Functional characterization of *Mt*CBP60B in plant endosymbioses

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Abstract

Plants perceive a range of microbes at the root-soil interface, which induces cellular responses in a microbe-specific way. Legume plants, such as *Medicago truncatula,* respond to signals from symbiotic nitrogen-fixing rhizobial bacteria or arbuscular mycorrhizal fungi with the induction of nuclear calcium oscillation. This nuclear calcium release is required for activation of downstream genes involved in the symbiont colonisation process and organogenesis of nodule or arbuscule structures. Generation of these calcium oscillations is controlled by a suite of nuclear-localised ion channels, including the cation channel DMI1. In this study, we begin to describe the activity and function of the DMI1 interacting partner, CALMODULIN BINDING PROTEIN60 B (CBP60B). Here, it is suggested that CBP60B is involved in the colonisation of root tissues by symbiont partners. It was found that the formation of symbiosis specific organs was reduced in the mutant *cbp60b*-1. In symbiont inoculated tissues, *CBP60B* was shown to be highly expressed at the nodule apex, in cells infected by rhizobia, as well as in AM containing cortical tissues. In non-colonised tissues, expression was limited to the root apical meristem and the vascular-associated pericycle cells, sites involved in rapid cell division, potentially implicating CBP60B in root organogenesis. Additionally, *cbp60b-1* mutants were less susceptible to colonisation by the fungal pathogen *Fusarium graminearum* compared with the wild-type. Related proteins AtCBP60a, AtCBP60g and AtSARD1 are known to be involved in immune signalling. These results demonstrate that CBP60B may function in the relative modulation of symbiosis and immune signalling in response to microbe interaction.

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AM	Arbuscular Mycorrhiza
AMF	Arbuscular Mycorrhizal Fungi
CaM	Calmodulin
CBP	Calmodulin Binding Protein
CBP60B	CALMODULIN BINDING PROTEIN 60 B
CFP	Cyan Fluorescent Protein
CNGC15	CYCLIC NUCLEOTIDE-GATED ION CHANNEL PROTEIN 15
CO	Chitooligosaccharides
CSP	Common Symbiosis Pathway
DBD	DNA Binding Domain
DMI1	DOESN'T MAKE INFECTIONS 1
ENOD11	EARLY NODULIN 11
ETI	Effector-Triggered Immunity
FsK	*Fusarium solani S*train K
GAL	β-galactosidase
GFP	Green Fluorescent Protein
GUS	β-glucuronidase
HRP	Horseradish peroxidase
IT	Infection Thread
LCM	Laser-Capture Microdissection
LCO	Lipochitooligosaccharide
MAMP	Microbe-Associated Molecular Patterns
NADP dpt ox	Nicotinamide Adenine Dinucleotide Phosphate–dependent oxidoreductase
NFN	Nitrogen-Fixing root Nodule
NFP	NOD FACTOR PERCEPTION
NLS	Nuclear Localisation Signal
PAM	Peri-Arbuscular Membrane
PAMP	Pathogen-Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PPA	Prepenetration Apparatus
PRR	Pattern-Recognition Receptors
PTI	Pattern-Triggered Immunity
QRT-PCR	Quantitative Reverse Transcription PCR
RAM	Root Apical Meristem
RGS	Regulator of G-protein Signalling
RNS	Root Nodule Symbiosis
ROS	Reactive Oxygen Species
RT-PCT	Reverse Transcription PCR
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SARD1	SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1
UBC9	UBIQUITIN-CONJUGATING ENZYME 9
WGA	Wheat Germ Agglutinin
YFP	Yellow Fluorescent Protein

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1. Introduction

Plants interact with many microorganisms at the root-soil interface. While some microorganisms act as plant pathogens, others are able to form intra- or extracellular mutualistic symbioses with the plant. Key to these symbiotic interactions is the exchange of materials between symbiont and host, whereby the plant is provided with key nutrients in exchange of a carbon source. Two of the major classes of root endosymbionts include the arbuscular mycorrhizal fungi (AMF), which aid the plant in uptake of phosphorus, sulphur and other key nutrients, and the nitrogen-fixing bacteria, which are able to form root nodule symbiosis (RNS) with the legume family of plants.

