_{20} to bioactive GA_1 or GA_5 (Méndez *et al.*, 2014).

1.13 A possible role for GA in nodulation

Endogenous GA levels in the roots and nodules of various legume species have been measured in the past; however, these studies were conducted using the technology of the time, which was often unreliable. Therefore, few studies have made use of techniques such as high performance liquid chromatography (HPLC) or ultra performance liquid chromatography (UPLC) coupled with mass spectrometry (MS) (Hayashi *et al.*, 2014).

Studies of endogenous GA in both *Phaseolus lunatus* (lima bean) and *Vigna unguiculata* (cowpea) were conducted using a combination of HPLC and gas chromatography-mass spectrometry. *P. lunatus* nodules were found to contain GA_1, GA_3, GA_{19}, GA_{20}, G_{29} and GA_{44}. The presence of GA_4 was specifically investigated in these nodules but it was not found (Dobert *et al.*, 1992a). In *V. unguiculata* nodules, GA_4, GA_7, GA_{17} and GA_{53} were specifically not found to be present while GA_{20}, GA_{44}, GA_{29}, GA_1 and GA_{19} were found in respective descending order of abundance. It is also likely, though not definitive, that GA_3 was present also (Dobert *et al.*, 1992b).

GA_1 is the main bioactive GA present in *P. sativum* (pea) roots, with GA_{20} also found to be present (Yaxley *et al.*, 2001). In the taproot tip GA_{20}, GA_1, GA_{29} and GA_8 were found. GA_3 and GA_4 were not found in any root samples (Yaxley *et al.*, 2001). GA_{19}, GA_{20}, GA_1, GA_{29} and GA_8 have also been found in *P. sativum* roots via Gas Chromatography/Mass Spectrometry using Selected Ion Monitoring (GC/MS-SIM) (Weston *et al.*, 2009).

In contrast to *V. unguiculata* and *P. lunatus*, *P. sativum* was found to not possess either GA_3 in its roots or nodules, while all three species were devoid of GA_4. It is hypothesised that the differences in presence of bioactive GA types is related to the development of indeterminate or determinate nodules, as *V. unguiculata* and *P. lunatus* both form determinate nodules, whilst *P. sativum* forms indeterminate nodules (Hirsch, 1992).

The most recent work measuring endogenous GA levels in *G. max* roots, amazingly some 33 years ago, was by Williams *et al.* (1982) via thin layer chromatography (TLC). It was shown that GA levels were higher in nodules than in the roots.

Little other work has been carried out to measure GA levels in soybean roots, and certainly no modern techniques have been used. Here, we measure the endogenous GA levels in soybean roots 16 hours post-inoculation (hpi) and 6 days post-inoculation (dpi), inoculated either with WT *B. japonicum* (USDA110) or its NF deficient mutant *nodC*$^{-}$.

As mentioned above, tight regulation of GA levels is important in maintaining normal growth and development. This seems to be especially true in nodulation and has been demonstrated through the study of GA biosynthesis mutants and through application of GA and GA inhibitors (Ferguson *et al.*, 2005a; Ferguson *et al.*, 2011; Ferguson *et al.*, 2014; Maekawa *et al.*, 2009). These studies suggest that GA is not an absolute promoter or inhibitor of nodulation, but that optimal concentrations of the hormone differ by plant species and growth conditions.

The results of Maekawa *et al.* (2009) showed that high concentrations of exogenous GA_3 can have a negative effect on nodule number in *Lotus japonicus*. Similarly, combined application of GA_3 and Uniconazole P (Uni P), a GA biosynthesis inhibitor, minimised the negative effect of the exogenous GA_3, presumably because the total GA concentration was not elevated to high inhibitory levels. High GA concentration not only inhibited nodule number, but the number of infection threads formed per plant.

In *P. sativum* the *na* mutant has reduced GA content of the whole plant and forms few to no nodules, with those that form being aberrant (Ferguson *et al.*, 2005a). Application of exogenous GA_3 at concentrations of 10^{-6} M to *na* plants restored the WT phenotype. Not only this but small doses of GA_3, 10^{-9} M and 10^{-6} M, to the NA plants resulted in increased nodule number with 10^{-9} M leading to a higher increase. Application of an exceedingly high concentration of GA_3, 10^{-3} M, saw reduced nodule number for both *na* and NA plants as compared to the numbers obtained at 10^{-6} M (Ferguson *et al.*, 2005a). Double mutants of *na* and supernodulating mutants *sym28*, *sym29* and *nod3* produced aberrant nodules in supernodulating numbers (Ferguson *et al.*, 2011). This suggests that low endogenous GA is not completely inhibitory of nodule initiation, but higher concentrations are needed for successful nodule development (Ferguson *et al.*, 2011).

Lievens *et al.* (2005) utilised exogenous application of GA biosynthesis inhibitor chlormequat chloride (CCC) on *Sesbania rostrata* at various stages post- and pre-inoculation to determine when GA is required for nodule development. CCC applied pre-inoculation resulted in almost complete inhibition of nodulation while applications 1, 2, 3 and 4 dpi had progressively less

inhibition. Application of 10^{-5} M exogenous GA_3 post-inoculation and CCC treatment showed partial rescue of nodulation (Lievens *et al.*, 2005).

These physiological studies lead Hayashi *et al.* (2014) to conclude that GA is required not only at specific levels for successful nodulation, levels which differ depending on legume species, but it is also involved in both nodule initiation and nodule maturation.

The high throughput RNA-seq study by Hayashi *et al.* (2012) identified several GA biosynthesis genes which are up-regulated in *G. max* roots in the ZON. These genes include a GA20ox (*GmGA20ox a,* Glyma04g42300.1), GA3ox (*GmGA3ox 1a,* Glyma15g01500.1) and GA2ox (*GmGA2ox*, Glyma02g01330.1) (Hayashi *et al.*, 2012, 2014).

This thesis aims to further build on the current knowledge of early nodulation genes as well as the role of GA in nodulation. Molecular techniques such as silencing, over-expression and promoter studies will be carried out to characterise the early nodulation genes *GmGA20ox a*, *GmGA3ox 1a*, *GmGA2ox* and *GmTIR-NBS-LRR.* Additionally, endogenous GA levels in soybean roots will be studied for the first time using modern techniques.

Chapter 2 General Methods

2.1 Plant and rhizobia growth

All experiments were untaken utilising either WT soybean (*G. max* (L.) Merr. cv. Bragg) or supernodulating mutant variety *nts382*. Plants were either grown in temperature controlled glass houses or environmentally-controlled growth cabinets (L/D = 16/8 h; T = 28/25°C and 70% humidity). Seeds were surface sterilised with 70% ethanol for 20 seconds and rinsed with water 6 times before being planted in sterile grade 2 vermiculite. Plants were watered and given B&D nutrient solution (Broughton *et al.*, 1971) three times a week, as well as 1 mM KNO_3.

All rhizobia inoculations were using either *Bradyrhizobium japonicum* strain USDA110 or NF deficient mutant strain *nodC*$^-$. Cultures were grown in YMB medium (Vincent, 1970) at 28°C, shaking, for 3 days. Cultures were diluted to an OD600 ≈ 0.1 with water before being used for inoculation.

Transformation of soybean by the hairy root method was carried out as described in Kereszt *et al.* (2007). This includes growing transformed plants in growth cabinets inside well-sealed seedling trays with lids. Plants were inoculated approximately 1 week following the transfer of plants from growth trays into pots.

2.2 Histochemical β-glucuronidase (GUS) staining for promoter studies

Plants were grown and transformed using the hairy root method as stated in chapter 2.1. Three controls were used for this study regardless of which gene was the focus. A positive control was used in the form of a β-glucuronidase (GUS) vector carrying the 35S promoter derived from the cauliflower mosaic virus (CaMV), it was termed p35S::GUS. A negative control in the form of a GUS vector including no promoter to drive the GUS gene was used, it was termed no promoter::GUS. These two control vectors were created by Ms Dongxue Li (CILR, The University of Queensland). The third control differed depending on the gene in question.

The pGmTIR-NBS-LRR::GUS vector carried a 2620 kb promoter fragment located directly upstream of *GmTIR-NBS-LRR* and cloned via primers; 5'-aagcttATTGAAACTATGGTTGAGTTCCCATC-3'with restriction enzyme *Hind*III and 5'-agatctACCATGATTGTATTGTAGTAGCACTGC-3' with restriction enzyme *Bgl*II. The pGmGA3ox 1a::GUS vector carried a 2926 kb promoter fragment located directly upstream of *GmGA3ox 1a* and cloned via primers; 5'-ggtaccTTTCCACTTTGCTATGTTGCTCAATTA-3' with restriction enzyme *Bam*HI and 5'-

agatctACCATAATAGTGTGGAACAAATAGTGACCA-3' with restriction enzyme *Bgl*II. The pGmGA20ox a::GUS vector carried a 2648 kb promoter fragment located directly upstream of *GmGA20ox a* and cloned via primers; 5'-ggtaccTTTCCACTTTGCTATGTTGCTCAATTA-3' with restriction enzyme *Hin*dIII and 5'-agatctACCATCGTCTCACGTTAATTGTGTT-3' with restriction enzyme *Bgl*II. Plants were transformed using a GUS vector carrying the gene of interest's promoter, but were then inoculated with *nodC*$^{-}$ *B. japonicum*.

Following harvest (which occurred at various time points), roots were vacuum infiltrated on ice with 0.5% paraformaldehyde for 30 minutes. Roots were then washed twice in 100 mM sodium phosphate buffer before being shaken for 30 minutes in clean 100 mM sodium phosphate buffer at room temperature. Roots were then immersed in GUS solution (final pH 7, 5 ml 100 mM potassium ferricyanide, 5 ml 100 mM potassium ferrocyanide, 200 ml 0.5 M sodium phosphate buffer, 1 ml Triton-X, 10 ml 0.5 M EDTA in 1 L MiliQ water) for 24 hours at 37°C for staining. Post staining, roots were fixed through an ethanol gradient, being shaken for 1 hour at 10%, 25%, 50% and 70% ethanol with each concentration being changed to fresh solution after 30 minutes. Samples were then kept at 4°C.

2.3 Endogenous GA content analysis of plants and rhizobia

Plants were grown as per chapter 2.1. Following this, the plants were harvested at various time points with the appropriate amount of root being excised and frozen in liquid nitrogen. Roots samples were pooled to create multiple biological replicates, samples were then ground whilst frozen and freeze dried at -80°C, 100 µbar for 48 hours. Samples were then handled by Dr Dana Tarkowska (Palacky University and Institute of Experimental Botany ASCR) who analysed the samples using the technique described in Urbanová *et al.* (2013).

2.4 Vector Construction – promoter::GUS fusion

Two promoter::GUS vectors, utilising the binary vector pCAMBIA1305.1 (GenBank: AF354045) and containing either the promoter of *GmGA3ox 1a* or *GmTIR-NBS-LRR,* were constructed by Dr Satomi Hayashi (CILR, University of Queensland) as described in Hayashi (2014). However, these vectors contained a double 35S promoter driving a hygromycin resistance gene, which were bidirectional causing over-expression of the GUS gene. Ms Dongxue Li (CILR, The University of Queensland) created a modified pCAMBIA1305.1 vector which contained no 35S promoters. In this study the promoter of both genes was excised from the original pCAMBIA1305.1 vectors by double digest and ligated into the modified

pCAMBIA1305.1 vector with T4 DNA ligase (New England Biolabs). Following promoter sequence verification, the new vectors were electroporated into *A. rhizogenes* K599 and selected via antibiotic resistance to rifampicin and kanamycin. *A. rhizogenes* carrying either of the binary vectors was then used in hairy root transformation as described in chapter 2.1.

Chapter 3 Characterisation of a Nodulation Specific TIR-NBS-LRR

3.1 Abstract

The *GmTIR-NBS-LRR* gene is nodulation-specific in its expression and thought to be involved in host range specificity as other TNL *G. max* genes are. Through over-expression, RNAi and histochemical promoter studies we aimed to further characterise this gene. No phenotypic changes occurred in any of the experiments, which was likely due to the TVs and possible APs of the gene whose existence was unknown at the time of vector construction. Investigation into the nature of the TVs will allow for clearing characterisation of this gene moving forward.

3.2 Introduction

Control of legume host specificity in nodulation is a process that shares its mechanisms with plant defence (Yang *et al.*, 2010). Both symbiotic and pathogenic bacteria use surface polysaccharides or secreted proteins to evade or suppress host defences (D'Haeze *et al.*, 2004; Jones *et al.*, 2008; Deakin *et al.*, 2009; Soto *et al.*, 2009). Such polysaccharides have also been identified as being involved in infection thread formation (Jones *et al.*, 2008). NF also plays a significant role in host range as its strain-specificity allows for recognition by the compatible Nod-factor receptor (NFR) (Geurts *et al.*, 1997; Perret *et al.*, 2000; Limpens *et al.*, 2003; Radutoiu *et al.*, 2003; Radutoiu *et al.*, 2007). Introducing the *Lotus japonicus* NFR1 and NFR5 genes into *Medicago truncatula* allowed the modified plant to nodulate with *L. japonicus'* symbiont (Radutoiu *et al.*, 2003; Radutoiu *et al.*, 2007).

Type III secretion systems (T3SS) are also common to both symbiotic and pathogenic bacteria. In rhizobia that possess T3SS, effectors called nodulation out proteins (Nops) are delivered into the host cells (Deakin *et al.*, 2009). If the correct *R* gene is not present in the plant then the effectors can disrupt the host's defence and metabolism by acting on the cytoskeleton or intracellular signalling (Cornelis, 2000). Intriguingly, T3SSs are compulsory for diseases but not for determining the symbiotic host range, which is dependent on genotype (Yang *et al.*, 2010). It is presumed that legumes possess a T3SS recognition mechanism that allows nodulation to progress when compatible effectors are recognised (Yang *et al.*, 2010). Understanding of the various methods of compatible rhizobia recognition by the plant may allow for the modification of superior nitrogen-fixing bacteria to carry preferred T3SSs or similar in order to out-compete inferior but highly competitive native rhizobia in the field (Yang *et al.*, 2010). *B. japonicum* USDA110 has been shown to possess genes which are known to form a T3SS (Göttfert *et al.*, 2001; Kaneko *et al.*, 2002a,b). It is unknown how Nops are involved in nodulation; however, it is proposed that the legume plant must possess the correct *R* gene to recognise and respond to the cocktail of effectors (Skorpil *et al.*, 2005).

Plant defence can also involve a gene-for-gene interaction between pathogen avirulence (*Avr*) genes and plant resistance (*R*) genes. The R gene recognises its specific *Avr* counter-part and triggers a hypersensitive response intended to halt pathogen growth. Many R genes are from the NBS-LRR gene family and either carry a TIR domain or a coiled-coil (CC) domain (Ameline-Torregrosa *et al*., 2008). The NBS domain is responsible for binding and ATP and GTP hydrolysis (Tameling *et al*., 2002) while the LRR is normally involved in protein-protein interactions and likely responsible in part for the specificity of the *R* gene (Kobe *et al*., 1995;

Leister *et al.,* 2000). Lastly, the TIR domain interacts with effector molecules (Axtell *et al*., 2003). TNL genes *Rj2* and *Rfg1* are known to enforce symbiosis specificity, *Rj2* with nodulation specific *B. japonicum* strains and *Rfg1* with *S. fredii* (Yang *et al*., 2010). Both *Rj2* and *Rfg1* enforce specificity by inhibiting nodulation by various strains. It does not appear that they act by allowing nodulation of a compatible strain, thus they act to negatively-regulate nodulation by incompatible rhizobia (Yang *et al*., 2010).

It is possible that the *GmTIR-NBS-LRR* gene plays a similar role to *Rj2* or *Rfg1*. This gene was identified to be highly NF dependently up-regulated in an RNA-seq study carried out by Hayashi *et al.* (2012). Quantitative reverse-transcription PCR (qRT-PCR) analysis identified maximum expression at 24 hpi and a tapering off of expression at 48 hpi onwards (Figure 3-1). qRT-PCR analysis of the gene's expression in multiple tissues of *G. max* found that it was almost exclusively nodule specific with highest expression found at 3 wpi (Figure 3-2; Hayashi *et al.*, 2012). This indicates that *GmTIR-NBS-LRR* plays a role in early nodulation, possibly in symbiont recognition, but also possibly has a role in the mature nodule.

In this chapter characterisation of *GmTIR-NBS-LRR* is carried out through molecular studies, including the use of the over-expression and RNA interference (RNAi) vectors created by Hayashi (2014; Table 3-1). Additionally, a modified version of the promoter::GUS vector pGmTIR-NBS-LRR::GUS is used in a histochemical study to determine the cell specific expression of this TNL gene (Table 3-1).

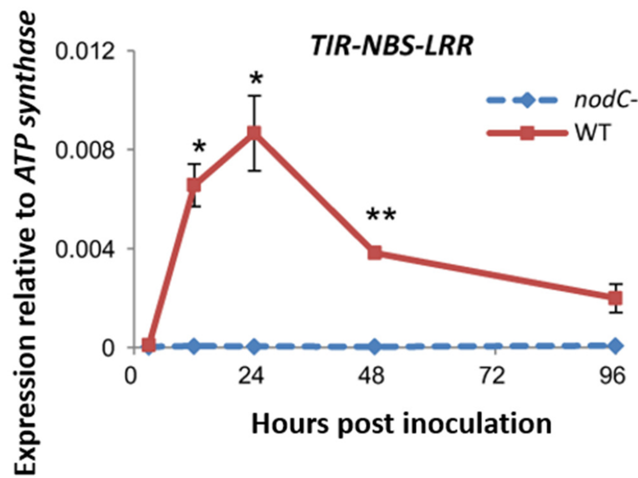


Figure 3-1 - qRT-PCR measurement of expression of *GmTIR-NBS-LRR*, 0, 12, 24, 48 and 96 hpi with either WT (red line) or *nodC*⁻ (blue line) *B. japonicum* (Hayashi, 2014).

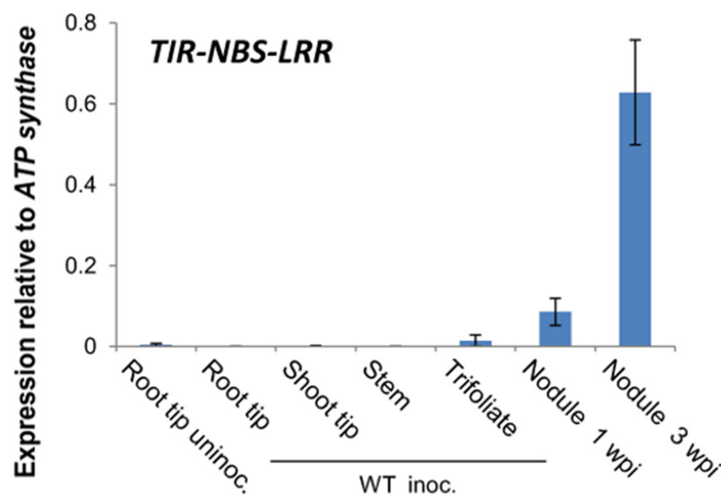


Figure 3-2 - qRT-PCR analysis of expression of *GmTIR-NBS-LRR* in various tissues of *G. max* (Hayashi et al. 2012).

Table 3-1 Primers and restriction enzyme sites of the constructs made by Dr Satomi Hayashi (CILR). Lowercase letters represent the RE site, bold letters represent the start codon of the GUS gene, underlined letters represent the nucleotide added to the sequence in order to keep a distance between the promoter and translational start site (Hayashi 2014).

Construct	Primers	Restriction Enzyme Site
***GmTIR-NBS-LRR* over-expression**	5'-ctcgagTGCTACTACAATACAATCATGGCAAATG-3'	*Xho*I
	5'-ggtaccTGCTAGTTAACCTGAGACCATAAGATGTTT-3'	*Kpn*I
GmTIR-NBS-LRR RNAi	(S) 5'-ctcgagAACTTACTGGGCTTAACTATCTTCACATTG-3'	*Xho*I
	(S) 5'-ggtaccTAGTAGATTGTTTAATGTGTGCTGGGAGAG-3'	*Kpn*I
	(A) 5'-tctagaACTTACTGGGCTTAACTATCTTCACATTG-3'	*Xba*I
	(A) 5'-aagcttAGTAGATTGTTTAATGTGTGCTGGGAGAG-3'	*Hin*dIII
pGmTIR-NBS-LRR::GUS	5'- aagcttATTGAAACTATGGTTGAGTTCCCATC-3'	*Hin*dIII
	5'-agatct<u>A</u>C**CAT**GATTGTATTGTAGTAGCACTGC-3'	*Bgl*II

3.3 Methods

3.3.1 *GmTIR-NBS-LRR* RNAi

Supernodulating *nts382* plants were grown, inoculated with *B. japonicum* USDA110 and transformed as outlined in chapter 2.1. Silencing of *GmTIR-NBS-LRR* was done utilising the *GmTIR-NBS-LRR* RNAi vector created by Dr Satomi Hayashi (CILR, The University of Queensland; Hayashi, 2014), chapter 3.2. Supernodulating plants were used to magnify the possible effects of gene silencing. Plants were harvested at 3 wpi with nodule number and root weight recorded on an individual root basis.

3.3.2 *GmTIR-NBS-LRR* over-expression

Supernodulating *nts382* plants were grown, inoculated with *B. japonicum* USDA110 and transformed as outlined in chapter 2.1. Over-expression of *GmTIR-NBS-LRR* was done utilising the *GmTIR-NBS-LRR* over-expression vector created by Dr Satomi Hayashi (CILR, The University of Queensland; Hayashi, 2014), chapter 3.2. Supernodulating plants were used to magnify the possible effects of over-expressing the gene. Plants were harvested at 3 wpi with nodule number and root weight recorded on an individual root basis.