1.1. Arbuscular mycorrhizal fungi

The AMF, a group of soil-borne fungi belonging to the phylum *Glomeromycota* (Schüßler, Schwarzott and Walker, 2001) are able to form a symbiotic relationship with ~80% of extant land plants (Wang and Qiu, 2006). AMF mutualism enhances the uptake of micronutrients, such as phosphates, sulphur and nitrogen, from the soil in return for a supply of plant photosynthates but can also be shown to improve tolerance to fungal pathogens (Jung *et al.*, 2012) and abiotic stress (Chitarra *et al.*, 2016; Lenoir, Fontaine and Lounès-Hadj Sahraoui, 2016). It has been suggested that this ancient endosymbiosis first emerged ~450 million years ago as fossil records show AMF-like infection in early terrestrial plants (Remy *et al.*, 1994; Parniske, 2008). This endosymbiotic relationship is thought to have played a crucial role in plant colonisation of land, perhaps by making nutrients more readily available. Indeed, three highly-conserved symbiosis genes*, DOESN'T MAKE INFECTIONS 1 (DMI1), DOESN'T MAKE INFECTIONS 3 (DMI3) and INTERACTING PROTEIN OF DMI3* (*IPD3*)*,* have been vertically inherited into almost all extant terrestrial plant lineages, including liverworts (Wang *et al.*, 2010). As obligative biotrophs, AMF must inhabit a host plant in order to survive and reproduce. AMF spores in the soil germinate and undergo exploratory hyphal growth to find a suitable plant host, processes that can both be positively stimulated by the presence of plant-derived factors, most notably strigolactones (Akiyama, Matsuzaki and Hayashi, 2005), which induces chemotaxic hyphal branching by activating mitochondria in the fungal cells (Besserer *et al.*, 2006). Strigolactone exudation into the soil is enhanced under nitrogen and phosphate starvation conditions (Yoneyama *et al.*, 2012). In host proximity, AMF produce mycorrhization (Myc) factors to initiate the signalling required for AM colonisation. These Myc factors comprise chitin-derived lipochitooligosaccharides (LCOs) and shorter chain chitooligosaccharides (COs) (Maillet *et al.*, 2011; Genre *et al.*, 2013; Feng *et al.*, 2019). LCOs/COs are detected at the root cell surface by membrane-bound LysM receptor-like kinases (Malkov *et al.*, 2016), which activate downstream signals as part of the common symbiosis pathway, triggering gene expression changes in the host

plant essential for fungal colonisation. Amongst these changes in gene expression is the upregulation of a nicotinamide adenine dinucleotide phosphate–dependent oxidoreductase (NADP dpt Ox) in response to non-sulphated LCO detection, a change used to mark early AM colonisation (Maillet *et al.*, 2011; Charpentier *et al.*, 2016). Although the Myc factor receptors are yet to be properly characterised, studies in *Oryza sativa* suggest that the AMF symbiosis requires the receptor CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) (Zhang *et al.*, 2015; Carotenuto *et al.*, 2017).

Once in contact with a potential host, the AMF forms a flattened infection structure, known as the appressorium at the root epidermis, which applies turgor pressure to the root surface enabling penetration of hyphae into the plant tissue (Howard *et al.*, 1991). Hyphal penetration is controlled by the plant through formation of prepenetration apparatus (PPA), a cytoskeletal construct that directs progression of hyphae through the root epidermis to the inner-cortical layer of cells, helping to keep the invading fungi compartmentally separated from the host cytoplasm (Genre *et al.*, 2005, 2008). Using fluorescent microscopy, Genre *et al.* were able to show that the nucleus of epidermal plant cells localises to the site of AM appressorium contact, recruiting the requisite cytoskeletal components that allow for assembly of the PPA (Genre *et al.*, 2005). Subsequent migration of the nucleus through the epidermal cell generates a channel in the cytoplasm that acts as an invagination for fungal entry. Transcriptomic analysis of AMF infected roots during the formation of the PPA indicates that the fungal infection process is dependent on key symbiotic genes involved in the common symbiosis pathway (to be described later), including the calmodulin-dependent protein kinase DMI3 (Siciliano *et al.*, 2007). Following entry into the inner-cortex, hyphae grow longitudinally and develop into highly branched intracellular arbuscule structures, which, with the plant-derived peri-arbuscular membrane (PAM), act as the main surface for nutrient exchange (George, Marschner and Jakobsen, 1995; Sieh *et al.*, 2013). Growth of these intracellular arbuscule structures within the inner-cortical cells requires a drastic overhaul of cellular structure. Typically, such inner-cortical cells are composed predominantly of a large vacuole, which is important for its structural role in the root. However, upon colonisation there is a drastic reorganisation of the cytoskeleton (Genre and Bonfante, 1998, 1999) and duplication of subcellular components, such as mitochondria and endoplasmic reticulum. In addition, the nucleus of the colonised cell becomes enlarged, due to an endoreduplication event (Carotenuto *et al.*, 2019). Generation of the PAM itself is genetically demanding for newly colonised cells, requiring the activity of many genes downstream of AM perception. In the model legume *Medicago truncatula*, cellular colonisation requires the protein VAPYRIN, which is known to localise to mobile VAPYRIN-bodies. These VAPYRIN-bodies are thought to play a key role in the secretory pathway involved in the membrane dynamics of the PAM (Bapaume *et al.*, 2019), where metabolite exchange is facilitated by transporters at the plant-fungal interface. For example, acquisition

of phosphates is mediated by PHOSPHATE TRANSPORTER 4 (PT4), which is localised to the PAM (Harrison, Dewbre and Liu, 2002).

Like any plant organogenesis event, the plant developmental program of arbuscule formation is heavily influenced by the activity of phytohormones (Gutjahr, 2014). As AM colonisation is known to result in enhanced root branching, the role of auxin signalling in arbuscule formation has long been discussed (Fusconi, 2014). Treatment of *M. truncatula* and *Solanum lycopersicum* (tomato) roots with synthetic auxin has been found to result in decreased arbuscule abundance (Etemadi *et al.*, 2014), implicating auxin signalling in arbuscule development. More recently, gibberellin (GA) signalling has been tied to the establishment and maintenance of AM symbiosis (McGuiness, Reid and Foo, 2019). Studies in *Lotus japonicus* found that, not only is AM colonisation inhibited by GA application, but expression of *REDUCED ARBUSCULAR MYCORRHIZATION1* (*RAM1*) and *RAM2,* genes required for hyphal entry into the host root, is impaired in the GA treated plants (Takeda *et al.*, 2015).

1.2. Root nodule symbiosis

Root nodule symbiosis (RNS) constitutes a broad range of mutualistic interactions between plant roots, primarily plants belonging to the legume family (*Fabaceae*), and nitrogen-fixing bacteria, which are internalised into the root tissue, forming macroscopic nodule structures and converting atmospheric nitrogen into a form usable by the plant (Oldroyd and Downie, 2008). Though nodulating plant species are found mostly in the *Fabaceae* family, the nitrogen-fixing root nodule (NFN) clade actually contains plants belonging to four plant orders; Fabales, Fagales, Curcubitales and Rosales, simplified to FaFaCuRo (Soltis *et al.*, 1995). However, scattered within the NFN clade, only 10 out of the 28 families are able to form RNS. Recent phylogenomic studies, involving genome sequencing of nodulating and non-nodulating members of the NFN clade, have shown that this scattered distribution of nodulating species is most likely the result of independent losses of RNS, rather than RNS evolving multiple times (Griesmann *et al.*, 2018). Nodules can be divided into two types based on ontogeny as well as histology: legume-type nodules and actinorhizal-type nodules. Legume-type nodules are specific to legume species of the Fabaceae (order Fabales) as well plants in the genus *Parasponia* (order Rosales), which form RNS with the gram-negative bacteria, Rhizobia. Actinorhizal-type nodules, however, are formed through RNS with Frankia bacteria. By far the most well studied of these RNS are those formed between legumes and rhizobia, which can again be collected into two major groups; those that form determinate nodules (e.g. *Lotus*, soybeans) and those that form indeterminate nodules (e.g. clovers, *Medicago*).