3.3.3 GmTIR-NBS-LRR promoter study

A pGmTIR-NBS-LRR::GUS vector was created as described in chapter 2.4 utilising the following primers and restriction enzymes to excise the promoter, digest the modified pCAMBIA1305.1 vector and verify the sequence; 5'- aagcttATTGAAACTATGGTTGAGTTCCCATC-3'with restriction enzyme *Hin*dIII and 5'-agatctACCATGATTGTATTGTAGTAGCACTGC-3' with restriction enzyme *Bgl*II.

Plants were originally grown under growth lights on the laboratory bench, this lead to unsuccessful development of hairy roots most likely due to excess heat generated from the lighting. Newly transformed plants were then grown under glasshouse conditions; however, this led to unsuccessful hairy root formation most likely due to insufficient humidity. Successful growth and transformation was finally achieved through following growth and transformation conditions detailed in chapter 2.1. After multiple attempts at germinating the supernodulating *nts382* seeds without success, the sterilisation step detailed in chapter 2.1 was removed. Controls are described in chapter 2.2. Plants were then harvested at 12 hpi, 48 hpi or 3 wpi and root were subjected to histochemical staining as described in chapter 2.2. Supernodulating plants were used to obtain an increased number of infection events.

3.4 Results

3.4.1 *GmTIR-NBS-LRR* RNAi

When comparing *GmTIR-NBS-LRR* RNAi silenced hairy roots to control roots, no significant differences were found (p>0.05) (Figure 3-3). To gain an accurate portrayal of nodule number, nodule number/root fresh weight was examined to account for root size.

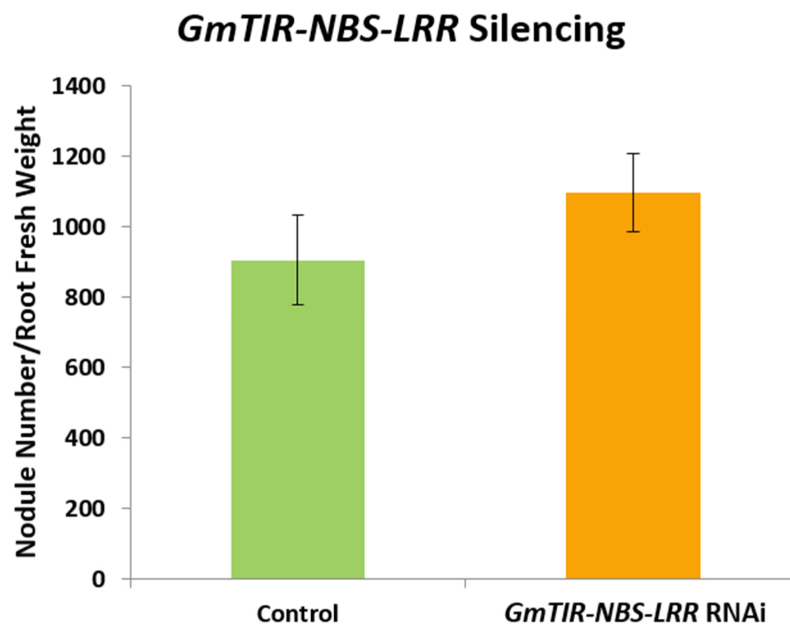


Figure 3-3 - Comparison of nodule number of control and *GmTIR-NBS-LRR* RNAi silenced hairy root transformed supernodulating *nts382* soybean mutants. Nodule number is analysed on an individual root fresh weight basis. Confidence intervals were used in the formation of error bars. This graph shows no significant difference and the transformed roots (P>0.05).

3.4.2 GmTIR-NBS-LRR over-expression

When comparing 35S::GmTIR-NBS-LRR over-expressing hairy roots to control roots, no significant differences were found ($p>0.05$) (Figure 3-3). To gain an accurate portrayal of nodule number, nodule number/root fresh weight was examined to account for root size.

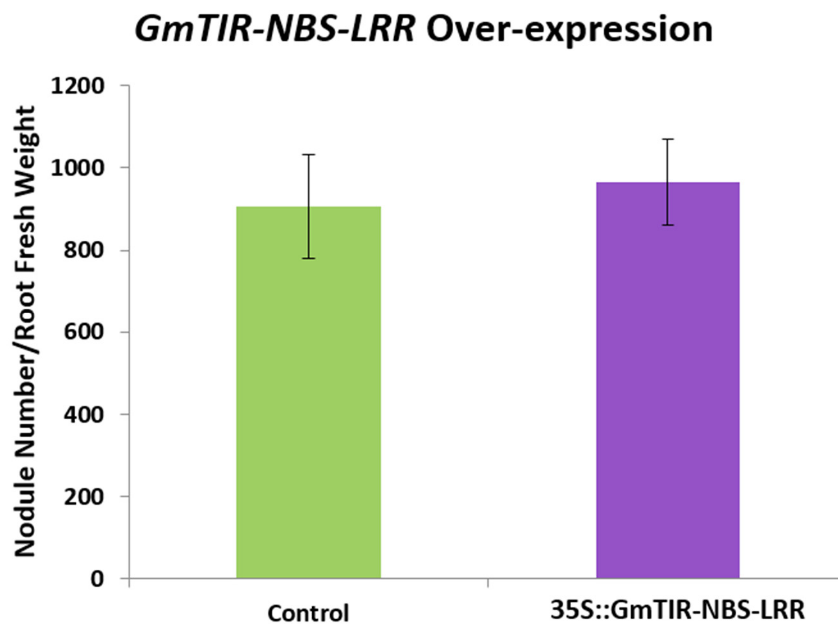


Figure 3-4 - Comparison of nodule number of control and 35S::GmTIR-NBS-LRR hairy root transformed supernodulating *nts382* soybean mutants. Nodule number is analysed on an individual root fresh weight basis. Confidence intervals were used in the formation of error bars. This graph shows no significant difference and the transformed roots (P>0.05).

3.4.3 *GmTIR-NBS-LRR* promoter study

Supernodulating hairy root plants transformed with the GmTIR-NBS-LRR::GUS vector did not show any GUS staining at any time point (results not shown). Control over-expressing hairy roots transformed with the 35S::GUS vector did show GUS staining in all cell types (results not shown).

3.5 Discussion

After the conclusion of the above studies, it was discovered by Hayashi (2014) that the sequence for *GmTIR-NBS-LRR* which had been obtained from Phytozome (http://phytozome.jgi.doe.gov/pz/portal.html) and subsequently used to construct the over-expression and RNAi vectors, and to inform the proposed promoter region for the promoter::GUS vector, had been re-annotated. Originally it was thought that *GmTIR-NBS-LRR* had a 3781 bp genomic sequence, 2667 bp CDS and a protein sequence 888 amino acids long. The new annotation, now named Glyma12g03040.2 in the Phytozome database, contains an additional exon, 834 bp long. This means that the previously predicted stop codon is actually positioned in an intron.

Further investigation by Hayashi (2014) found that an additional transcript variant (TV), *Glyma12g03040.3*, is transcribed. This sequence is truncated due to its transcription through the 2nd intron which produces a premature stop codon. The protein sequence of *Glyma12g03040.3* is only 543 bp long. This TV lacks the LRR domain but does possess the TIR and NBS domains. The primers originally used by Hayashi *et al.* (2012) to measure expression of the *GmTIR-NBS-LRR* gene by qRT-PCR amplified all three transcripts. New primers measuring *Glyma12g03040.1/2* and *Glyma12g03040.3* separately found *Glyma12g03040.3*, transcription levels to be 50 fold lower than that of *Glyma12g03040.1/2*.

It is quite common for TNLs to have multiple transcript variants (Zhang *et al.*, 2003; Zhang *et al.*, 2007). Truncated genes, alternative splicing (AS) and alternative promoters (AP) are all possible methods or combinations of methods for achieving the various variants (Xin *et al.*, 2008). TNLs often have alternative transcripts which lead to truncated genes containing only the TIR-NBS domains (Jordan *et al.*, 2002). The results of the over-expression, RNAi and promoter::GUS studies, coupled with the new information from Hayashi (2014), point to *GmTIR-NBS-LRR* likely possessing both AS and AP.

Over-expression of one variant might not affect change at the phenotypic level as ratios of TV are often important to achieving a working complex. The *RPS4* gene, a TNL in *A. thaliana*, requires both its full transcript and its truncated alternative transcripts to convey full resistance to the *Pseudomonas syringae* pv tomato strain DC3000 (Zhang *et al.*, 2003). Additionally, the *GmTIR-NBS-LRR* sequence in the over-expression vector does not possess the newly discovered fifth exon and so is unlikely to be producing a functional product. Thus, seeing no phenotypic change in nodulation is due to ineffective over-expression of the target gene. Once

the TVs of *GmTIR-NBS-LRR* are better understood, over-expression of the gene could be repeated after varying the up-regulated sequence.

Similarly, as there was no knowledge of the TVs prior to the creation of the RNAi vector, the sequence used targets only the *Glyma12g03040.1/2* sequence as it is located in the 4th exon. As the truncated *Glyma12g03040.3* stop prematurely in the 2nd exon, its transcript abundance would likely have been unaffected. Truncated TVs can convey some degree of resistance in defence related TNLs, it is possible that the unaffected truncated transcript, coupled with the impact of using supernodulating plants, was able to compensate for the possibly silenced *Glyma12g03040.1/2* transcript, thus resulting in no phenotypic change to nodule number.

It is often the case that gene with transcript variants also have AP (Xin *et al.*, 2008). An AP can change tissue specificity, developmental activity, transcription activity and the presence of TV. Given that no GUS staining was observed in roots transformed with the pGmTIR-NBS-LRR::GUS vector, it is possible that *GmTIR-NBS-LRR* also possesses APs. This lack of knowledge when altering the pGmTIR-NBS-LRR::GUS vector likely resulted in a lack of functional promoter sequence being included in the vector. Once more is known about the likely nature of the *GmTIR-NBS-LRR* promoter, multiple pGmTIR-NBS-LRR::GUS vectors containing various lengths of sequence can be created and used to identify the possible APs.

A fast neutron mutant for *GmTIR-NBS-LRR* is now available in the SoyBase database (http://soybase.org; Grant *et al.*, 2010). Phenotypically characterising this mutant will provide great insight not just into the role of *GmTIR-NBS-LRR* in nodulation but also to its various TVs and how they might interact. Over-expression of the differing *GmTIR-NBS-LRR* transcripts in this mutant of could provide a wealth of knowledge as to how these transcript interact and to the existence of additional variants.

Carrying out an RNase protection assay would likely grant further insight into the complex transcription of *GmTIR-NBS-LRR,* allowing for mapping of transcription start sites, studying of intron-exon junctions, alternative promoters and identifying small transcript differences (Emery, 2007).

Chapter 4 Examining Gibberellins' Role in Nodulation

4.1 Abstract

This chapter examines the role of GA in nodulation through molecular and physiological studies. Silencing of *GmGA20ox a* showed no difference in nodule number, likely due to redundancy amongst GA20-oxidases. Histochemical GUS staining of both *GmGA20ox a* and *GmGA3ox 1a* confirmed the specificity of *GmGA20ox a* and more general nature of *GmGA3ox 1a*. Both sets of results align with previous qRT-PCR analysis of the expression of the two genes. *GmGA20ox a* was shown to be expressed in the cortex of the infection site and associated root hairs in the early stages of nodulation; developing nodules showed expression in the nodule primordium and cortex while mature nodules had greatly reduced expression in the inner cortex of the nodule. *GmGA3ox 1a* was expressed during early nodulation in the phloem of all roots, including in the elongation zone of the root tip. Later this expression was greatly reduced and concentrated in phloem that is extending into developing lateral roots. *GmGA3ox 1a* was expressed in the nodule primordium of developing nodules, faintly in the vascular of older, still developing nodules and sporadically in the scleroid layer of mature nodules. These histochemical results support the theory that GA is important in early nodulation events and in the development of emerging nodules, but not so important in mature nodules. *GmGA2ox* was classified as a Class II GA2-oxidase by phylogenetic analysis. GA produced by rhizobia in culture is unlikely to affect the GA content of inoculated roots. Endogenous GA levels were successfully measured in *G. max* roots and it was found that GA_3 is the most abundant bioactive GA in whole roots.

4.2 Introduction

A high throughput RNA-seq study revealed several GA biosynthesis genes which are up-regulated in *G. max* roots in the ZON behind the developing root tip within 48 hours of inoculation with *B. japonicum* (Hayashi *et al.*, 2012). These same genes are not induced when the plants are inoculated with *nodC*⁻ rhizobia. . Thus, it is highly likely that these genes are specifically involved in GA production as a response to rhizobia-produced Nod factor and successful infection events. These genes include those encoding a GA20ox (*GmGA20ox a,* Glyma04g42300.1), GA3ox (*GmGA3ox 1a,* Glyma15g01500.1) and GA2ox (*GmGA2ox*, Glyma02g01330.1) (Hayashi *et al.*, 2012; Hayashi, 2014). A similar GA20ox (*SrGA20ox1*) was found in *S. rostrata* to be up-regulated 8 hpi and shown to be NF dependent (Lievens *et al.*, 2005). *GmGA20ox a* and *GmGA3ox 1a* were both found by qRT-PCR to be most highly up-regulated at 12 hpi with a major decrease in expression at 48 hpi (Figure 4-1). The expression of *GmGA3ox 1a* increased again at 96 hpi. *GmGA2ox's* expression shows its greatest increase in expression at 24 hpi with its major decrease also occurring at 48 hpi similar to *GmGA20ox a* and *GmGA3ox 1a* (Hayashi, 2014; Figure 4-1). *GmGA2ox* also has a slight increase in expression at 96 hpi in the same way that *GmGA3ox 1a* increases.

Both GA20oxs and GA3oxs are involved in the biosynthesis of bioactive GA_4, GA_1 and GA_3 (Figure 1-1). Class I and II GA2oxs are involved in the deactivation of GA_9, GA_4, GA_{20} and GA_1 (Figure 1-1). It is proposed that *GmGA20ox a* and *GmGA3ox 1a* are up-regulated at 12 hpi to provide an increase in endogenous GA and that 12 hours later at 24 hpi, *GmGA2ox* is up-regulated in order to tightly control the level of endogenous GA (Hayashi, 2014).

GmGA20ox a was shown through qRT-PCR of various *G. max* tissues to be nodule-specific (Figure 4-2; Hayashi *et al.*, 2012). Interesting, it seems that after the initial reduction in expression at 96 hpi, expression increases again in 1 week-old nodules or 1 week post-inoculation (1 wpi). However, expression at 3 wpi is extremely low (Hayashi *et al.*, 2012). In roots, *GmGA3ox 1a* expression was specific to inoculated tissue, but not specific to roots over all. *GmGA3ox 1a* showed more general expression than *GmGA20ox a*, as it was found in the stem, shoot tip and trifoliate (Figure 4-2; Hayashi *et al.*, 2012). Similar to *GmGA20ox a*, *GmGA3ox 1a's* expression is up-regulated at 1 wpi; dissimilarly, it is still up-regulated at 3 wpi, but at less than half the expression level of 1 wpi (Hayashi *et al.*, 2012).

To carry out a histochemical promoter::reporter gene assay of both *GmGA20ox a* and *GmGA3ox 1a*, GUS vectors were constructed by Dr Satomi Hayashi (Hayashi, 2014).

Approximately 2-3 kb upstream of the translation start site of each gene was selected as the promoter region, amplified and cloned into the binary vector pCAMBIA 1305.1 (GenBank: AF354045; Hayashi, 2014; Table 4-1). These constructs were then electroporated into *A. rhizogenes* strain K599 and could then be used for hairy root mediated soybean transformation (Kereszt *et al.*, 2007; Hayashi, 2014).

It was later discovered that the pCAMBIA 1305.1 vector's CaMV35Sx2 promoter which is used to induce hygromycin resistance, was also inducing increased *GUS* expression independent of the *GmGA20ox a* or *GmGA3ox 1a* promoter (Hayashi, 2014). This led to inaccurate results. Ms Dongxue Li (CILR, The University of Queensland) removed the CaMV35Sx2 promoter from the pGmGA20ox a::GUS construct so that only the *GmGA20ox a* promoter was driving *GUS* expression (Hayashi, 2014).

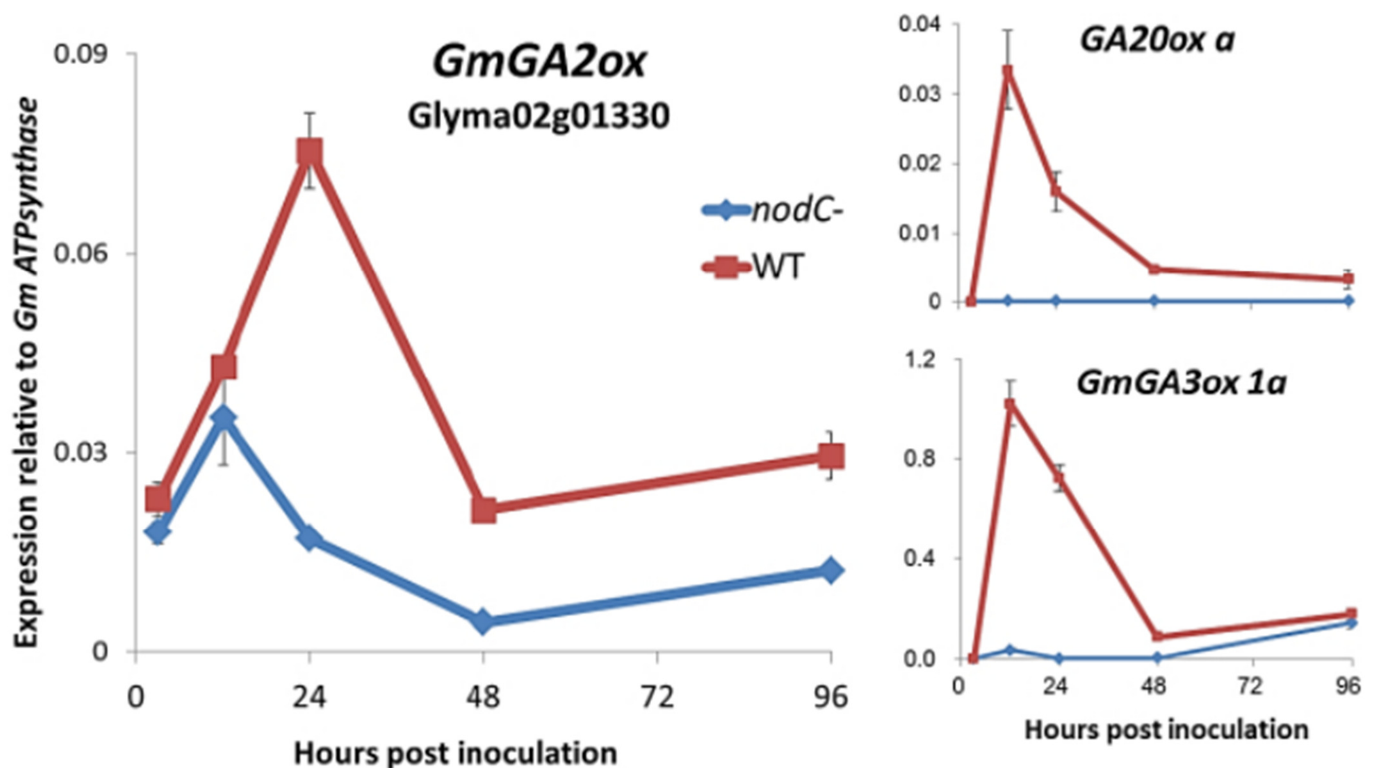


Figure 4-1 - qRT-PCR measurement of expression of *GmGA2ox*, *GmGA20ox a* and *GmGA3ox 1a* 0, 12, 24, 48 and 96 hpi with either WT (red line) or *nodC*- (blue line) *B. japonicum* (Hayashi, 2014).

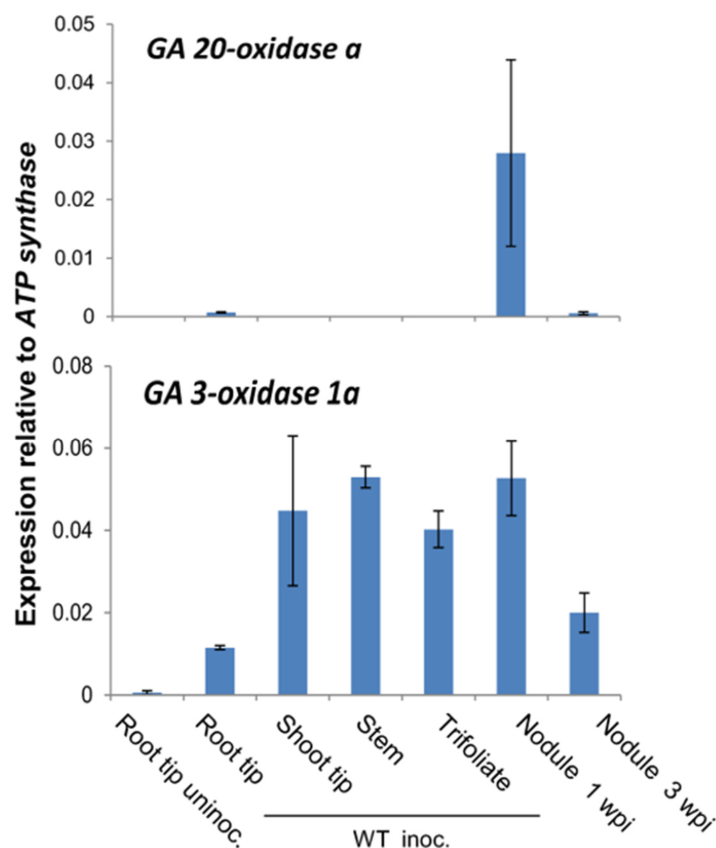


Figure 4-2 - qRT-PCR analysis of expression of *GmGA20ox a* and *GmGA3ox 1a* in various tissues of *G. max* (Hayashi et al. 2012).

An RNAi vector was contrasted by Dr Hayashi to silence *GmGA20ox a* (Hayashi, 2014). A 344 bp region of the coding sequence was amplified with two primer sets, sense (S) and anti-sense (A), each with differing restriction enzyme (RE) sites (Table 4-1). These two target sequences were amplified and cloned into the pKannibal vector (GenBank: AJ311873.1; Wesley *et al.*, 2001; Hayashi *et al.*, 2014). The RNAi construct was excised from the pKannibal vector and subsequently ligated into the phosphatase-treated binary integration vector p15SRK2 (Kereszt *et al.* unpublished; Hayashi 2014). This construct was then transferred into *A. rhizogenes* K599 via tri-parental mating and could then be used for hairy root mediated soybean transformation (Kereszt *et al.*, 2007; Hayashi, 2014).

Table 4-1 - Primers and restriction enzyme sites of the constructs made by Dr Satomi Hayashi (CILR). Lowercase letters represent the RE site, bold letters represent the start codon of the GUS gene, underlined letters represent the nucleotide added to the sequence in order to keep a distance between the promoter and translational start site (Hayashi 2014).

Construct	Primers	Restriction Enzyme Site
pGmGA20ox a::GUS	5’-ggtaccTTTCCACTTTGCTATGTTGCTCAATTA-3’	*Hind*III
	5’-agatctAC**CAT**CGTCTCACGTTAATTGTGTT-3’	*Bgl*II
pGmGA3ox 1a::GUS	5’-ggtaccTTTCCACTTTGCTATGTTGCTCAATTA-3’	*Bam*HI
	5’-agatctAC**CAT**AATAGTGTGGAACAAATAGTGACCA-3’	*Bgl*II
GmGA20ox a RNAi	(S) 5’-ctcgagCACATGATCAAATGGACACTTTCT-3’	*Xho*I
	(S) 5’- ggtaccTTCTTCAAACAAGTCCCTATAATGCAAC-3’	*Kpn*I
	(A) 5’-tctagaGCACATGATCAAATGGACACTTTCT-3’	*Xba*I
	(A) 5’-aagcttCTTCAAACAAGTCCCTATAATGCAAC-3’	*Hind*III

A nodulation study was conducted using WT Bragg control hairy roots and *GmGA20ox a* RNAi hairy roots (Hayashi, 2014). No significant difference in nodule number was found between the two groups. When expression of *GmGA20ox a* was measured via qRT-PCR in both control and *GmGA20ox a* RNAi hairy roots, no expression data could be obtained repeatedly. This was due to the low transcript abundance of *GmGA20ox a* in whole roots. As discussed above, this gene is highly expressed in the ZON but not in the whole root itself, which was the tissue sampled (Hayashi, 2014).

Here, we aimed to continue to characterise these early nodulation GA biosynthesis genes and investigate the role of GA in nodulation as a whole. The *GmGA20ox a* RNAi vector mentioned above was used to transform *nts382* supernodulating plants to determine, if an overall increase in gene expression would amplify the effect of silencing. The new *GmGA20ox a::GUS* construct, without the interfering CaMV35Sx2 promoter was used to transform *G. max* plants and study the expression of the gene at various times post inoculation. The pGmGA3ox 1a::GUS construct altered to remove the interfering CaMV35Sx2 promoter was used in a similar histochemical *promoter::reporter gene* assay. A vector to over-express *GmGA2ox* was constructed and used to study the possible physiological differences that increased *GmGA2ox* expression causes.

Additionally, to study the role of GA at the hormone level, whole root tissue, inoculated with WT or *nodC*⁻ *B. japonicum* was harvested and the endogenous GA level measured by Dr Dana Tarkowska (Palacky University and Institute of Experimental Botany ASCR). Further to this initial study, the ZON was harvested from plants inoculated with WT or *nodC*⁻ *B. japonicum*

and the endogenous GA level measured as it is hypothesised that the greatest difference in GA level will be seen when comparing the ZONs. In order to examine the possible role of *B. japonicum* in affecting the level of GA measured, whole roots inoculated with WT or *nodC*$^{-}$ *B. japonicum* or a mock inoculation were harvested and the GA level measured. In addition, the *in vitro* levels of GA from WT and *nodC*$^{-}$ *B. japonicum* and a mock culture were measured.

4.3 Methods

4.3.1 GmGA2ox Phylogenetic Analysis

GmGA2ox's amino acid sequence was obtained from Phytozome (http://www.phytozome.net/soybean). All other amino acid sequences were obtained through NCBI (http://www.ncbi.nlm.nih.gov/). The amino acid sequences of multiple GA2-oxidases were compared to generate a phylogenetic tree. GenBank accession numbers of proteins investigated are in parentheses: *A. thaliana*; AtGA2ox1 (CAB41007), AtGA2ox2 (CAB41008), AtGA2ox3 (CAB41009), AtGA2ox4 (AAG51528), AtGA2ox6 (AAG00891), AtGA2ox7 (AAG50945), AtGA2ox8 (CAB79120), *Lactuca sativa*; LsGA2ox1 (BAB12442), *Oryza sativa*; OsGA2ox1 (BAB40934), OsGA2ox2 (BAC16751), OsGA2ox3 (BAC16752), OsGA2ox4 (AAU03107), OsGA2ox5 (BAC10398), OsGA2ox6 (CAE03751), *Phaseolus coccineus*; PcGA2ox1 (CAB41036), *P. sativum*; PsGA2ox1 (AAF08609), PsGA2ox2 (AAD45424), *Spinacia oleracea*; SoGA2ox1 (AAN87571), SoGA2ox2 (AAN87572), and SoGA2ox3 (AAX14674). Pairwise identities were found through alignment using Geneious. The alignment was used to generate a phylogenetic tree using Genebee (http://www.genebee.msu.su/services/phtree_reduced.html).

4.3.2 *GmGA2ox* over-expression

To examine *GmGA2ox*'s possible role in nodulation, an over-expression study was conducted. The *GmGA2ox* gene sequence of 1071bp was retrieved from Phytozome (http://www.phytozome.net/soybean). The sequence was amplified from soybean nodule cDNA using the following primers; 5'-ccgcggATGGTTGCCCCTTGTCCAACATC-3' with restriction enzyme *Sac*II and 5'-tctagaTCAGGGAGAAGCAGGTGCGAGAT-3' with restriction enzyme *Xba*I. Following ligation with T4 DNA ligase (New England Biolabs) into the pGEM-T (Promega) cloning vector, the sequence of the gene was verified. Post verification, the gene was excised by double digest using the restriction enzymes *Sac*II and *Xba*I, similarly the binary vector pORE (AY562538) underwent double digest of by *Sac*II and *Xba*I. *GmGA2ox* was then ligated with T4 DNA ligase (New England Biolabs) into pORE. Following additional sequence verification, the *GmGA2ox* over-expression vector was electroporated into *A. rhizogenes* K599 and selected via antibiotic resistance to rifampicin and kanamycin. *A. rhizogenes* carrying either of the binary vectors was then used in hairy root transformation as described in chapter 2.1. A vector of pORE carrying no soybean genes was similarly electroporated into *A. rhizogenes* K599 and used to transform plants via hairy root transformation to act as a control.

WT Bragg plants were transformed, inoculated with USDA110 and grown as in chapter 2.1. Plants were checked for nodulation 3 wpi but no nodules formed on the control or overexpressing plants. Plants were re-inoculated with fresh rhizobia. 3 wpi. The plants were harvested but both treatments still failed to nodulate. The plants were also now too old to attempt a third inoculation and produce reliable results.

4.3.3 *GmGA20ox a RNAi*

Supernodulating *nts382* plants were grown, inoculated with USDA110 and transformed as in chapter 2.1, utilising the *GmGA20ox a* RNAi vector created by Dr Satomi Hayashi (CILR, The University of Queensland; Hayashi, 2014), chapter 4.2 Supernodulating plants were used to magnify the possible effects of gene silencing. Plants were harvested at 3 wpi with nodule number and root weight recorded on an individual root basis.

4.3.4 *GmGA20ox a* promoter study

The pGmGA20ox a::GUS vector described in chapter 4.2, created by Dr Satomi Hayashi (CILR, The University of Queensland; Hayashi, 2014), was used in hairy root transformation as described in chapter 2.1 of supernodulating *nts382* plants.

Plants and *B. japonicum* were grown as described in chapter 2.1, with controls as described in chapter 2.2. Plants were then harvested at 16 hpi, 24 hpi, 48 hpi, 1 wpi or 3 wpi and underwent histochemical staining as described in chapter 2.2. Supernodulating plants were used as per chapter 3.3.1.

4.3.5 *GmGA3ox 1a* promoter study

A pGmGA3ox 1a::GUS vector was created as described in section 2.4 utilising the following primers and restriction enzymes to excise the promoter, digest the modified pCAMBIA1305.1 vector and verify the sequence; 5'-ggtaccTTTCCACTTTGCTATGTTGCTCAATTA-3' with restriction enzyme *Bam*HI and 5'-agatctACCATAATAGTGTGGAACAAATAGTGACCA-3' with restriction enzyme *Bgl*II. WT Bragg plants were grown and transformed as described in chapter 2.1, with controls as described in chapter 2.2. Plants were harvested at 12 hpi, 48 hpi or 3 wpi and underwent histochemical staining as described in chapter 2.2.

4.3.6 Endogenous GA levels in whole roots

WT Bragg plants were grown as outlined in chapter 2.1 and were inoculated with either USDA110 or *nodC- B. japonicum,* either 16 hours or 4 days prior to harvest. At 14 days-old

the plant's tap root was harvested. Root tips, considered to be the newest 1 cm of root, were removed prior to freezing. Chapter 2.3 details the rest of the procedure that followed.

4.3.7 Endogenous GA levels in the ZON

WT Bragg [lants were grown as in 2.1 and were inoculated with either USDA110 or *nodC*$^-$ *B. japonicum,* either 18 h or 36 h prior to harvest of 6 day-old plants. Only the ZON, representing the 5 cm section of root located 1 cm behind the root cap was harvested. The samples were then treated as described in chapter 2.3.

4.3.8 Endogenous GA levels in whole roots examining rhizobia's effect

WT Bragg plants were grown as in 2.1 and were inoculated with either USDA110 or *nodC*$^-$ *B. japonicum* or a mock inoculation consisting of YMB diluted with water. Inoculation took place either 18 h or 36 h prior to harvest of 6 day-old plants. The plant's tap root was harvested, excluding root tips, considered to be the newest 1 cm of root. Chapter 2.3 details the rest of the procedure followed.

4.3.9 GA levels present in *B. japonicum* culture

B. japonicum strains USDA110 and *nodC*$^-$, as well as mock culture containing no bacteria but simply YMB were grown as in Chapter 2.1. Samples of each culture type were not diluted but centrifuged at 8,000 rpm for 20 min at 4°C. The supernatant was kept and freeze-dried at -80°C, 100 µbar for 48 h. Samples were then handled by Dr Dana Tarkowska (Palacky University and Institute of Experimental Botany ASCR), who analysed the GA content using the technique described in Urbanová *et al.* (2013).

4.4 Results

4.4.1 GmGA2ox Phylogenetic Analysis

GmGA2ox was found via phylogenetic analysis to be most closely related to Class II GA2-oxidases of other species, having 56.0%, 48.8%, 45.7%, 43.1%, 76.7% and 58.3% pairwise identity to AtGA2ox4, AtGA2ox6, OsGA2ox1, OsGA2ox2, PsGAox2 and SoGA2ox2 respectively (Figure 4-3). When amino acid sequences of multiple GA2-oxidases were analysed, and a phylogenetic tree was generated using Genebee, GmGA2ox was found to cluster with other Class II GA2-oxidases (Figure 4-3).

4.4.2 *GmGA2ox* over-expression

The *GmGA2ox* over-expression hairy root plants and the corresponding control failed to nodulate. As a result, it was not possible to assess the effects of GmGA2ox over-expression on nodulation.

Figure 4-3 - Unrooted phylogenetic tree of known GA2-oxidases generated using Genebee (http://www.genebee.msu.su/services/malign_reduced.html). GenBank accession numbers of proteins are in parentheses: *A. thaliana*; AtGA2ox1 (CAB41007), AtGA2ox2 (CAB41008), AtGA2ox3 (CAB41009), AtGA2ox4 (AAG51528), AtGA2ox6 (AAG00891), AtGA2ox7 (AAG50945), AtGA2ox8 (CAB79120), *L. sativa*; LsGA2ox1 (BAB12442), *G. max*; GmGA2ox, *O. sativa*; OsGA2ox1 (BAB40934), OsGA2ox2 (BAC16751), OsGA2ox3 (BAC16752), OsGA2ox4 (AAU03107), OsGA2ox5 (BAC10398), OsGA2ox6 (CAE03751), *P. coccineus*; PcGA2ox1 (CAB41036), *P. sativum*; PsGA2ox1 (AAF08609), PsGA2ox2 (AAD45424), *S. oleracea*; SoGA2ox1 (AAN87571), SoGA2ox2 (AAN87572), and SoGA2ox3 (AAX14674).

4.4.3 *GmGA20ox a* RNAi

Nodule number/root fresh weight was examined to account for root size. When comparing *GmGA20ox a* RNAi silenced hairy roots to control roots, no significant differences were found (p>0.05) (Figure 4-4).

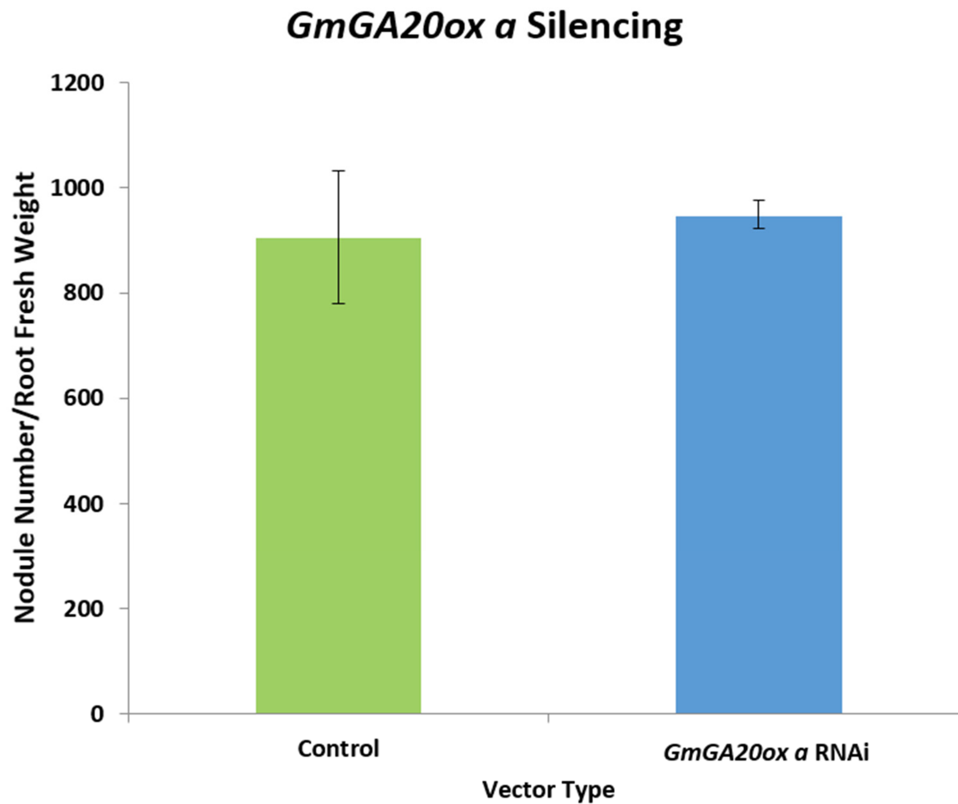


Figure 4-4 - Comparison of nodule number of control and *GA20ox a* RNAi silenced hairy root transformed supernodulating *nts382* soybean mutants. Nodule number is analysed on an individual root fresh weight basis. Confidence intervals were used in the formation of error bars. This graph shows no significant difference and the transformed roots (P>0.05).

4.4.4 GmGA20ox a promoter study

Three controls were used in the histochemical analysis of *GmGA3ox 1a* expression, including; no promoter::GUS plants and 35S::GUS plants (results not shown) as described in chapter 2.2. The third control consisted of plants transformed with the pGmGA20ox a::GUS vector but inoculated with *nodC⁻ B. japonicum,* which therefore did not nodulate.

Neither the no promoter::GUS nor *nodC⁻* inoculated control saw GUS staining in any tissue, at any time point (Figure 4-5). At 16 hpi GUS staining was seen at the sites of developing nodules and in the root hairs associated with those developing nodules. The GUS staining was not present in the epidermis but was present in the cortex (Figure 4-6).

GUS staining was observed exclusively in nodule related tissues, in tissues that were the site of early nodule development, in root hairs that were associated with a developing nodule and within nodules themselves (Figure 4-5 and Figure 4-6). 1 week old nodules had GUS staining present in the nodule primordia whilst mature nodules of 3 weeks of age had no staining in the infection zone, but faint staining in the inner cortex of the nodule at the north and south positions within the nodule, not the entire inner cortex (Figure 4-6). No staining was observed at the site of developing lateral roots (results not shown) or in the root tip.

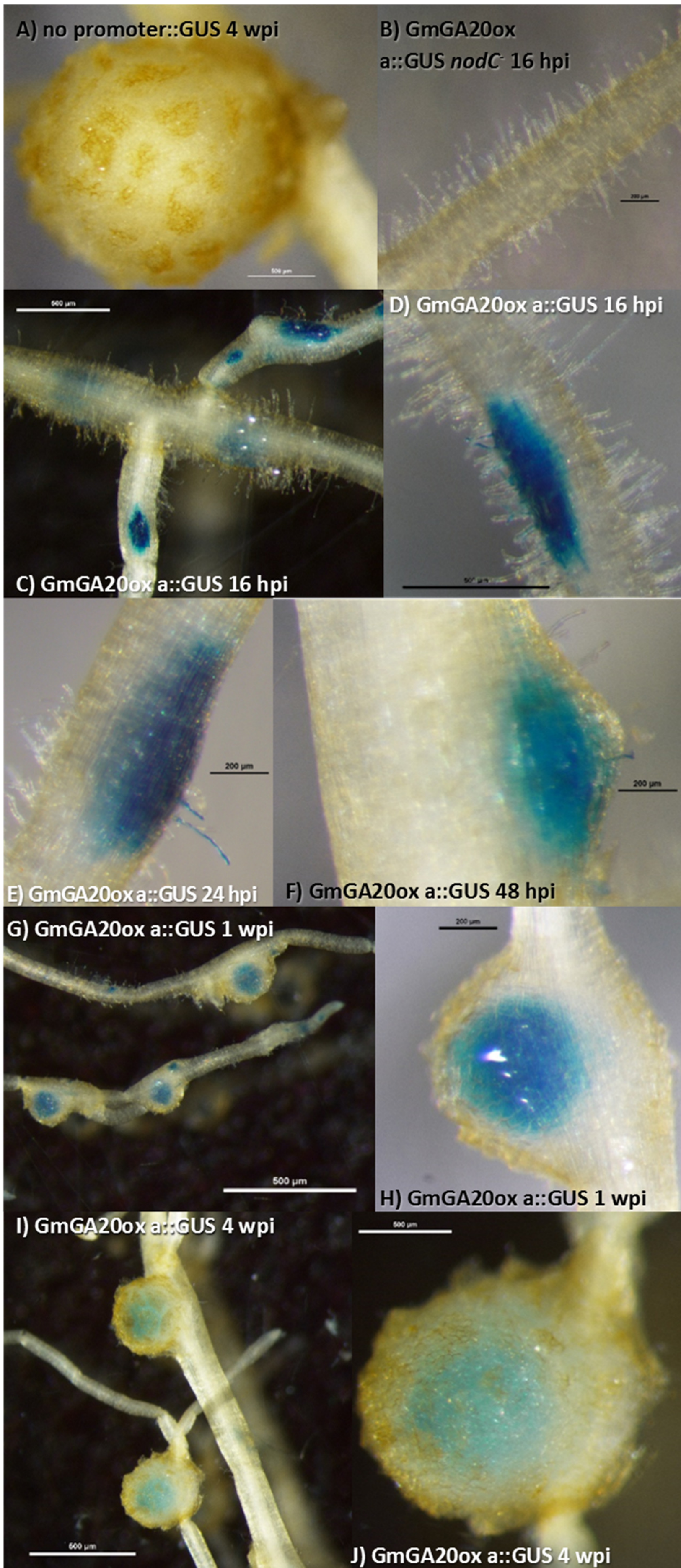


Figure 4-5 - Photos of *G. max* hairy roots carrying the GmGA20ox a::GUS vector inoculated with *B. japonicum* strains USDA110 or the non-nodulating mutant *nodC*⁻ and harvested at 12 hpi, 48 hpi, approximately 1 wpi or 3 wpi. No promoter::GUS roots shown are representative of all no promoter::GUS roots from all time points in regards to a lack of visible GUS staining, not in root or nodule development. *nodC*⁻ roots shown are representative of *nodC*⁻ from all time points. A) no promoter::GUS nodule 4 wpi with USDA110. B) GmGA20ox a::GUS root 4 wpi with *nodC*⁻. C-J) GmGA20ox a::GUS roots/nodules inoculated with USDA110 and harvested at: C & D) 16 hpi. E) 24 hpi. F) 48 hpi. G) 1 wpi. H) 1 wpi. I) 4 wpi. J) 4 wpi