Initiation of legume-rhizobia interaction is dependent on reciprocal signalling between the root and the soil-borne bacteria. Legume roots are able to influence rhizobia by secretion of a flavonoid compounds into the soil immediately surrounding the plant, termed the rhizosphere. These flavonoid compounds are detected by rhizobia, interacting with NodD Lys-R type transcriptional regulators which in turn bind with *nod*-box promoter regions and activates *nod* gene expression (Fisher *et al.*, 1988; Cooper, 2004). Here, flavonoids detection offers a level of specificity between symbiont and host, as NodD proteins interact with secreted flavonoids in a legume-species specific manner (Peck, Fisher and Long, 2006; Wang, Liu and Zhu, 2018). Upregulation of *nod* genes is required for the biosynthesis and secretion of nodulation (Nod) factors, LCO molecules required for host recognition of invading rhizobia (Schultze and Kondorosi, 1996). Perception of Nod-LCOs at the plasma membrane is mediated by LysM receptor-like kinases (Madsen *et al.*, 2003), functioning as a heterodimer (Radutoiu *et al.*, 2003; Moling *et al.*, 2014). In the model legume *Medicago truncatula*, two likely LysM receptors have been identified, LYSM DOMAIN RECEPTOR-LIKE KINASE 3 (LYK3) (Limpens *et al.*, 2003) and NOD FACTOR PERCEPTION (NFP) (Amor *et al.*, 2003; Arrighi *et al.*, 2006).

To initiate infection, rhizobia attach to tip root hair cells, which curl to entrap the bacteria, forming an infection chamber. Here, the bacteria proliferate, elevating Nod factor levels above a threshold required for further infection (Haag *et al.*, 2013). Rhizobia are directed through the root tissue *via* intracellular tubular structures known as infection threads (ITs), which transverse the root cortex, growing from the tip in a manner analogous to root hair or pollen tube development (Pawlowski and Bisseling, 1996), towards the developing nodule primordia, a group of mitotically activate inner cortical cells induced by rhizobia perception (Rae, Bonfante-Fasolo and Brewin, 1992). As the activation of this nodule primordia only requires the perception of Nod-LCO's at the root surface, and no active uptake of rhizobacteria, it is important for nodule development that IT formation is coordinated with development and differentiation at the nodule primordia. This spatiotemporal coordination of these distinct processes is thought to involve auxin and cytokinin plant hormone signalling (Oldroyd and Downie, 2008). More specifically, CYTOKININ RESPONSE 1 histidine kinase (MtCRE1), a cytokinin receptor, was found to be required for nodule organogenesis through a systemic RNAi approach (Gonzalez-Rizzo, Crespi and Frugier, 2006), suggesting that perception of cytokinin is essential for this process. In fact, exogenous application of cytokinin is known to induce nodule primordia formation (Heckmann *et al.*, 2011). Cytokinin signalling is also required to regulate the expression of *NODULE INCEPTION* (*NIN),* an early symbiotic transcription factor responsible for the coordination of a variety of nodulation programs (Vernié *et al.*, 2015). It is suggested that cytokinin functions by regulating auxin transport in the root, as auxin is implicated in key processes during early nodule organogenesis, including the control of cell cycle (Kondorosi, Redondo-Nieto and

Kondorosi, 2005) as well as the differentiation of peripheral vascular tissues found in legume-type nodules (Takanashi, Sugiyama and Yazaki, 2011; Guan *et al.*, 2013). The transport of auxin through the root, and subsequent dispersal of auxin, is dependent on the distribution of auxin efflux carrier proteins known as PINs, which have been studied extensively in *Arabidopsis thaliana* (Billou *et al.*, 2005). Cytokinin perception is thought to block the polar auxin transport at the point of rhizobia infection by disrupting auxin movement (Ng *et al.*, 2015; Kohlen *et al.*, 2018), perhaps *via* interference with PIN protein activity (Huo *et al.*, 2006). Induction of pseudo-nodules in *M. truncatula* roots deficient in early signalling genes, including *NIN*, was seen following the application of a synthetic auxin inhibitor (Rightmyer and Long, 2011), placing the auxin response downstream of cytokinin signalling. However, this cytokinin sensitivity is only observed in nodule-forming legume species but not in non-legumes, such as actinorhizal-type nodule forming plants (Gauthier-Coles, White and Mathesius, 2019). Interestingly, recent study also suggests a role of callose degradation, producing symplastic domains capable of transport of a key mobile signal (Gaudioso-Pedraza *et al.*, 2018). The passage of the IT to the nodule primordium is mediated by the pre-infection thread (PIT), changes in cytoskeletal organisation analogous to PPA in AMF infection (Yokota *et al.*, 2009). Rhizobia become entrapped in the nodule primordia cells through an endocytosis-like process (Brewin, 2004) and differentiate into nitrogen-fixing bacteroids (Oldroyd *et al.*, 2011). Within the host cell, rhizobia are separated from the cytosol by a specialised membrane known as the peribacteroid membrane (PBM) which acts as the main plant-bacteria interface for nutrient exchange (Bolaños *et al.*, 2004).