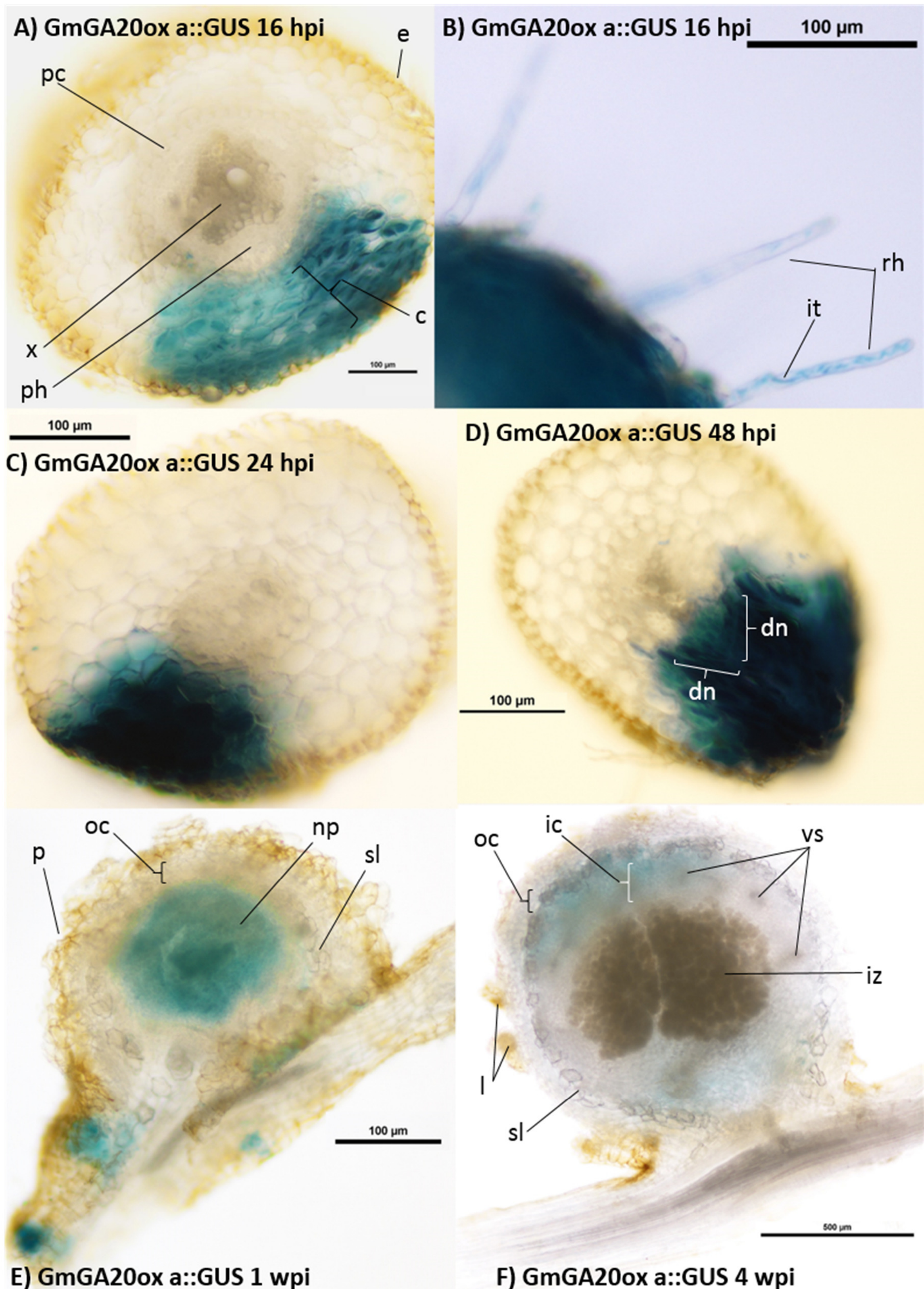


Figure 4-6 - Photos of *G. max* hairy roots carrying the GmGA20ox a::GUS vector inoculated with *B. japonicum* strains USDA110, harvested at 16 hpi, 24 hpi, 48 hpi, 1 wpi or 4 wpi. A) Transverse of root 16 hpi; p – pericycle, e – epidermis, x – xylem, ph – phloem, c - cortex. B) Close up of root hairs (rh) with infection thread (it) 16 hpi. C) Transverse of root 24 hpi. D) Transverse of root 48 hpi; dn – developing nodule. E) Longitudinal section of nodule approximately 1wpi; oc – outer cortex, p – periderm, np – nodule primodrium, sl – scleroid layer. F) Longitudinal section of nodule 4 wpi; oc – outer cortex, p – periderm, in – infected zone, sl – scleroid layer, ic – inner cortex, vs – vascular strand.

4.4.5 GmGA3ox 1a promoter study

Three controls were used in the histochemical analysis of *GmGA3ox 1a* expression; no promoter::GUS plants and 35S::GUS plants (results not shown) as described in chapter 2.2. The third control consisted of plants transformed with the pGmGA3ox 1a::GUS vector but inoculated with *nodC*$^{-}$ *B. japonicum,* which therefore did not nodulate.

Neither the no promoter::GUS nor *nodC*$^{-}$ inoculated control saw GUS staining in any tissue, at any time point. The GUS staining observed in GmGA3ox 1a::GUS transformed roots was exclusively in the vasculature at 12 and 48 hpi, further, expression was localised to the phloem and not present in the xylem (Figure 4-7). This phloem expression continued into the elongation region of the root tip. At 12 hpi the expression of GmGA3ox 1a::GUS is much greater and wider spread than expression at 48 hpi (Figure 4-8). Although expression was exclusively in the phloem at 12 hpi, expression could be seen all along the root, including in lateral roots and at the junction between root and lateral root (Figure 4-8). However, at 48 hpi expression was greatly reduced and was seen only at the site of developing lateral roots were it concentrated in the phloem extending into the developing root (Figure 4-7).

Despite roots being harvested at 3 wpi, nodules that were less than 3 week old could still be observed, however their exact age is unknown. In developing nodules likely less than a week old, GUS staining could be observed in the nodule primordium (Figure 4-8 and Figure 4-7). Nodules that were not fully mature showed no staining in the nodule primordium but did show GUS in the vasculature of the nodule. Mature nodules showed very faint GUS staining which was sporadically present in the scleroid layer (Figure 4-7).

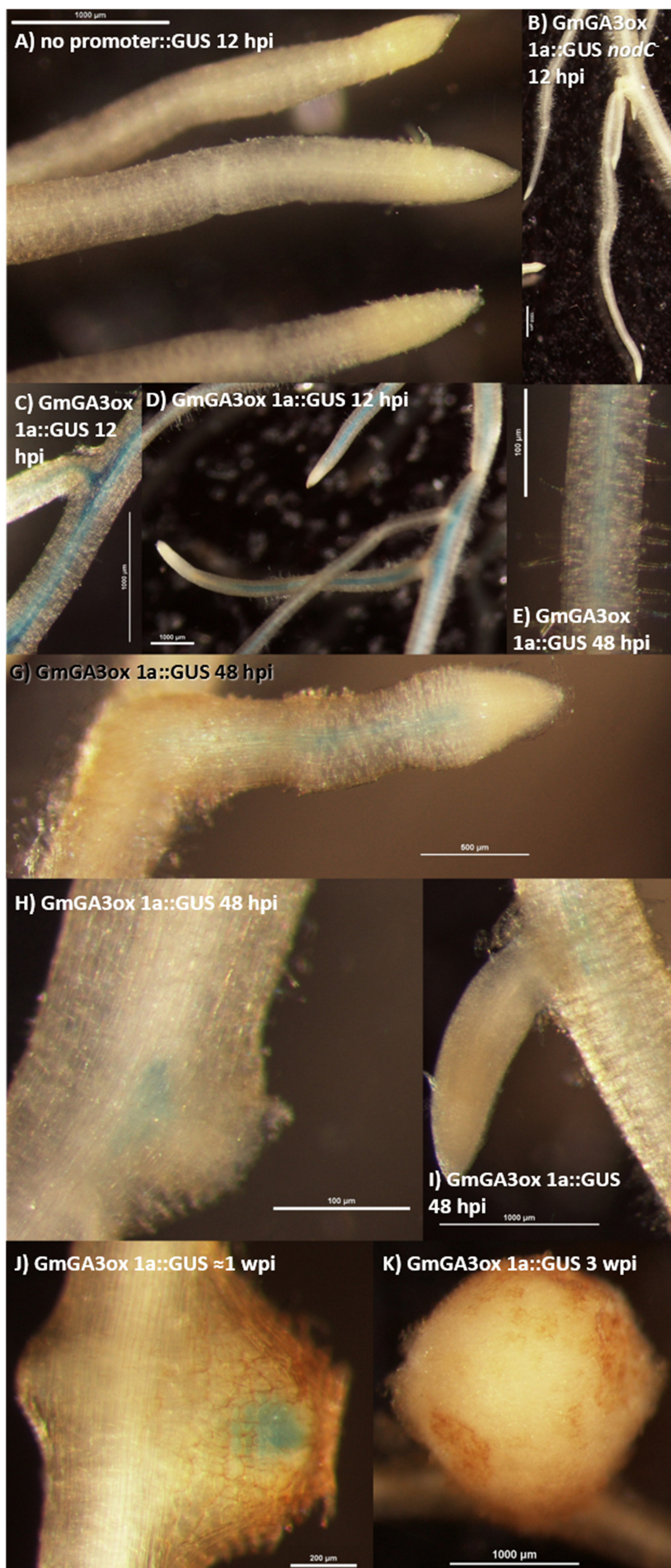


Figure 4-7 - Photos of *G. max* hairy roots carrying the GmGA3ox 1a::GUS vector inoculated with *B. japonicum* strains USDA110 or the non-nodulating mutant *nodC*- and harvested at 12 hpi, 48 hpi, approximately 1 wpi or 3 wpi. No promoter::GUS roots shown are representative of all no promoter::GUS roots from all time points in regards to a lack of visible GUS staining, not in root or nodule development. *nodC*- roots shown are representative of *nodC*- from all time points. A) no promoter::GUS roots 12 hpi with USDA110 B) GmGA3ox::GUS roots 12 hpi with *nodC*-. C-K) GmGA3ox::GUS roots/nodules inoculated with USDA110 and harvested at: C & D) 12 hpi. E-I) 48 hpi. J) Approximately 1wpi. K) 3 wpi.

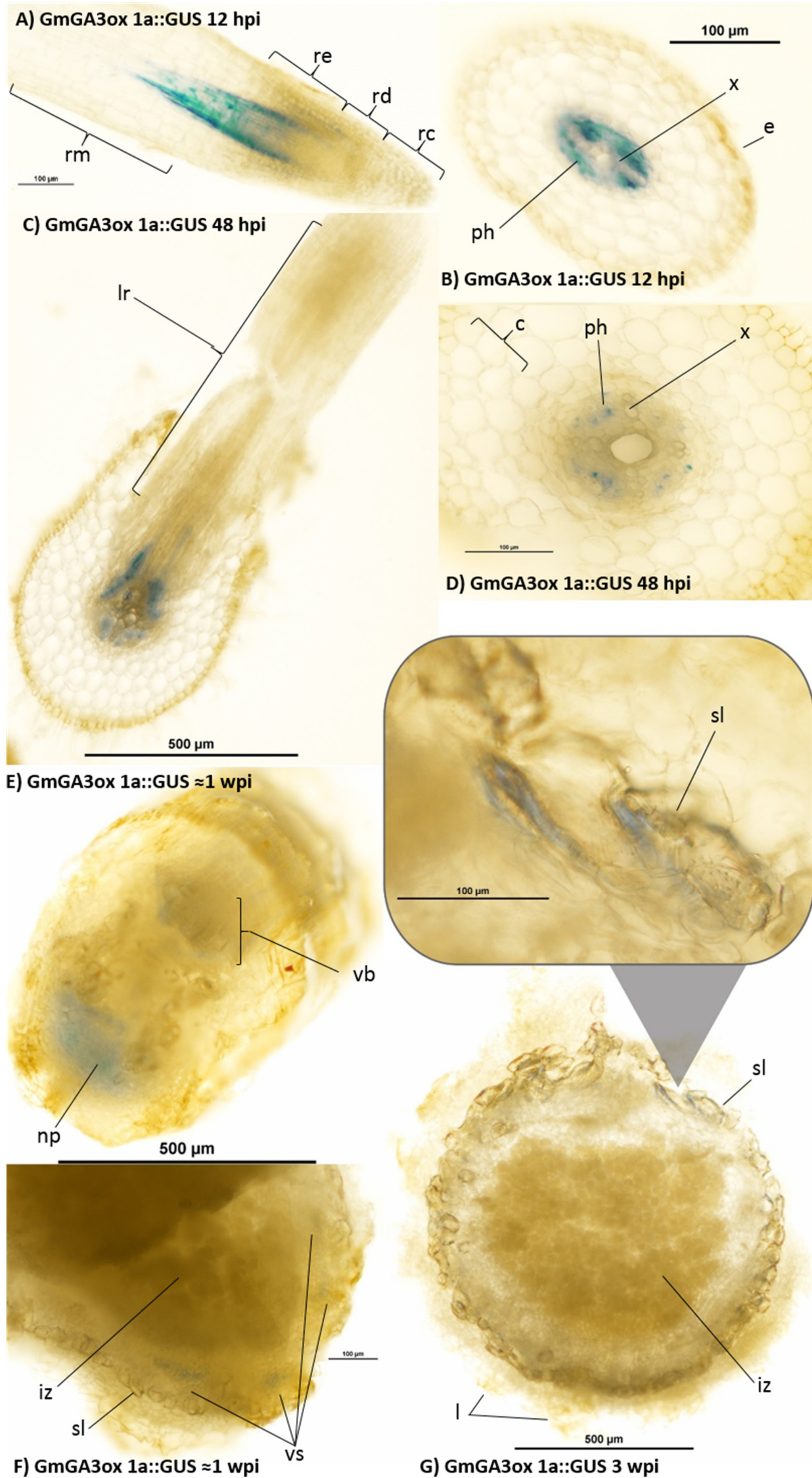


Figure 4-8 - Photos of *G. max* hairy roots carrying the GmGA3ox 1a::GUS vector inoculated with *B. japonicum* strain USDA110, harvested at 12 hpi, 48 hpi, approximately 1 wpi or 3 wpi. A) Longitudinal section of root tip 12 hpi; rm – region of maturation, re – region of elongation, rd – region of division, rc – root cap. B) Cross section of root 12 hpi; e – epidermis, x – xylem, ph – phloem. C) Cross section of root and lateral root (lr) 48 hpi. D) Cross section of root 48 hpi; c – cortex, x – xylem, ph – phloem. E) Cross section of nodule approximately 1 wpi, vb – vascular bundle, np – nodule primodrium. F) Cross section of nodule 3 wpi; vs – vascular strand, iz – infection zone, sl – scleroid layer. G) Cross section of nodule 3 wpi with close up of nodule scleroid layer (sl).

4.4.6 Endogenous GA levels in whole roots – 14 days old

When examining whole roots, endogenous GA_3 levels were at least 15 times higher than that of GA_1 or GA_4 (Figure 4-9). Levels of GA_4 were found to significantly double at 4 dpi regardless of inoculation treatment; however, no significant difference was found between inoculation treatments at either time point. GA_1 levels decreased significantly at 4 dpi with USDA110; however, no significant difference was found between treatments or between time points for *nodC*$^-$ treated roots.

The GAs involved in the GA_1 biosynthesis pathway were examined together. GA_{44} and GA_{29} were present at much greater levels than any of the other GAs in this pathway with GA_{29} having at least double the level of even GA_{44} (Figure 4-10). While the high levels of GA_{29} at both 18 hpi and 4 dpi were virtually unchanged, at 4 dpi with *nodC*$^-$ the level significantly increased by approximately 10 pg/mg dw. The levels of GA_{19}, GA_{20} and GA_1 were all similar to each other when comparing levels of the same treatment type, the level of GA_1 at 4 dpi with USDA110 is an exception as it was significantly lower than the same GA_{20} level but not GA_{19} (Figure 4-10). The level of GA_{53} significantly reduced at 4 dpi, regardless of inoculum. GA_{44}'s levels for *nodC*$^-$ vs USDA110 have opposing patterns as expression increased at 4 dpi with *nodC*$^-$ but decreased at 4 dpi with USDA110 when compared to their earlier 18 dpi counterparts.

GA_5 in the GA_3 biosynthesis pathway follows a very similar pattern to that of GA_{44}, the level of the GA increased at 4 dpi in *nodC*$^-$ inoculated roots by approximately 39% and 50% respectively and decreased in both GAs by approximately 40% at 4 dpi in USDA110 inoculated roots (Figure 4-11). GA_3 was present at significantly higher levels in USDA110 inoculated roots at both time points with the level significantly increasing between 18 hpi and 4 dpi.

Within the GA_4 biosynthesis pathway, GA_{51} was found at the highest levels across all treatments, at least twice as high as GA_{15}, the next most abundant GA (Figure 4-12). GA_{51} increased in abundance at 4 dpi regardless of inoculum. Levels of both GA_{24} and GA_9 decreased by at least 50% at 4 dpi in USDA110 inoculated roots but had no difference in level between time points for *nodC*$^-$ inoculated roots. GA_{34} saw no change in level across any of the treatments. GA_{15} had consistent levels when inoculated with USDA110 but saw an increase in level between 18 hpi and 4 dpi with *nodC*$^-$ (Figure 4-12).

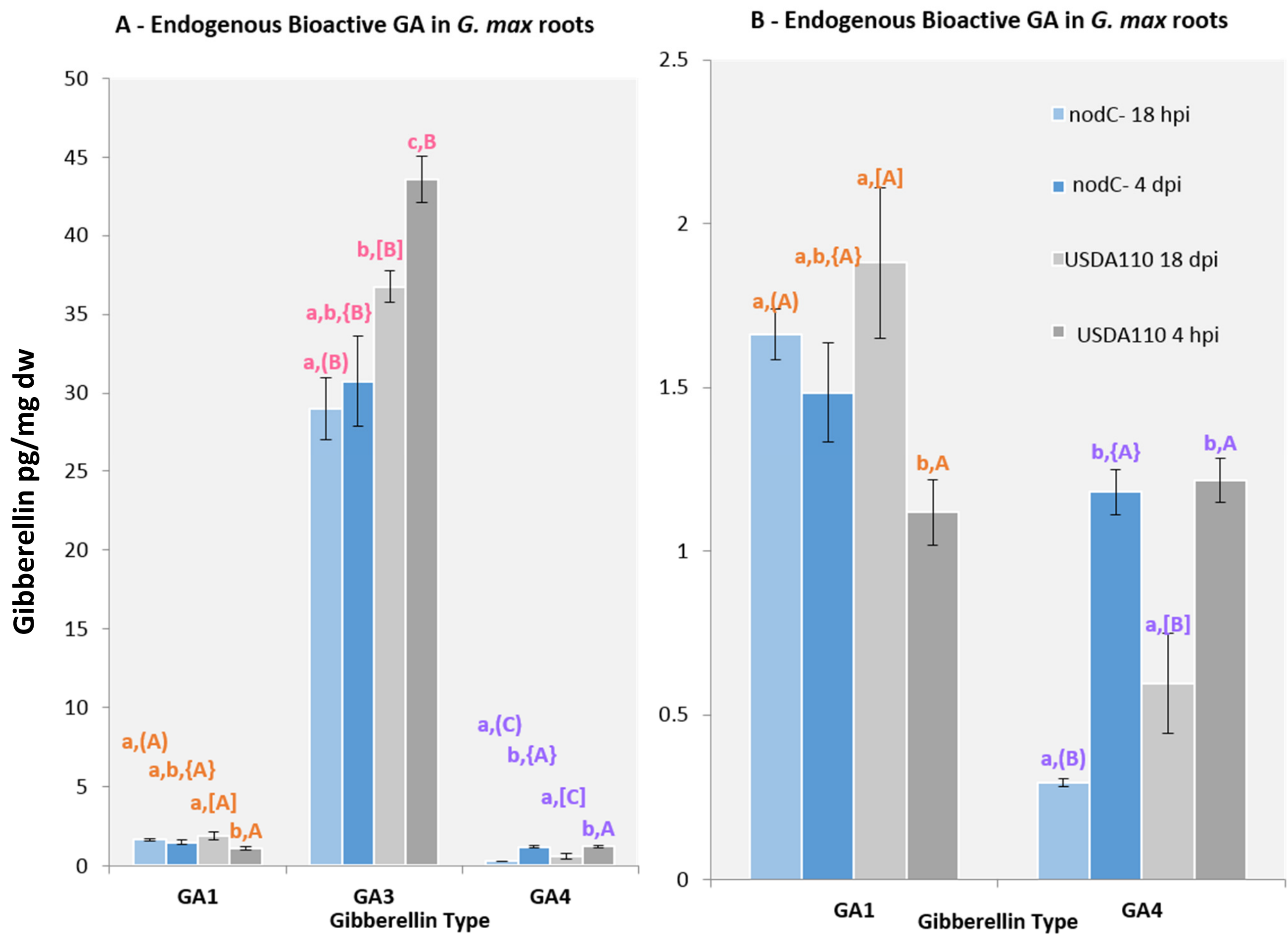
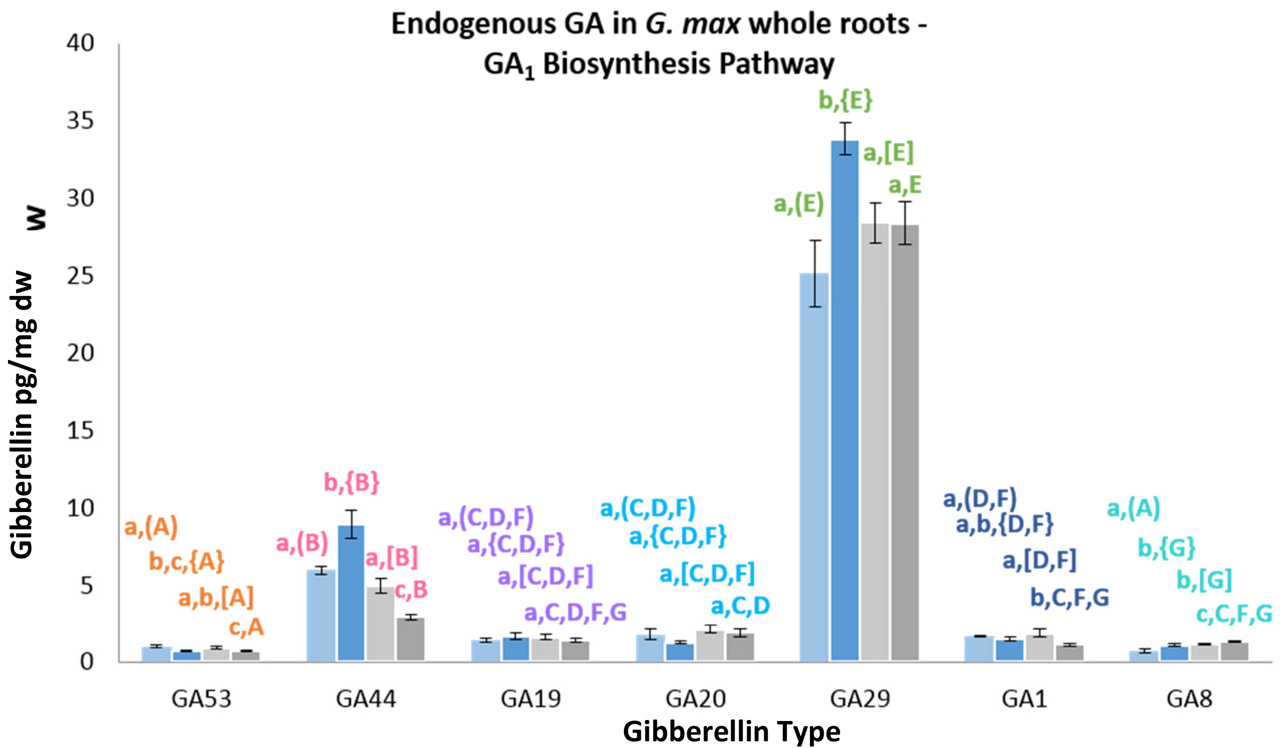


Figure 4-9 –Average pg/mg dw of bioactive endogenous GAs in whole *G. max* roots treated with *B. japonicum* strains USDA110 or the non-nodulating mutant *nodC*⁻ and harvested 18 hpi or 4 dpi. Error bars represent standard error. Different lowercase letters of the same colour represent a significant difference between treatment averages for the same GA type. Different uppercase letters of the same bracketing – (), {}, [] or none – represent a significant difference between averages of different GA types of the same treatment (inoculum and time point). (Student's t-test; P≤0.05).



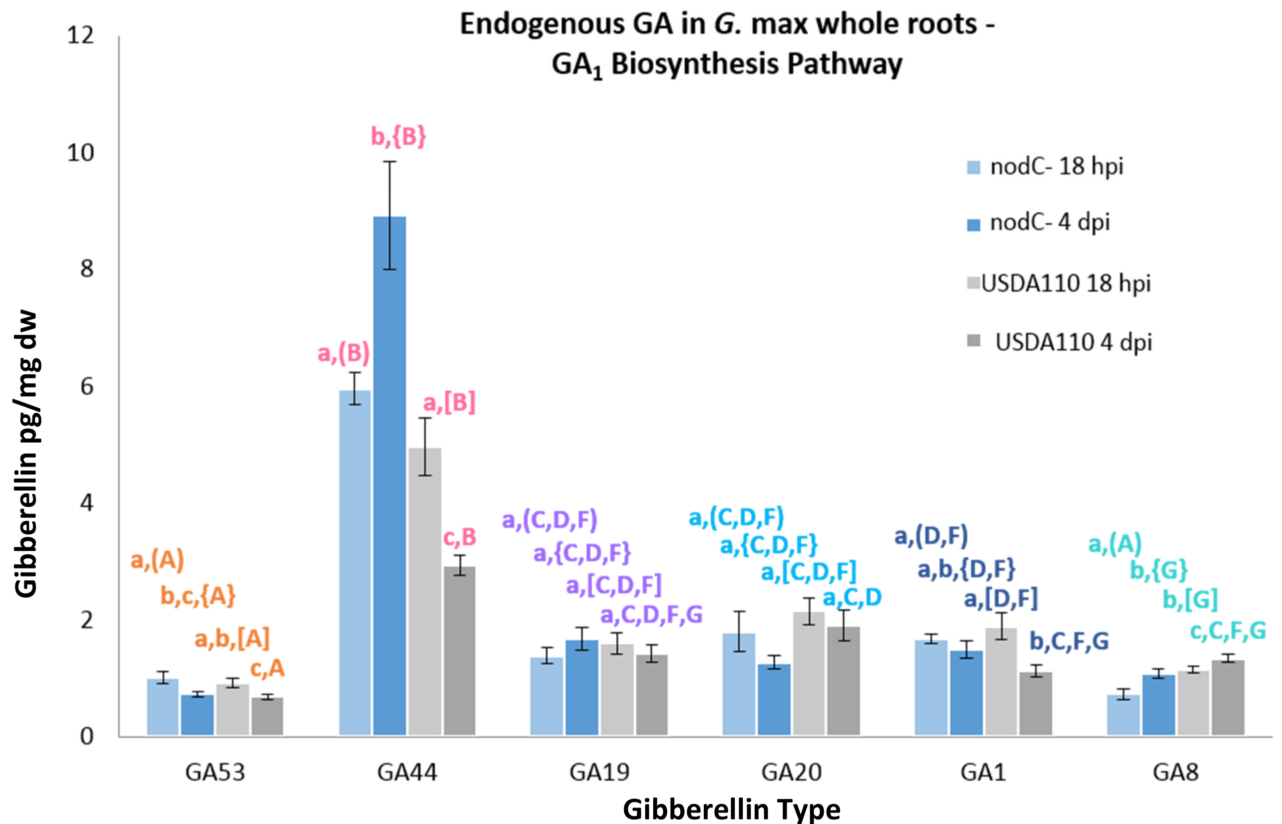
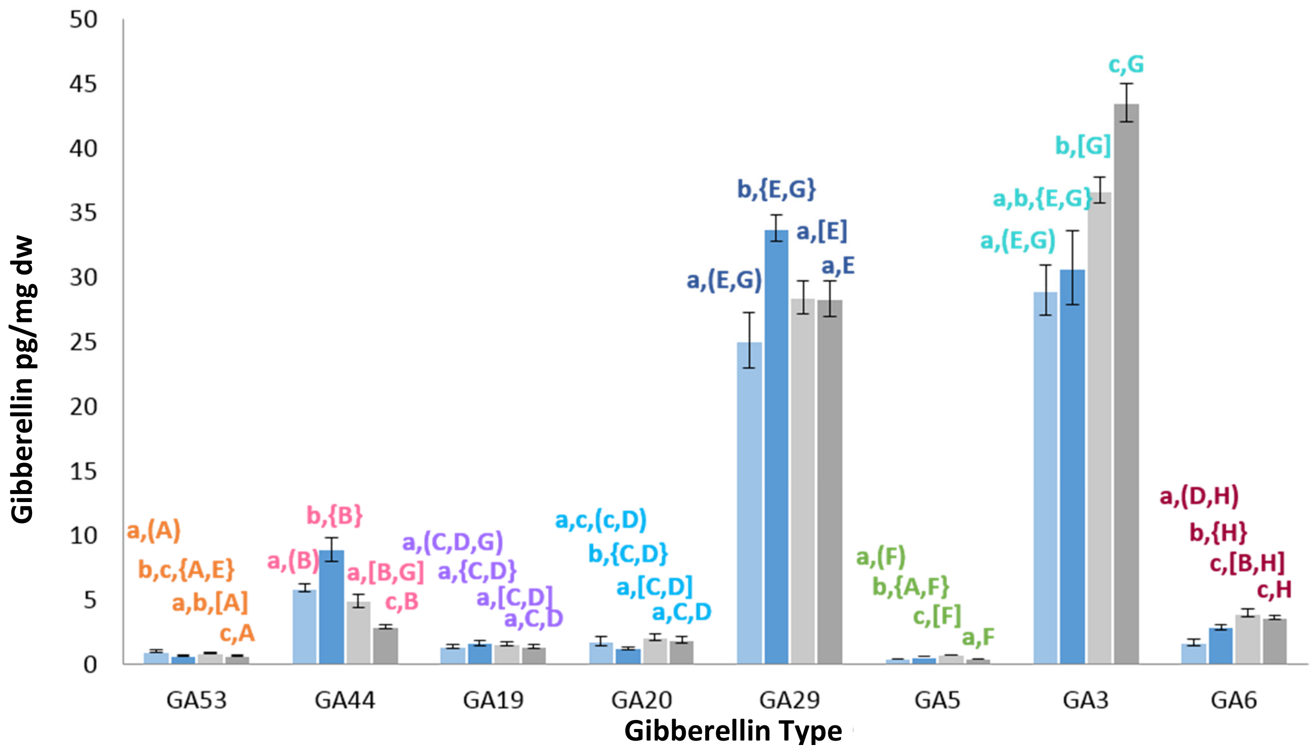


Figure 4-10 - Average pg/mg dw of endogenous GAs involved in GA_1 biosynthesis, in whole G. *max* roots treated with *B. japonicum* strains USDA110 or the non-nodulating mutant *nodC*⁻ and harvested 18 hpi or 4 dpi. Error bars represent standard error. Different lowercase letters of the same colour represent a significant difference between treatment averages for the same GA type. Different uppercase letters of the same bracketing – (), {}, [] or none – represent a significant difference between averages of different GA types of the same treatment (inoculum and time point). (Student's t-test; P≤0.05).



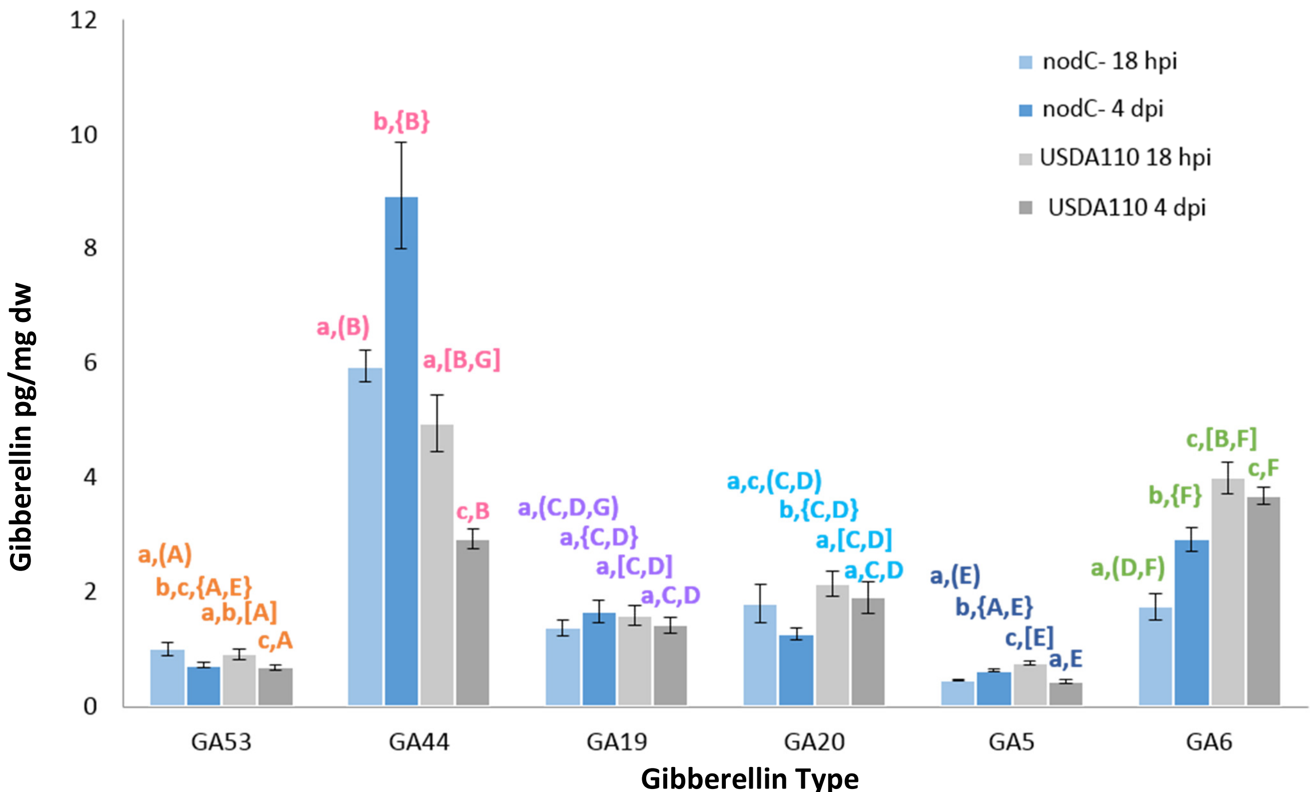
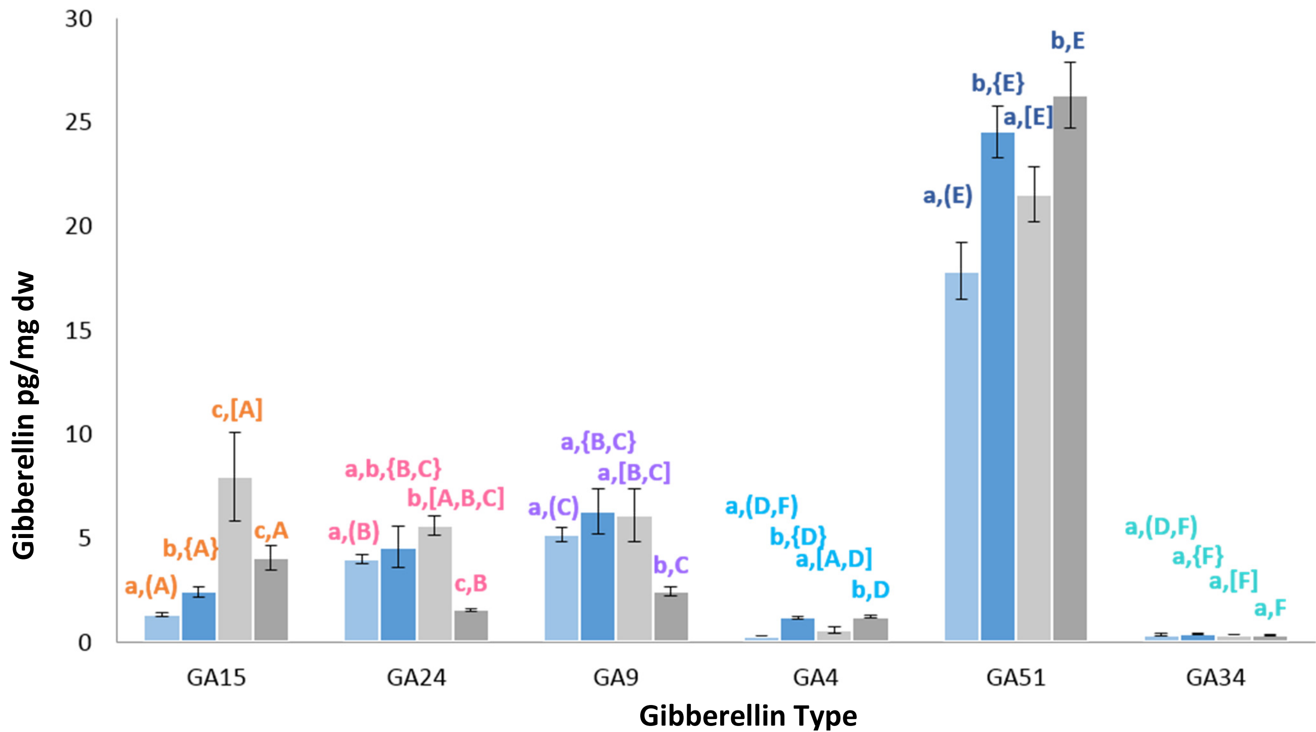


Figure 4-11 - Average pg/mg dw of endogenous GAs involved in GA_3 biosynthesis, in whole G. *max* roots treated with *B. japonicum* strains USDA110 or the non-nodulating mutant *nodC*⁻ and harvested 18 hpi or 4 dpi. Error bars represent standard error. Different lowercase letters of the same colour represent a significant difference between treatment averages for the same GA type. Different uppercase letters of the same bracketing – (), {}, [] or none – represent a significant difference between averages of different GA types of the same treatment (inoculum and time point). (Student's t-test; $P \leq 0.05$).



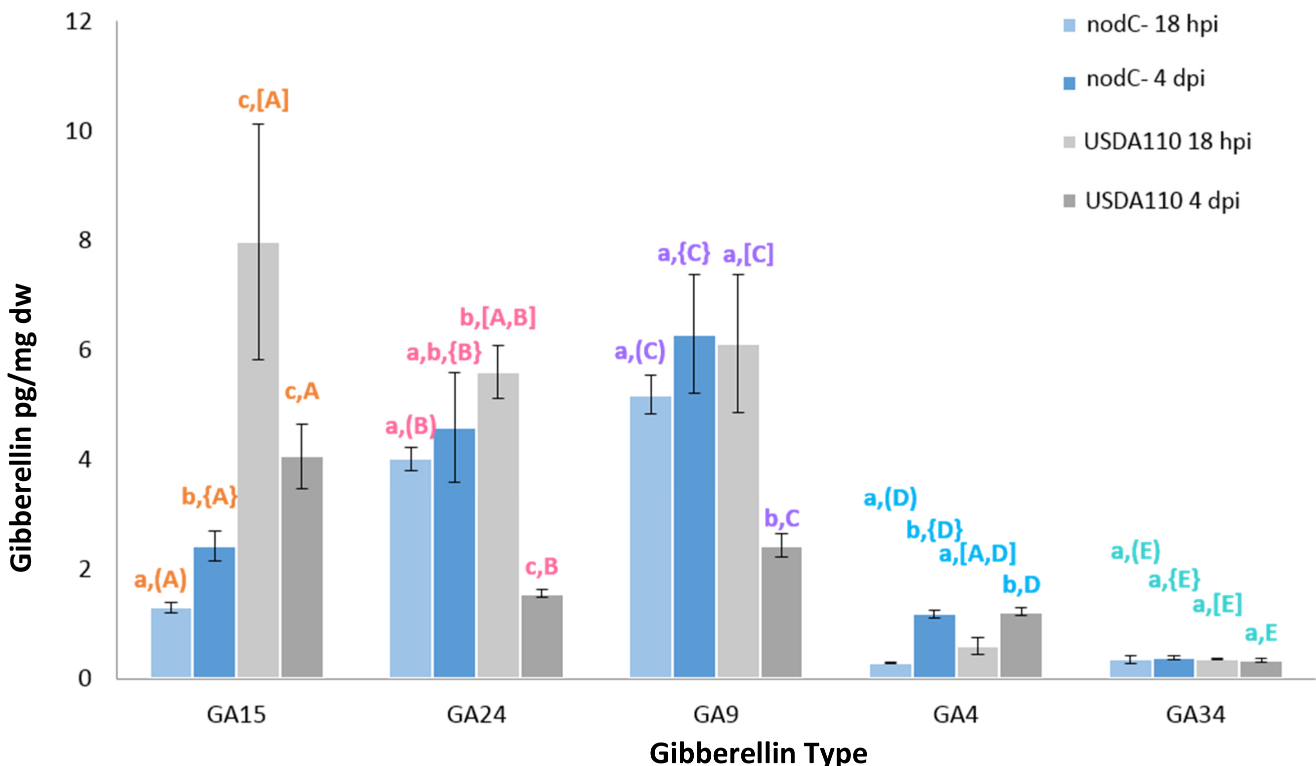


Figure 4-12 - Average pg/mg dw of endogenous GAs involved in GA_4 biosynthesis, in whole G. *max* roots treated with *B. japonicum* strains USDA110 or the non-nodulating mutant *nodC*⁻ and harvested 18 hpi or 4 dpi. Error bars represent standard error. Different lowercase letters of the same colour represent a significant difference between treatment averages for the same GA type. Different uppercase letters of the same bracketing – (), {}, [] or none – represent a significant difference between averages of different GA types of the same treatment (inoculum and time point). (Student's t-test; P≤0.05).