As described, legume-rhizobia nodules have either a determinate or indeterminate structure. Determinate nodules, such as those in the model species *L. japonicus*, are typically spherical in shape and lack a persistent meristem. Mature indeterminate nodules, however, like in *Medicago truncatula*, have a cylindrical shape due to the presence of an apical meristem. While this meristem is important for the overall shape of these nodules, using a detailed fate mapping approach Xiao *et al*. were able to show that the non-infected basal cells of indeterminate nodules in *M. truncatula* were actually derived directly from the pericycle and endodermal cells of the root (Xiao *et al.*, 2014), which divide early in nodule primordia development, producing only a few cell layers before cell division is arrested. As the nodule develops, distinctive zones within the indeterminate nodule are formed (Vasse *et al.*, 1990); the bacteroid-free meristematic Zone I (ZI), Zone II (ZII)/ the infection zone (IZ) and Zone III (ZIII) containing nitrogen-fixing symbiosomes. ZII itself is comprised of distal and proximal regions; the former is a non-infected cell differentiation region, while the latter is important for plant and bacterial cell enlargement and endoreduplication events (Roux *et al.*, 2014).

1.3. The Common Symbiosis Pathway

Through a series of genetic studies in legumes, a series of components required for nodulation were identified. These components included; the membrane bound SYMBIOSIS RECEPTOR-LIKE KINASE (SYMRK) (Schauser *et al.*, 1998; Madsen *et al.*, 2003), three nuclear-porins; NUCLEOPORIN 85 and 133 (NUP85 &NUP133) and NENA (Kanamori *et*