4.4.7 Endogenous GA levels in ZON

GA_{44} increases significantly and to the same level in both *nodC*$^-$ and USDA110 inoculated roots at 36 hpi (Figure 4-15). GA_{20} and GA_{29} both increase significantly and similarly in both treatments at 36 hpi (Figure 4-15). GA_1 at 18 hpi in *nodC*$^-$ and 36 hpi in USDA110 inoculated roots has a large variation in the data resulting in a large standard error (Figure 4-14). As a result, the level of GA_1 at all time points and across both treatments is statistically similar. The level of GA_3 does not change between 18 and 36 hpi in *nodC*$^-$ inoculated roots but does decrease between 18 and 36 hpi in USDA110 inoculated roots. At 18 hpi in USDA110 inoculated roots, GA_3 is significantly more abundant than at any other time point in any treatment. GA_3 is significantly more abundant in USDA110 18 hpi roots than GA_1 (Figure 4-13).

GA_6 follows a similar pattern between 18 and 36 hpi in both *nodC*$^-$ and USDA110 inoculated roots as it significantly increases at 36 hpi (Figure 4-15). GA_5 does not change significantly across treatments or time point (Figure 4-15). GA_{19} levels do not change significantly in *nodC*$^-$ roots across time points but does significantly increase at 36 hpi in USDA110 roots compared to the levels at 18 hpi (Figure 4-14).

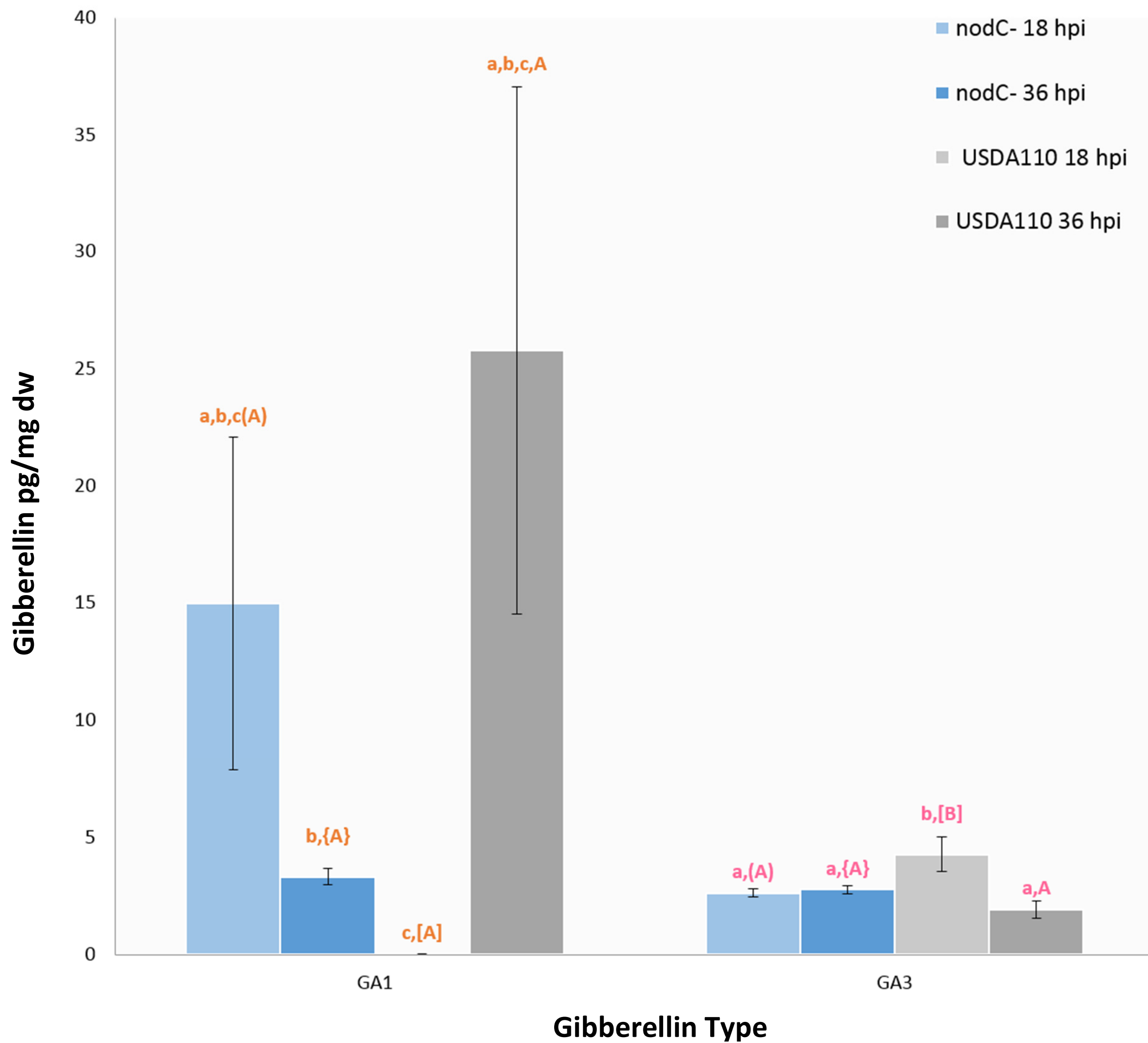


Figure 4-13 - Average pg/mg dw of bioactive endogenous GAs in ZON of G. *max* roots treated with *B. japonicum* strains USDA110 or the non-nodulating mutant *nodC*⁻ and harvested 18 hpi or 36 hpi. Error bars represent standard error. Different lowercase letters of the same colour represent a significant difference between treatment averages for the same GA type. Different uppercase letters of the same bracketing – (), {}, [] or none – represent a significant difference between averages of different GA types of the same treatment (inoculum and time point). (Student's t-test; P≤0.05).

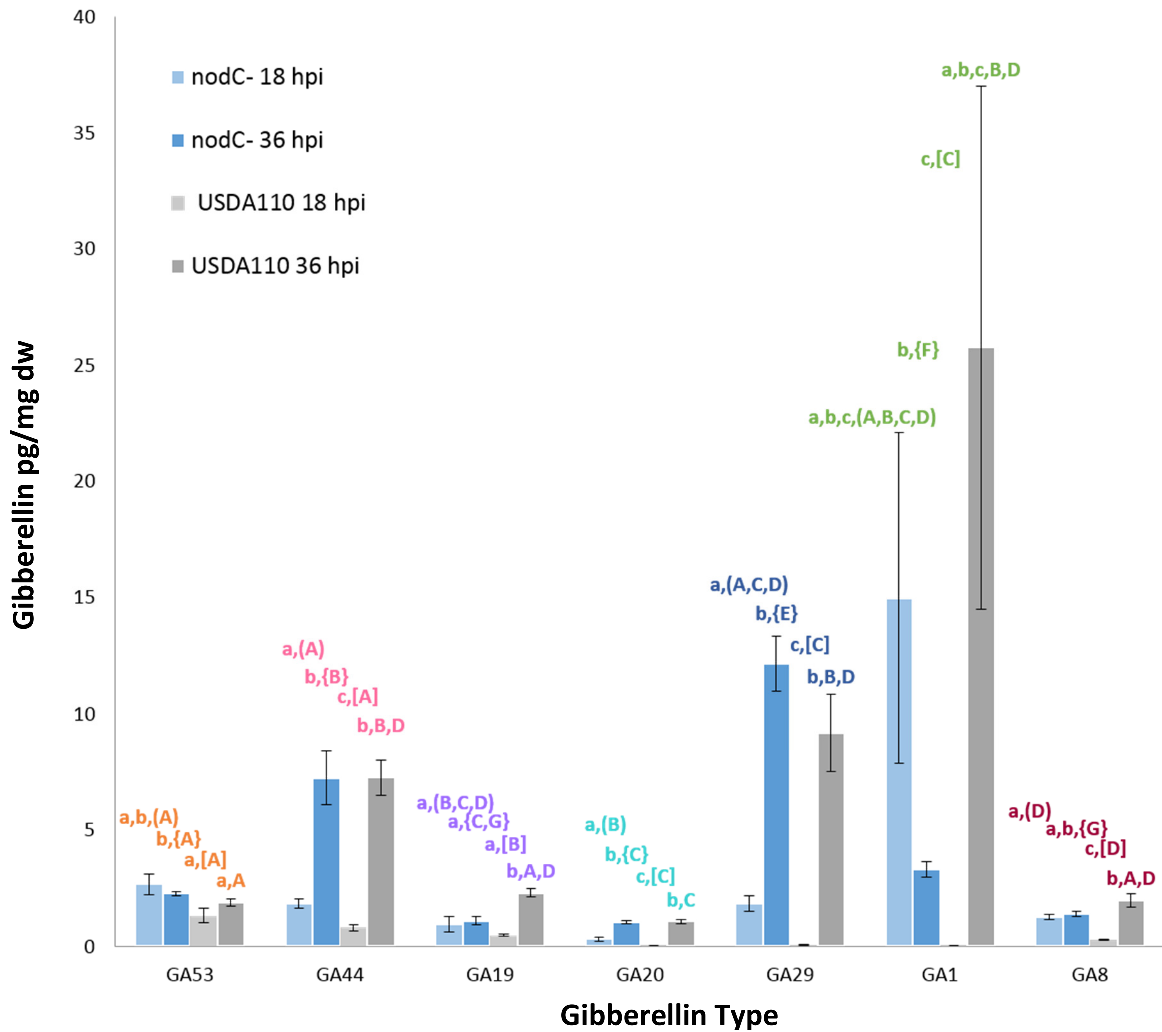


Figure 4-14 - Average pg/mg dw of endogenous GAs involved in GA_1 biosynthesis, in ZON of G. *max* roots treated with *B. japonicum* strains USDA110 or the non-nodulating mutant *nodC*$^-$ and harvested 18 hpi or 36 hpi. Error bars represent standard error. Different lowercase letters of the same colour represent a significant difference between treatment averages for the same GA type. Different uppercase letters of the same bracketing – (), {}, [] or none – represent a significant difference between averages of different GA types of the same treatment (inoculum and time point). (Student's t-test; $P \leq 0.05$).

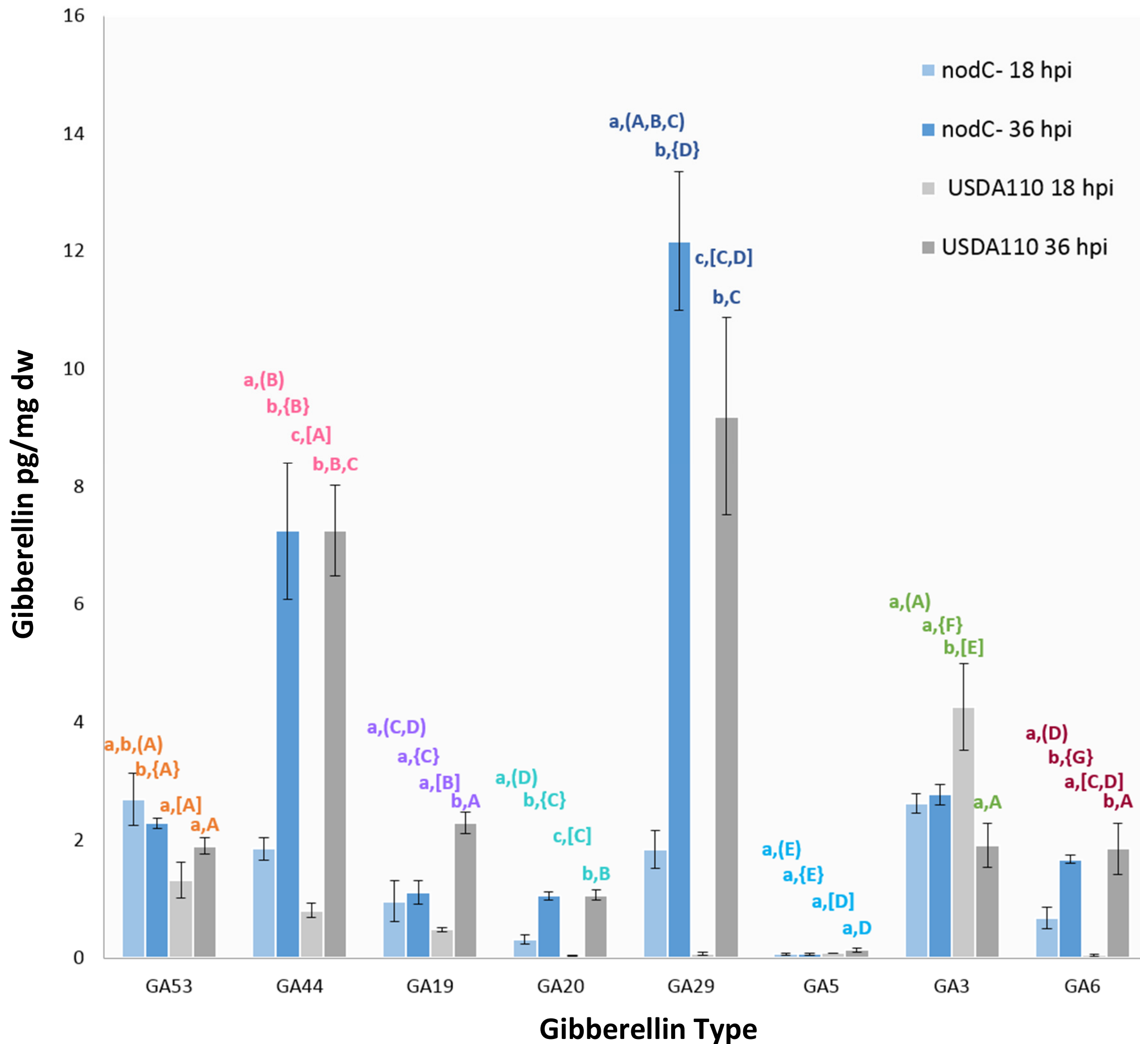


Figure 4-15 - Average pg/mg dw of endogenous GAs involved in GA_3 biosynthesis, in ZON of G. *max* roots treated with *B. japonicum* strains USDA110 or the non-nodulating mutant *nodC*⁻ and harvested 18 hpi or 36 hpi. Error bars represent standard error. Different lowercase letters of the same colour represent a significant difference between treatment averages for the same GA type. Different uppercase letters of the same bracketing – (), {}, [] or none – represent a significant difference between averages of different GA types of the same treatment (inoculum and time point). (Student's t-test; P≤0.05).

4.4.8 Endogenous GA levels in whole roots – 6 days old

GA_1 levels in *nodC*$^-$ inoculated roots appear to be highly elevated compared to both mock and USDA110 inoculated roots. However, due to the high error in this data, the 18 hpi levels of mock, USDA110 and *nodC*$^-$ are all statistically similar. At 36 hpi both *nodC*$^-$ and USDA110 inoculated roots have statically similar levels of GA_1. The level of GA_1 at 36 hpi is significantly increased in USDA110 roots compared to 18 hpi (Figure 4-17).

In 6 day old whole roots, GA_3 was more abundant in USDA110 inoculated roots than GA_1 at both 18 hpi and 36 hpi (Figure 4-16). At 18 hpi the mock inoculated roots had a statically similar level of GA_3 to the USDA110 roots; however, there was no increase in the mock roots at 36 hpi. *nodC*$^-$ inoculated roots had a much lower level of GA_3 at 18 hpi compared to USDA110, but increased to a statically similar level at 36 hpi. GA_3 was highly elevated at 36 hpi in USDA110 inoculated roots, compared to previous time points and both mock and *nodC*$^-$ inoculated roots. (Figure 4-16).

GA_{44} was at a statistically equal level at 18 hpi in all three treatments and increased significantly and similarly across treatments at 36 hpi (Figure 4-18). GA_6 was significantly lower in *nodC*$^-$ inoculated roots at both 18 and 36 hpi compared to USDA110 and mock inoculated roots. In mock inoculated roots the level of GA_6 does not significantly change between time points; however, in USDA110 inoculated roots there is a significant decreased between 18 and 36 hpi (Figure 4-18).

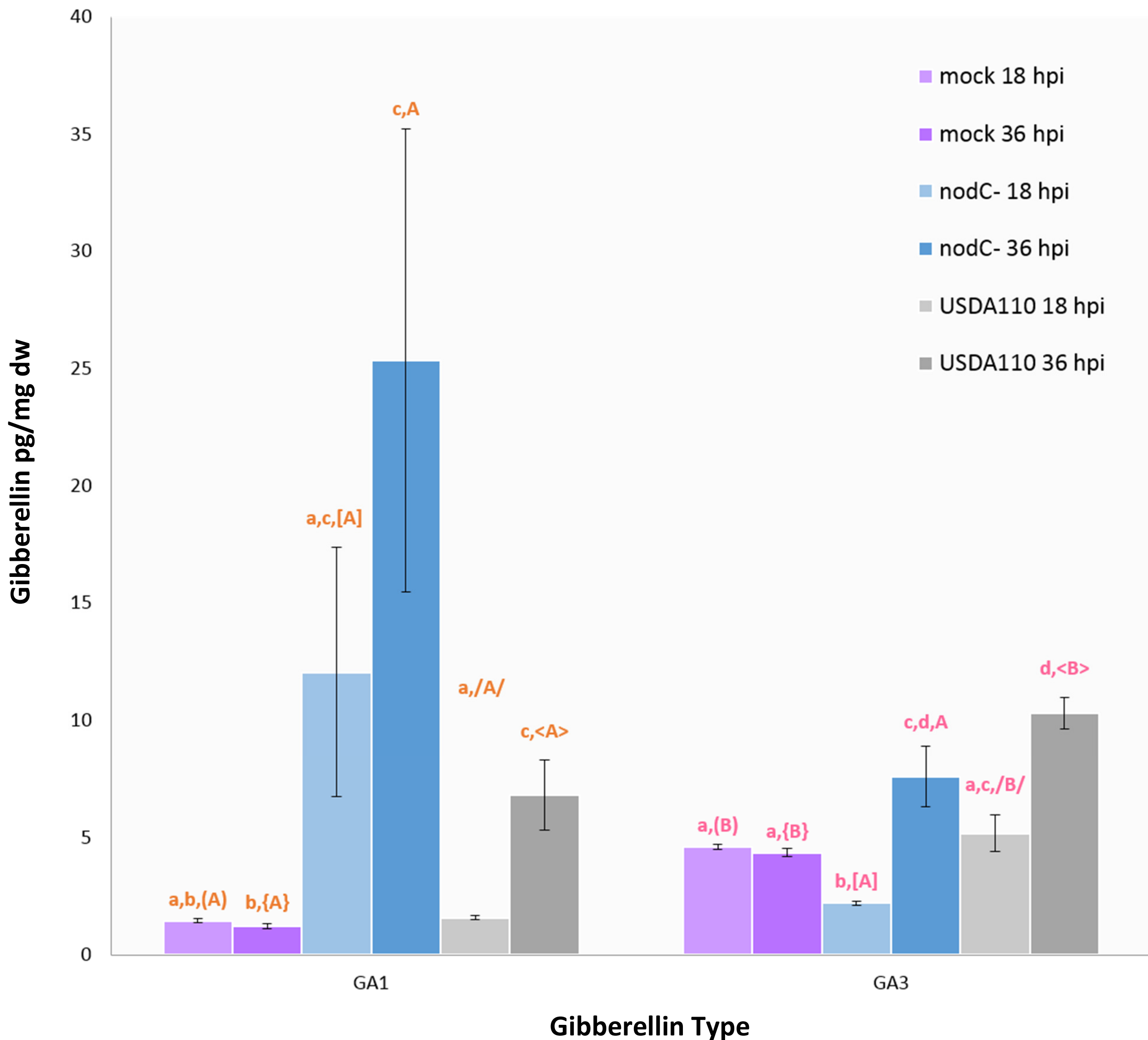


Figure 4-16 - Average pg/mg dw of bioactive endogenous GAs in whole *G. max* roots treated with a mock inoculation or *B. japonicum* strains USDA110 or the non-nodulating mutant *nodC*$^{-}$ and harvested 18 hpi or 36 hpi. Error bars represent standard error. Different lowercase letters of the same colour represent a significant difference between treatment averages for the same GA type. Different uppercase letters of the same bracketing – (), {}, [] or none – represent a significant difference between averages of different GA types of the same treatment (inoculum and time point). (Student's t-test; $P \leq 0.05$).

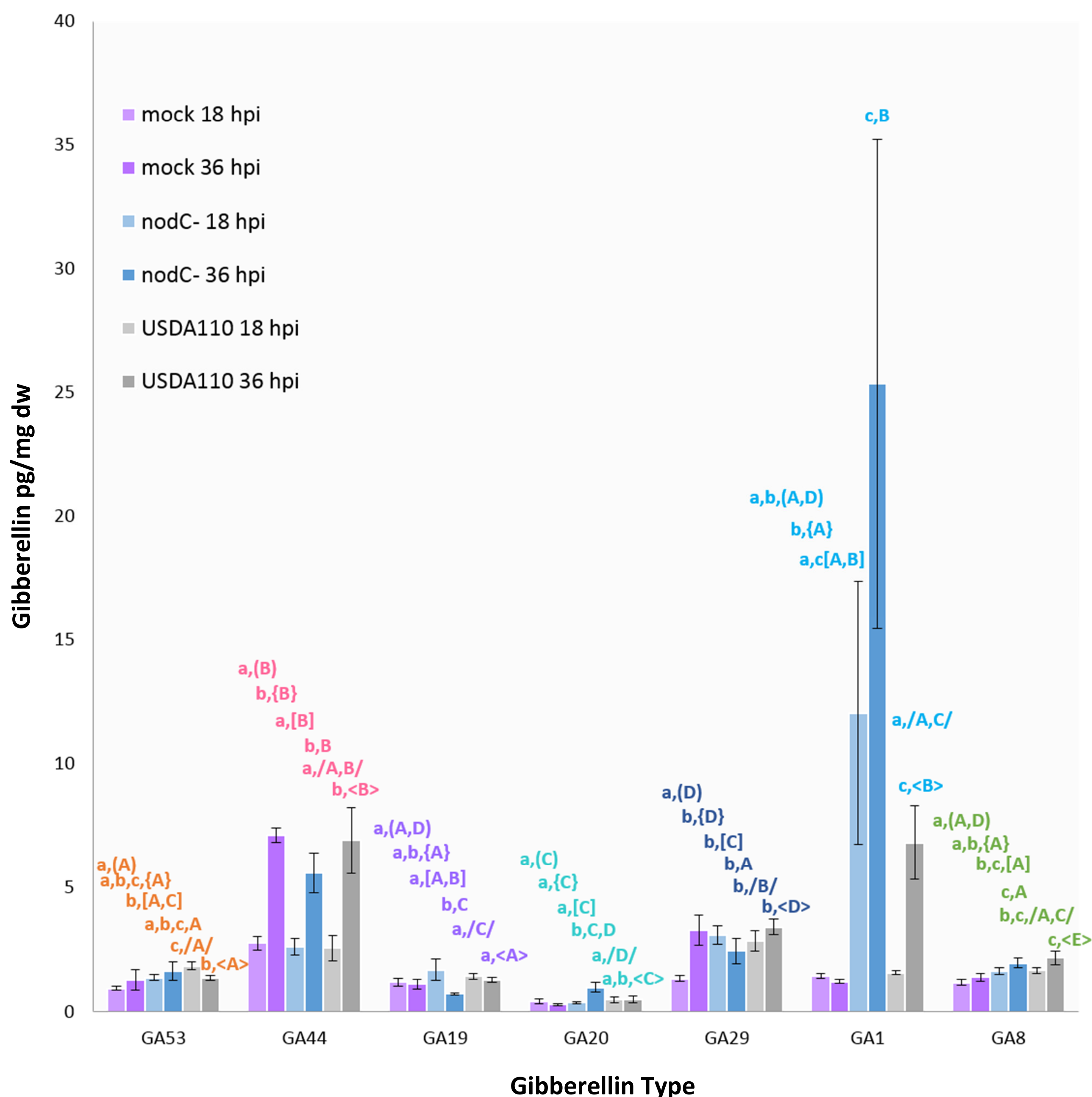


Figure 4-17 - Average pg/mg dw of endogenous GAs involved in GA_1 biosynthesis in whole *G. max* roots treated with a mock inoculation or *B. japonicum* strains USDA110 or the non-nodulating mutant *nodC*$^-$ and harvested 18 hpi or 36 hpi. Error bars represent standard error. Different lowercase letters of the same colour represent a significant difference between treatment averages for the same GA type. Different uppercase letters of the same bracketing – (), {}, [] or none – represent a significant difference between averages of different GA types of the same treatment (inoculum and time point). (Student's t-test; P≤0.05).

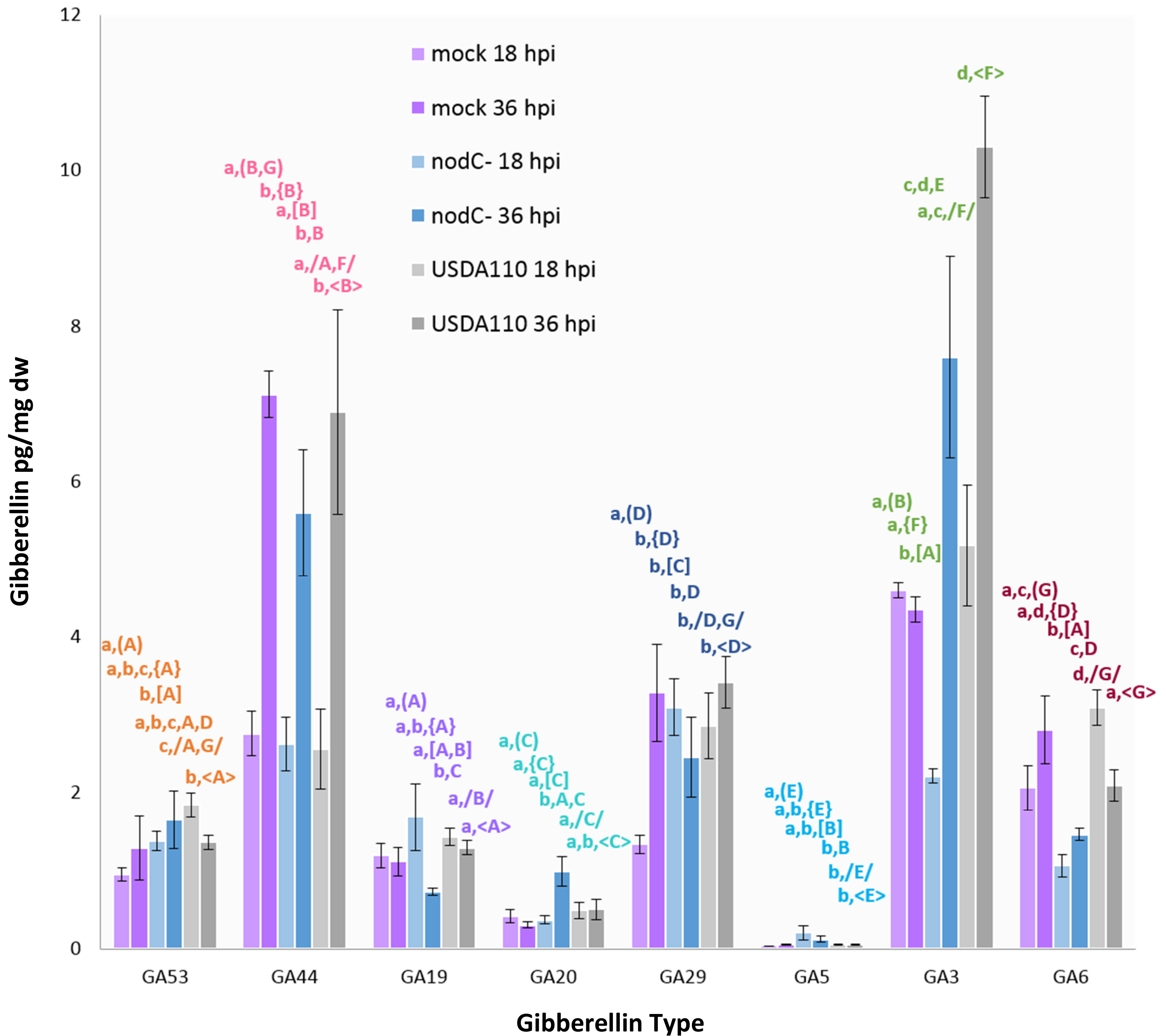


Figure 4-18 - Average pg/mg dw of endogenous GAs involved in GA_3 biosynthesis in whole *G. max* roots treated with a mock inoculation or *B. japonicum* strains USDA110 or the non-nodulating mutant *nodC*⁻ and harvested 18 hpi or 36 hpi. Error bars represent standard error. Different lowercase letters of the same colour represent a significant difference between treatment averages for the same GA type. Different uppercase letters of the same bracketing – (), {}, [] or none – represent a significant difference between averages of different GA types of the same treatment (inoculum and time point). (Student's t-test; P≤0.05).

4.4.9 GA levels in *B. rhizobium* culture supernatant

When comparing the *nodC*⁻ culture to the USDA110, most GA levels were statistically similar. Exceptions to this were GA_{53} and GA_8 which were lower in USDA110 and GA_3 which was higher in USDA110 (Figure 4-20 and Figure 4-21). The only GAs which exceeded 0.5 pg/mg dw in the *B. japonicum* cultures were GA_{44}, GA_{20} and GA_8, with none of these being greater than 1 pg/mg dw (Figure 4-20). The mock culture was only significantly lower than the *B. japonicum* cultures in GA_5. GA_{44}, GA_{29}, GA_{19}, GA_1, GA_8, GA_6 and GA_3 were all found in significantly greater quantity in the mock culture. The concentration of GA_1 was found to be significantly greater than that of GA_3 in all three cultures (Figure 4-19).

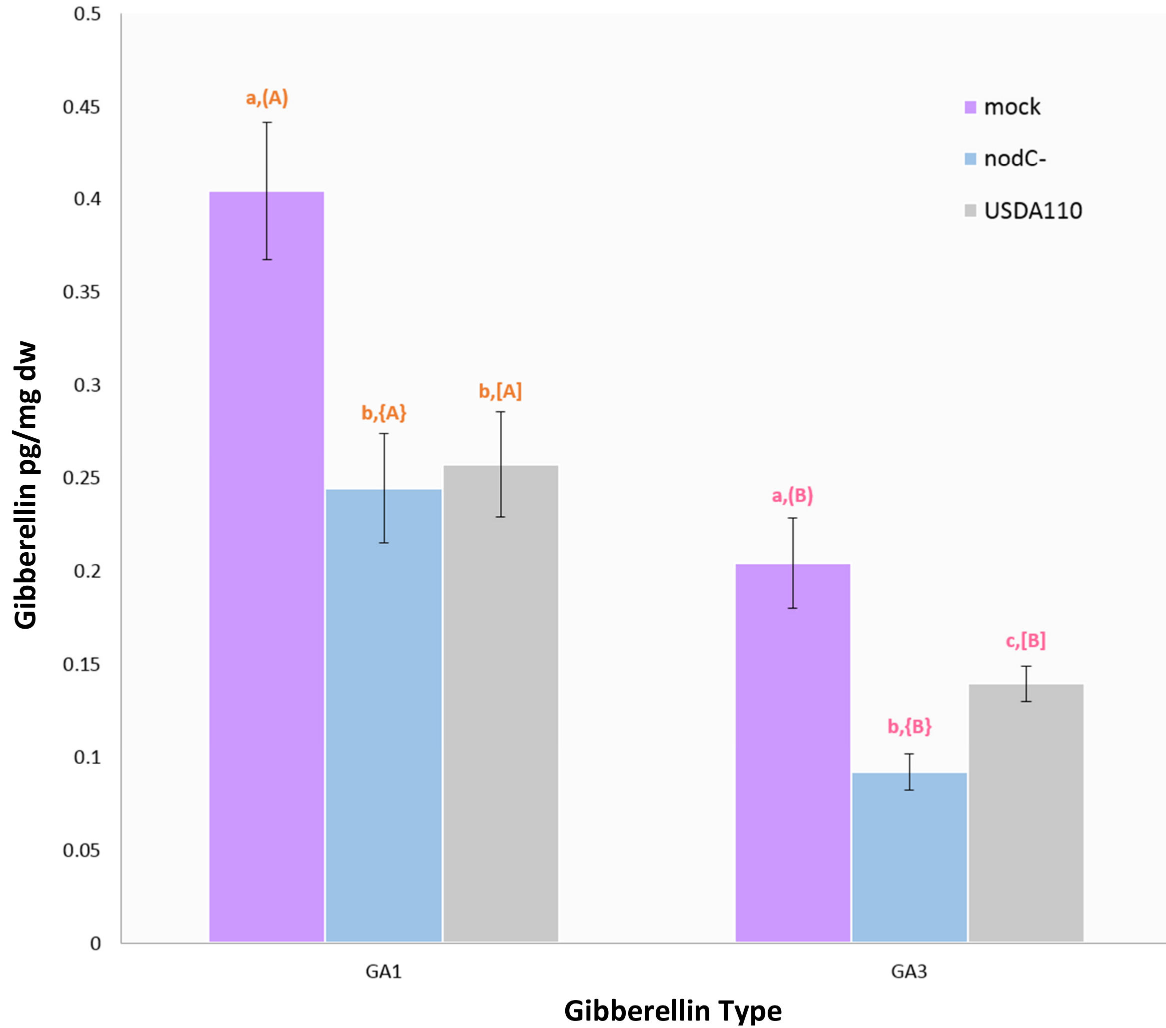


Figure 4-19 - Average pg/mg dw of bioactive GAs found in the freeze-dried supernatant of centrifuged culture containing either a mock culture of YMB or YMB inoculated *B. japonicum* strains USDA110 or the non-nodulating mutant *nodC*⁻. Error bars represent standard error. Different lowercase letters of the same colour represent a significant difference between treatment averages for the same GA type. Different uppercase letters of the same bracketing – (), {}, [] or none – represent a significant difference between averages of different GA types of the same treatment (inoculum and time point). (Student's t-test; P≤0.05).

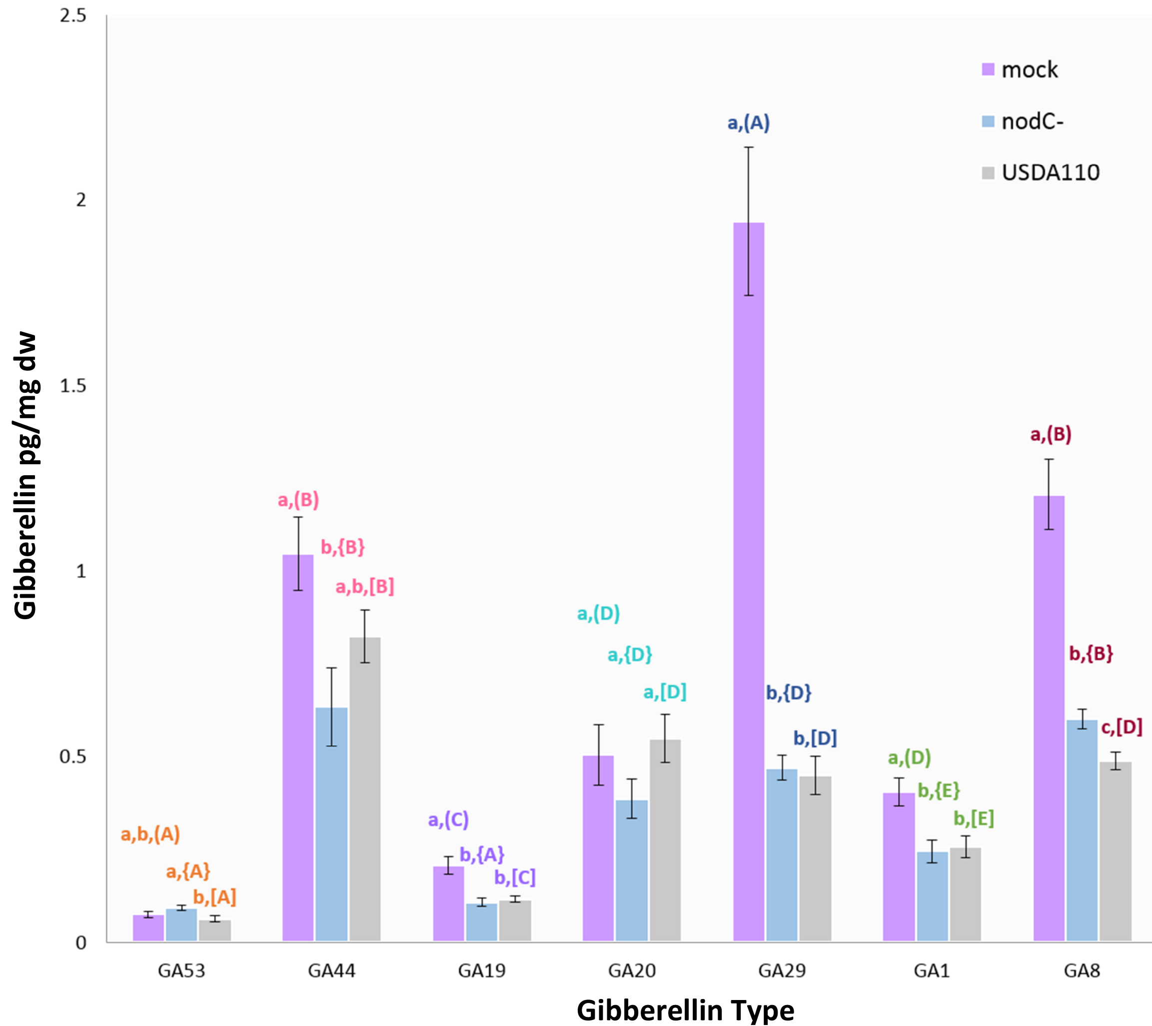


Figure 4-20 - Average pg/mg dw of GAs in involved in GA_1 biosynthesis found in the freeze-dried supernatant of centrifuged culture containing either a mock culture of YMB or YMB inoculated *B. japonicum* strains USDA110 or the non-nodulating mutant *nodC*$^-$. Error bars represent standard error. Different lowercase letters of the same colour represent a significant difference between treatment averages for the same GA type. Different uppercase letters of the same bracketing – (), {}, [] or none – represent a significant difference between averages of different GA types of the same treatment (inoculum and time point). (Student's t-test; P≤0.05).

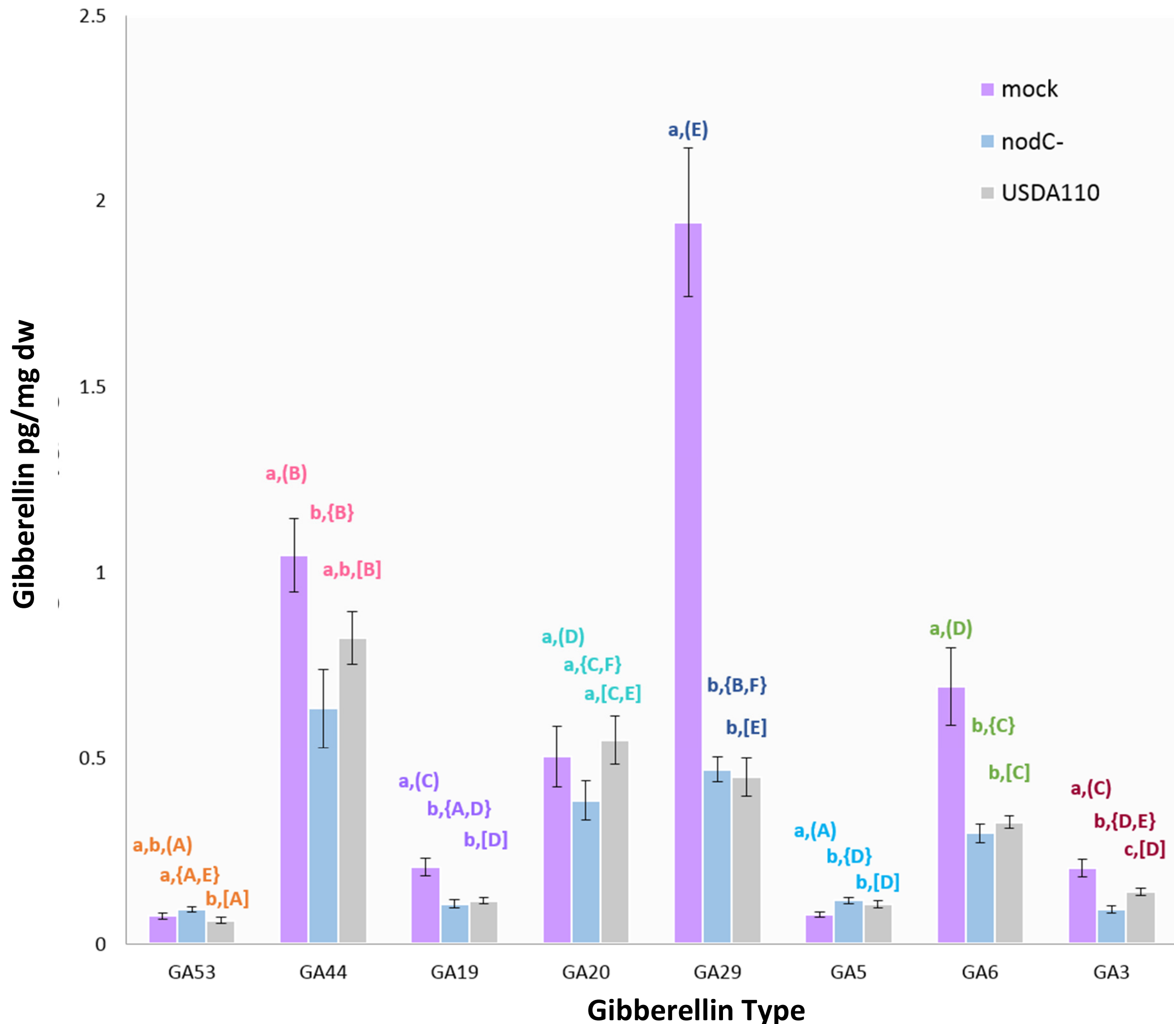


Figure 4-21 - Average pg/mg dw of GAs in involved in GA_3 biosynthesis found in the freeze-dried supernatant of centrifuged culture containing either a mock culture of YMB or YMB inoculated *B. japonicum* strains USDA110 or the non-nodulating mutant *nodC*⁻. Error bars represent standard error. Different lowercase letters of the same colour represent a significant difference between treatment averages for the same GA type. Different uppercase letters of the same bracketing – (), {}, [] or none – represent a significant difference between averages of different GA types of the same treatment (inoculum and time point). (Student's t-test; P≤0.05).