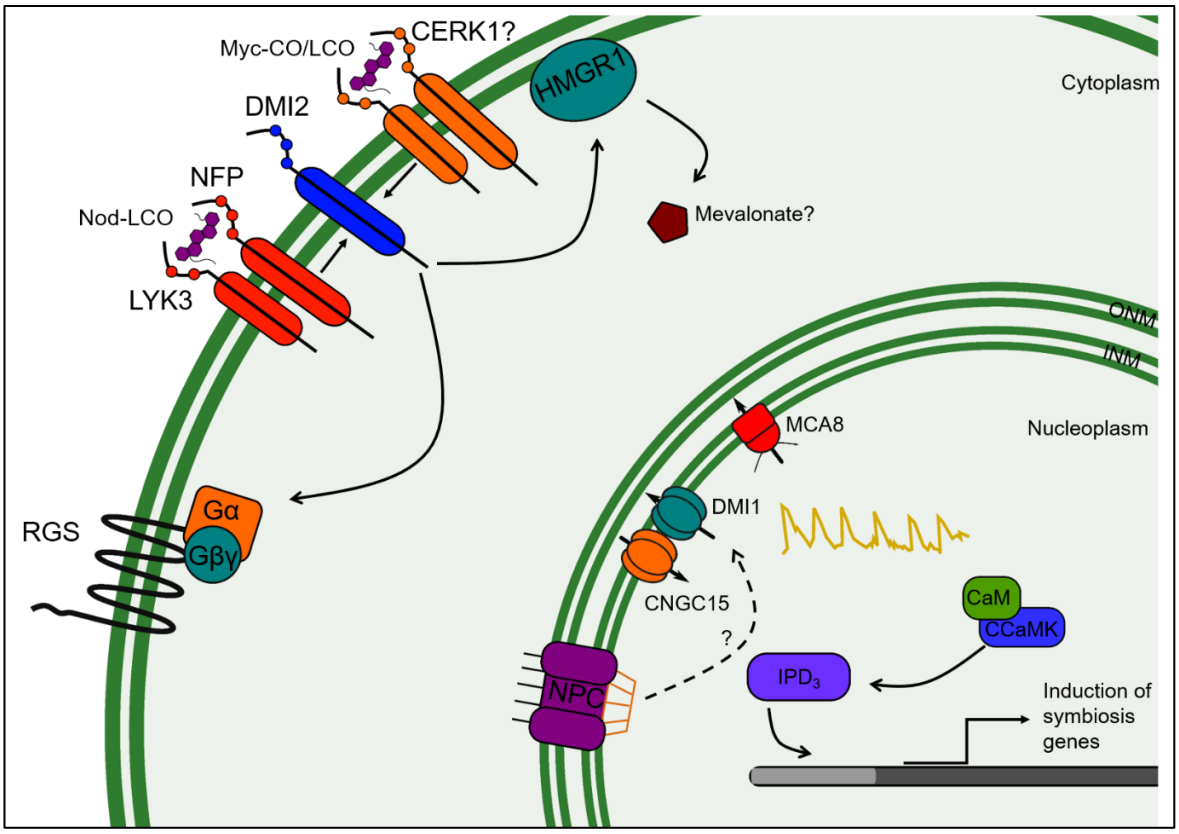

Figure 1 – Overview of the common symbiosis pathway

Perception of nodulation (Nod) factors (Nod-LCOs) and mycorrhization (Myc) factors (Myc-CO/LCOs) at the plasma-membrane is mediated by LysM receptor-like kinases. In *M. truncatula*, Nod factors are detected by LYK3 and NFP, while Myc factor receptors are yet to be characterised. Studies in rice suggest that CERK1 plays a role as the Myc factor receptor. LYK3/NFP interact with the co-receptor, DMI2. DMI2 is able to phosphorylate the Gα subunit of the heterotrimeric G-protein complex, preventing binding with Gβγ. DMI2 is also shown to interact with the key biosynthetic enzyme HMGR1. The Gβγ complex and mevalonate (the final product of HMGR1 activity) have been suggested as secondary messenger signals required for signal transduction from the plasma-membrane to the nucleus. One such secondary messenger, or another activated downstream, enters the nucleus *via* the nucleopore complex (NPC) and induces nuclear calcium influx through interaction with the CNGC15/DMI1 complex. MCA8 pumps calcium back into the nuclear envelope lumen to generate nuclear calcium oscillation (yellow). Oscillations are decoded by the calcium and calmodulin (CaM) dependent kinase, DMI3, which activates the transcription factor IPD_3, leading to the induction of symbiosis genes.

al., 2006; Saito *et al.*, 2007; Groth *et al.*, 2010), mevalonate biosynthetic enzyme HMG-COA REDUCTASE 1 (HMGR1) (Kevei *et al.*, 2007), nuclear-membrane localised cation channels; DMI1 (Ané *et al.*, 2004), CYCLIC NUCLEOTIDE-GATED ION CHANNEL PROTEIN 15 (CNGC15) (Charpentier *et al.*, 2016) and MCA8 (Capoen *et al.*, 2011), nuclear CALCIUM/CALMODULIN-DEPENDENT PROTEIN Kinase (CCaMK) (Lévy *et al.*, 2004) and the CCaMK substrate CYCLOPS (Yano *et al.*, 2008; Horváth *et al.*, 2011). Mutants in

these key symbiosis genes exhibited distinct nodulation phenotypes. For instance, nodule development was prematurely arrested in *cyclops* mutants (Yano *et al.*, 2008) and RNAi interference of HMGR1 resulted in a dramatic reduced number of nodules (Kevei *et al.*, 2007). Critically, mutants deficient in DMI1, SYMRK and CCaMK were unable to form RNS, they were also impeded in forming symbiotic relationships with AMF, leading to the development of the common symbiosis signalling pathway (CSP) model (Figure 1, Catoira *et al.*, 2000). Central to this pathway is the induction of nuclear calcium oscillations in root hair, epidermal and cortical cells in plant roots (Shaw and Long, 2003; Oldroyd and Downie, 2006; Genre *et al.*, 2013; J. Sun *et al.*, 2015). These oscillations are essential to endosymbiotic interactions, as they are required to activate downstream gene expression. Legumes defective in calcium oscillation generation are impaired in root nodule symbiosis (RNS) and arbuscular mycorrhizal (AM) symbiosis (Catoira *et al.*, 2000; Peiter *et al.*, 2007; Kosuta *et al.*, 2008).

The common symbiosis pathway is initiated through the perception of Nod and Myc factors at the root cell surface. While the Myc-LCO receptors remain to be characterised, structural similarity of Myc-LCOs to Nod factors suggests the existence of similar plasma-membrane receptors. Indeed, NFP is shown to be required to stimulate root branching in response to Myc-LCOs (Maillet *et al.*, 2011), but it is not essential for mycorrhization (Amor *et al.*, 2003). In *M. truncatula* the LysM plasma-membrane receptors interact with the receptor-like kinase DOESN'T MAKE INFECTIONS 2 (DMI2,known as SYMRK in *L. japonicus*), allowing for signal transduction from the cell surface to the nuclear membrane in order to induce nuclear localised calcium oscillations (Ried, Antolín-Llovera and Parniske, 2014), likely activated by a phosphorylation cascade.