4.5 Discussion

4.5.1 GA2ox discussion

GmGA2ox was found to have the most sequence similarity to Class II GA2-oxidases of other species. Class II and class I GA2-oxidases use C_{19}-GAs as substrates as opposed to class II GA2-oxidases which can use C_{20}-GAs (Yamaguchi, 2008). As GmGA2ox is a class II GA2-oxidase it can be assumed that it has a role in the later stages of the GA biosynthesis pathway, rather than an earlier role which would involve interacting with C_{20}-GA substrates. Thus, GmGA2ox likely converts GA_9 to GA_{51}, GA_4 to GA_{34}, GA_{20} to GA_{29} or GA_1 to GA_8 as these are all C_{19}-GAs.

When considering GmGA2ox's class II classification and the endogenous GA levels measured in the 14 day old whole root samples, we can begin to narrow down the likely role of GmGA2ox in the GA biosynthesis pathway. As the expression of *GmGA2ox* varies post inoculation, increasing at 12 hpi, drastically increased at 24 hpi and largely decreasing at 48 hpi followed by a slight increase again at 96 hpi, it would be expected that the GA substrate acted upon by this GA2-oxidase would similarly vary in level dependent upon inoculation type and time post inoculation. Therefore, it is unlikely that GmGA2ox acts on GA_4 to produce GA_{34} as the level of GA_{34} does not change at all, no matter the treatment type.

Additionally, GA_4's levels follow the same pattern over time regardless of inoculation type, this differs from *GmGA2ox's* expression pattern which is inoculum dependant. Although it is unlikely that GmGA2ox uses GA_4 as a substrate, it is difficult to narrow down its likely substrate any further as it is unknown what time frame should be considered to be reasonable between detecting a change in gene expression to expecting a change in endogenous GA level. Future experiments which harvest inoculated root tissue at multiple time points and use the same pooled root samples for both qRT-PCR measurement of gene expression and UPLC MS/MS measurement of endogenous GA level would give a better idea of the relationship between gene expression and GA level.

4.5.2 GA2ox Over-expression

To gain insight into *GmGA2ox's* role in nodulation, the hairy root transformation to over-express *GmGA2ox* requires repeating. This experiment failed to produce results due to a failure of the plants to nodulate. When carrying out the appropriate qRT-PCR confirmation of *GmGA2ox's* expression level, it is advised to simultaneously measure the expression level of *GmGA20ox a* and *GmGA3ox 1a* to test whether these genes' expression changes in response

to changes in *GmGA2ox's* expression. As previous studies have shown the importance of GA in nodulation lies in its level being kept at certain thresholds for different species and stages of nodule development (Ferguson *et al.*, 2005a; Ferguson *et al.*, 2011; Ferguson *et al.*, 2014; Maekawa *et al.*, 2009; Hayashi *et al.*, 2014), it is inferred that an increase in *GmGA2ox* would lead to a decrease in bioactive GA production. This could possibly trigger an increase in the expression of *GmGA20 a* and *GmGA3ox 1a* to compensate.

4.5.3 GA20ox RNAi

GA20-oxidases have a high level of redundancy within *A. thaliana* and *O. sativa* (Xu *et al.*, 1995; Sakamoto *et al.*, 2004). Given *G. max*'s duplicated genome, it is highly likely that redundancy also exists for *GmGA20ox a*. It is possible that it was due to redundancy that no phenotypic changes were observed in *GmGA20ox a* silenced hairy roots. It is also possible that silencing of *GmGA20ox a* was not successful. Recently, a fast neutron mutant for *GmGA20ox a* was added to the SoyBase database (http://soybase.org; Grant *et al.*, 2010). It is therefore recommended that future studies of *GmGA20ox a* utilise this resource, rather than repeat this silencing experiment or attempt to silence multiple GA20-oxidases at once. Characterising the mutant's phenotype would hopefully point to *GmGA20ox a's* role in nodulation. Further hairy root transformation could be utilised in the mutant to more easily allow for the silencing of an additional GA20-oxidase.

4.5.4 GmGA20ox and GmGA3ox promoter studies

The lack of GUS staining in the *nodC*$^-$ inoculated control for both pGmGA3ox 1a::GUS and pGA20ox a::GUS confirms the expression pattern measured through qRT-PCR by (Hayashi *et al.*, 2012; Figure 4-2), neither gene is expressed in response to inoculation with *B. japonicum* in the absence of NF. This further confirms that both genes are nodulation specific in their root expression.

In pGmGA20ox a::GUS transformed roots, the expression of *GmGA20ox a* was very nodule specific. *GmGA20ox a* is highly likely to be involved in the formation of the infection thread in the root hair given its strong expression in this cell during the early stages of nodulation. GA is known to play a role in cell elongation (Tanimoto, 2005) so it is possible that *GmGA20ox a* facilitates the elongation of the infection thread. It is proposed, given *GmGA20ox a*'s expression in the inner cortex of the root at the early stages of nodulation 16-48 hpi, that *GmGA20ox a* plays a role in cortical cell division of the developing nodule. *GmGA20ox a*'s role within maturing nodules seems to be in cell division of the nodule primordium. Within the

mature nodule *GmGA20ox a* is again working in the cortical cells specifically the inner cortex, however its reduced expression indicates a great reduction in expression and therefore role in these cells. As the expression is in the northern and southern inner cortical cells, it is again likely that *GmGA20ox a's* role is in few remaining cell divisions required for a mature nodule.

Unlike in pGmGA20ox a::GUS transformed roots, GUS staining in pGmGA3ox 1a::GUS transformed roots was not observed in the root hair at any time point. Thus it is unlikely that *GmGA3ox 1a* plays a major role in the formation of infection threads or root hair curling.

GmGA3ox 1a is not nodule specific in its expression. The widespread expression observed at 12 hpi in the phloem lends itself to the idea that *GmGA3ox 1a* plays a role in changes to the root in response to nodulation, but not actually involved in nodule development. The rapid decrease in staining at 48 hpi coupled with its clustering in the phloem extending into developing lateral roots is peculiar as there is no similar expression seen in the phloem of developing nodules, despite the similarity in the development of these two organs in their early stages. It is unclear what this staining pattern is indicating about *GmGA3ox 1a's* role in nodulation. GA is known to promote cell and root elongation, however, only within a small range of concentrations, similar to the way GA can promote or inhibit nodulation dependant on concentration (Tanimoto, 2012). As such, it is possible that *GmGA3ox 1a*'s expression is greatly up-regulated in order to increase the GA concentration of the roots and promote root elongation in response to nodulation. Assuming that increased gene expression correlates with increased endogenous GA level, the rapid decrease in expression is likely due to needing to tightly control the concentration of the GA produced. With this in mind it follows that newly formed lateral roots would display higher *GmGA3ox 1a* expression than the rest of the root system as new GA would be needed in these new cells.

In the mature nodule, faint *GmGA3ox 1a* expression was seen in the scleroid layer. The scleroid layer consists of cells with thicker cell walls, GA actually inhibits cell wall thickening (Tanimoto, 2005), it is possible that here *GmGA3ox 1a*'s is involved in maintaining the proper amount of cell thickening. Similarly, GA is often found in the region of elongation to inhibit root thickening, despite *GmGA3ox 1a* being expressed in this tissue, as its expression is nodulation dependant it is unlikely that that is a role for *GmGA3ox 1a.*

As both *GmGA20ox a* and *GmGA3ox 1a* are nodulation specific in their expression it was originally hypothesised that these biosynthesis genes may work in the same stream of the GA biosynthesis pathway. However, following the GUS expression study results which highlighted that *GmGA20ox a,* which is positioned up-stream in the biosynthesis pathway, is expressed solely in nodule related tissue but *GmGA3ox 1a*, whose role is down-stream, is expressed in tissue unspecific to nodulation, it seems less likely that they work in the same stream of the pathway.

It is speculated that other non-nodulation specific GA20-oxidases and GA3-oxidases work in the same stream as *GmGA20ox a* and *GmGA3ox 1a.* Other nodulation-specific GA20-oxidases and GA3-oxidases would likely have been identified through the RNA-seq study carried out by Hayashi *et al.* (2012). Thus it is possible that non-nodulation specific GA20-oxidases and GA3-oxidases are being expressed in similar cell types to *GmGA20ox a* and *GmGA3ox 1a* with the expression of these two nodulation specific genes acting as the limiting step in the biosynthesis.

4.5.5 Endogenous GA levels in whole roots – 14 day old

When considering the levels of endogenous GA present in whole roots of *G. max* post inoculation, GA_3 emerges as the main bioactive GA present. Both *P. lunatus* (lima bean) and *V. unguiculata* (cowpea) were found to possess GA_3 in their nodules (Dobert *et al.*, 1992a; Dobert *et al.*, 1992b) though it is unclear in both cases if GA_3 is the most abundant bioactive GA present. Neither GA_3 nor GA_4 were found in *P. sativum* (pea) root tissue (Yaxley *et al.*, 2001). GA_3 was not only present at much higher levels compared to the other bioactive GAs, but was more abundant in USDA110 inoculated roots and increased in concentration over time.

Neither GA_{29} nor GA_6, the two GAs along the GA_3 biosynthesis pathway which divert substrate from the biosynthesis of GA_3, increased in abundance over time when inoculated with USDA110, despite GA_3's level increasing. This could indicate that the level of GA_3 being produced does not require negative regulation at 4 dpi as an increase in level of either catabolite would indicate. However, when examining GA_{29} and GA_6 levels in *nodC*$^-$ inoculated roots, their levels do significantly increase at 4 dpi; this may be as a response to the lack of nodulation occurring and therefore a need to keep the GA_3 level from increasing.

The level of endogenous GA_4 found does increase 4 dpi, however this increase occurs independent of inoculum type. It would therefore seem likely that GA_4's increase is related to root growth and development, rather than to nodulation. The metabolite GA_{51}, a product of

deactivated GA_9, a GA_4 precursor, follows the same abundance pattern as GA_4. GA_{51} increases in abundance at 4 dpi regardless of inoculum type.

4.5.6 Endogenous GA levels in whole roots – ZON and Whole Root

The GA content of *B. rhizobium* culture was examined to determine the likelihood of measurement of endogenous root GA, particularly GA_3, being affected by rhizobia produced GA. GA_3 was of particular interest as it is not usually found in high concentrations in plant tissue comparative to GA_1 and GA_4. None of the GAs measured were found in great quantity in either the USDA110 or *nodC*⁻ cultures and for the most part the GA concentration in these cultures was lower than that of the mock culture containing just YMB. This was highly unexpected as previous studies had shown GA_3 production by *B. japonicum* (Boiero *et al.*, 2007). This experiment should be repeated with the inclusion of a water control to confirm the validly of the result from the mock culture. In the inoculated cultures, the concentration of GAs was quite low, particularly that of GA_3. It is unlikely that the measurement of endogenous GA in inoculated roots is greatly affected by the GA produced by rhizobia in culture.

The extent to which rhizobia produced GAs may be measured concurrently with endogenous root GAs, resulting in misleading results was tested. A harvest of whole roots inoculated with a mock culture containing no rhizobia was tested alongside a *nodC*⁻ treatment and USDA110 inoculation. It was hypothesised that GA levels in mock roots would be very similar to GA levels in *nodC*⁻ roots but both would differ from the levels found in USDA110 inoculated roots if it is true that endogenous GA increases during nodulation. It was also thought that if rhizobial GA production did affect the measurement of GA in the root, that the mock inoculated roots would have much lower GA levels than either the *nodC*⁻ or USDA110 roots.

Similar levels of GA were found in all three treatments for GAs: GA_{20}, GA_{44}, GA_{53}, GA_{29} and GA_5. This could be explained by considering the possibility that the mock or *nodC*⁻ were contaminated with nodulating rhizobia or that USDA110 plants did not receive viable USDA110 and therefore were not undergoing changes associated with nodulation. However, this assumption does not hold true when considering GA_1 and GA_3 levels at 36 hpi which are much greater in both *nodC*⁻ and USDA110 roots than mock inoculated roots. Further to this, GA_3 was significantly more abundant in USDA110 roots than *nodC*⁻ roots at both 18 and 36 hpi. It may well be that levels of intermediate GAs do not change in response to nodulation due to a possible increase in their metabolism. Thus, the only GA levels that increased absolutely during nodulation are bioactive GA_3 and GA_1. For this to hold true it must be accepted that

either the *nodC*$^-$ inoculated roots were contaminated with nodulating rhizobia or that an increase in bioactive GA is not NF dependant but a response to the rhizobia's surface proteins. This theory, however, is at odds with the clear NF dependent up-regulation of *GmGA20ox a* and *GmGA3ox 1a* seen in both the qRT-PCR of Hayashi *et al.* (2012) and the histochemical promoter study conducted in this thesis (sections 4.4.4 and 4.4.5). It is possible that the biosynthesis genes in question are involved in the production of GA_4, which was not measured in this study.

These results confirm what was seen in the previous whole root experiment with 14 day old roots, GA_3 is more abundant than bioactive GA_1 in *G. max* whole roots independent of rhizobia exposure. Further analysis of the material produced from this study of 6 day old whole roots may enable the measurement of GA_4 which may also be less abundant than GA_1 as was seen in the 14 day old root experiment. When comparing patterns of GA level changes between the whole root samples of 14 day old roots and 6 day old roots, it must be remembered that, although both contain a ZON, the proportion of ZON to non-ZON root tissue is much smaller in the older roots.

In both the 6 day old whole roots and the ZON samples, the GA_1 measurements were varied between biological replicates, resulting in a large standard error. It is therefore difficult to suggest, how GA_1 may be involved in nodulation with great confidence. Additional biological replicates should likely be added to future experiments where GA_1 is intended to be measured. Given the difficultly with measuring endogenous GA in *G. max* roots in the past (Hayashi, 2014), it is possible that GA_1 is particularly difficult to measure accurately.

When examining the levels of multiple GAs in the ZON no differences were found between the nodulating USDA110 and the non-nodulating *nodC*$^-$ inoculated roots, exceptfor a higher level of GA_{19} 36 hpi and GA_3 18 hpi in USDA110 inoculated roots.

It was expected that when examining the ZON, differences in GAs' concentrations between *nodC*$^-$ and USDA110 inoculated roots would be amplified if GA plays a major role in early nodulation. However, very few major differences were observed between the two treatments. GA_{19} increased significantly between 18 and 36 hpi and was significantly higher in concentration in USDA110 roots at the 36 hpi time point. Conversely, GA_3 was most highly concentrated in 18 hpi USDA110 roots and decreased to levels statically equal to *nodC*$^-$ at 36 hpi. GA_3's high concentration at 18 hpi is supported by the very low concentration of GA_6 at the same time point. As GA_6 is produced to decrease GA_3 production through diversion of GA_5,

GA_3's precursor, it follows that its concentration would be low when a high concentration of GA_3 is needed and increase when the concentration of GA_3 requires lowering. This pattern of concentration was seen here.

Conversely, GA_1 is most abundant at 36 hpi and very scarce at 18 hpi. Despite this, the difference between GA_1 and GA_3 in the ZON at 36 hpi is not statically significant due to the GA_1 measurement possessing a large standard error.

It may be that in the ZON GA_3 plays a role at 18 hpi and GA_1 a role at 36 hpi. This specificity could explain the vast differences in results of GA and GA biosynthesis inhibitor studies if different concentrations of different GAs are required at different points of both root and nodule development. GA_{29} is highly increased at 36 hpi in the ZON in both $nodC^-$ and USDA110 roots. The increase in GA_{29}, being a metabolite of the GA_1 and GA_3 precursor GA_{20}, in the ZON of both $nodC^-$ and USDA110 inoculated roots indicates that the concentration of both GA_1 and GA_3 is being regulated regardless of nodulation.

It is recommended that this ZON study is repeated and expanded to include further time points and a use of qRT-PCR to analyse the gene expression of the GA biosynthesis genes *GmGA20ox a*, *GmGA3ox 1a* and *GmGA2ox*. This would enable further conclusions to be drawn about the timing and relationship between gene expression and endogenous GA level. Additionally, some plants from each treatment should be grown for 2 wpi to be used as a sample of quality control. If the *nodC-* plants nodulate, then the samples harvested are not a representation of non-nodulating plants. Likewise, if USDA110 inoculated plants do not nodulate, then the samples were not representative of nodulating tissue.

Assuming all treatments were executed correctly, GA_{44} is almost certainly involved in root growth and development, independent of nodulation as in the ZON and 6 day old whole root experiment, all treatments, including the mock inoculation treatment, have a drastic but equal increase in endogenous GA_{44} content at 36 hpi.

Recently, fast neutron mutants for *GmGA20ox a*, *GmGA3ox 1a* and *GmGA3ox 1b* have been added to the SoyBase database (http://soybase.org; Grant *et al.*, 2010). In order to further understand not only the role of these individual genes in nodulation but the role of GA in general, endogenous GA levels in these mutants' roots should be measured. In addition to the characterisation of the mutants' physiology, the genes' expression levels should be measured via qRT-PCR, as well as the expression levels of related early nodulation genes, such as

GmGA2ox, whose expression may be altered where the mutated genes' expression is also abnormal.

Ultimately, the results of this study have led further credence to the role of GA in nodulation proposed by Hayashi *et al.* (2014) through the histochemical promoter analysis, that GA is necessary in the early stages of nodulation including infection thread formation and in the development of the nodule itself. However, GA does not play an important role in the mature nodule. Additionally, this study has established that GA_3 is more abundant in *G. max* whole roots than GA_1.

Chapter 5 General Discussion and Future Directions

This is the first study to measure the endogenous level of GA in soybean roots using reliable, modern techniques. The findings open up many avenues to further explore the role that GA plays in nodulation and root development. Importantly, being able to measure endogenous GA allows studies to examine what is actually occurring in the root, rather than to make inferences based on gene expression or application studies.

To understand how direct the relationship is between gene expression and endogenous GA content, a more detailed time course study could be conducted. By comparing the ZON of USD110 inoculated plants with *nodC*$^-$ inoculated plants via a combination of GA measurements and qRT-PCR studies (to measure *GmGA20ox a, GmGA3ox 1a* and *GAGm2ox* expression) at the same time points, it may be possible to establish a time window for the relationship between gene expression and GA content. Constructing a pGAGm2ox::GUS vector would allow for greater insight into which cell types this gene is acting and whether it is likely to be working down-stream of either of *GmGA20ox a* or *GmGA3ox 1a.*

Recently, fast neutron mutants for *GmGA20ox a*, *GmGA3ox 1a* and *GmGA3ox 1b* have been added to the SoyBase database (http://soybase.org; Grant *et al.*, 2010). These mutants will be an invaluable resource in researching GA's role in nodulation. Not only will the mutants be able to be phenotypically characterised, but the creation of double or even triple mutants could lead to a better understanding of whether and how these GA biosynthesis genes work within the same stream of the GA biosynthesis pathway.

Now that a successful method for measuring endogenous GA content of soybean roots has been established through collaboration, it could be capitalised on to measure the root GA content of the fast neutron mutants. If similar GAs are reduced in both *GmGA20ox a* and *GmGA3ox 1a* mutants then it is likely that they work within the same stream of the GA biosynthesis pathway.

Measuring endogenous GA levels allows for the role of GA biosynthesis genes to be better understood, as an unchanged GA level may indicate a level of redundancy or that the gene in question is not essential for GA biosynthesis. Comparing differences in GA levels of mutant plants to WT, coupled with the phenotypic analysis of the mutant plants, will allow for further conclusions to be made about the role of GAs in nodulation.

Neither over-expressing nor silencing of the *GmTIR-NBS-LRR* gene resulted in a phenotypic change to nodulation. Given that this gene is now known to be transcribed in multiple ways, it

is clear that a new approach needs to be taken to its characterisation. A fast neutron mutant for *GmTIR-NBS-LRR* has also been added to the SoyBase database (http://soybase.org; Grant *et al.*, 2010). Phenotypically characterising this mutant will provide great insight into the role of *GmTIR-NBS-LRR* in nodulation. Over-expression studies in this mutant of the differing *GmTIR-NBS-LRR* transcripts could provide insight into the role of these transcripts and their importance.

Inoculating a *GmTIR-NBS-LRR* mutant that has been transformed to over-express one of the various TVs, with different strains of rhizobia (and *B. japonicum* in particular) would allow for testing of the theory that *GmTIR-NBS-LRR* is involved in modulating host specificity.

Particularly for *GmGA20ox a* and *GmTIR-NBS-LRR*, the use of a mutant is a much more robust system than the use of hairy root transformation. Despite hairy root transformation being a flexible and useful tool, the transformation is inconsistent and differs on an individual root basis. The use of a mutant will give a much more consistent basis for future studies. One advantage of the hairy root transformation method is that only the roots and nodules are manipulated in their gene expression, genes which are expressed in other tissues are not affected. When considering the *GmGA20ox a* and *GmTIR-NBS-LRR* genes, they are expressed almost exclusively in the roots and nodules, therefore hairy root transformation has no advantage over the use of a mutant in this case.

This study further confirms the mechanism for GA's role in nodulation proposed by Hayashi *et al.* (2014) in which GA is vital for early nodulation events including the formation of the infection thread and for in the development of the nodule, but that its role subsides in the mature nodule. It has also provided a basis upon which to build in the investigation of the role of multiple early nodulation genes, including three within the GA biosynthesis pathway; *GmGA20ox a*, *GmGA3ox 1a* and *GmGA2ox* and *GmTIR-NBS-LRR*. Showing that bioactive GA_3 is the most abundant bioactive GA in whole *G. max* roots is an exciting result as it differs greatly from many other legume species. This establishes a basis for examining the role of GA in nodulation from a newly improved physiological perspective.

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