Although the activation mechanism of the nuclear cation channels is yet to be characterised, it is proposed that a secondary messenger is required to diffuse from the plasma-membrane DMI2 to the nuclear membrane to interact with DMI1 and/or CNGC15 to modulate activity (Charpentier, 2018). As nucleoporin genes are required for successful symbiosis, such secondary messengers may enter the nuclear-envelope through the nucleopore complex (NPC) in order to influence DMI1/CNGC15 activity (Charpentier, 2018). One proposed pathway of plasma-membrane to nucleus signal transduction, demonstrated in soybean, involves SYMRK (DMI2) mediated phosphorylation of the Gα subunit of the heterotrimeric G-protein complex upon symbiont perception. Phosphorylated Gα is no longer able to bind the Gβγ complex, allowing for downstream signalling and activation of nuclear calcium channels either through direct action or through activation of a secondary messenger (Choudhury and Pandey, 2019). Alternatively, the organic compound mevalonate has also been suggested as a potential secondary messenger (Venkateshwaran *et al.*, 2015). Mevalonate is the final product of the biosynthetic enzyme (HMGR1), a known interactor of DMI2 that is required to establish symbiosis between *M. truncatula* and *S. meliloti* (Kevei

et al., 2007). Direct application of mevalonate has been shown to induce nuclear calcium oscillations and expression of key symbiosis gene *EARLY NODULIN 11* (*ENOD11*) in a DMI1 dependent manner (Venkateshwaran *et al.*, 2015).

The generation of calcium oscillations at the nuclear membrane is controlled by three nuclear-localised components; a cation permeable channel DMI1 (Ané *et al.*, 2004), a calcium channel belonging to the cyclic nucleotide gated channels family, CNGC15 (Charpentier *et al.*, 2016), and a calcium ATPase, MCA8 (Capoen *et al.*, 2011). Interaction between DMI1 and CNGC15 suggests simultaneous activation of these channels, which is shown through mathematical modelling to be sufficient to produce sustained nuclear calcium oscillations (Charpentier *et al.*, 2016). Calcium oscillations are decoded by the nuclear-localised CCaMK, (known as DMI3 in *M. truncatula*) which is activated through either directly or indirect (*via* calmodulin-mediated calcium binding (Miller *et al.*, 2013)), influencing downstream gene expression by interacting with gene regulator CYCLOPS (Known as IPD_3 in *M. truncatula*, Yano *et al.*, 2008; Horváth *et al.*, 2011), which in turn influences transcription factors *NODULATION SIGNALING PATHWAY 1* and *2* (*NSP1 & NSP2*) and *REQUIRED FOR ARBUSCULAR MYCORRHIZATION 1 (RAM1)* (Oldroyd, 2013). Also induced is the aforementioned *NIN*, which plays a critical role in IT formation and initial cell divisions prior to nodule formation. It is clear that the generation and interpretation of these nuclear calcium oscillations are important for activation of the root endosymbiosis program. Application of mastoparan, a peptide toxin derived from wasp venom, which acts as a heterotrimeric G-protein agonist (Higashijima *et al.*, 1988; Ross and Higashuima, 1994), to legume roots is known to elicit calcium spiking (Tucker and Boss, 1996). Calcium oscillations induced by mastoparan application in *dmi1* and *dmi2* mutants can upregulate the expression of the early nodulation gene *ENOD11* (Sun *et al.*, 2007). Therefore, modulation of the nuclear calcium oscillations through interactions with the nuclear calcium machinery, such as DMI1, could result in varied responses to symbiotic partners.

Most calcium spiking measure in early symbiosis refers to nuclear calcium oscillations in the root hair cells. However, it has been suggested that such calcium spiking is also important at deeper levels within the root tissue and is implicated more closely with the organogenesis of nodule or arbuscule structures. Significantly, Sieberer *et al.* were able to use live cell imaging to show that nuclear calcium spiking in the outer cortical cell layers was associated with the progression of symbiont through the root tissue, with symbiont colonised cells exhibiting rapid calcium oscillations whereas nuclear calcium was observed to oscillate at a lower frequency in cells about to be colonised (Sieberer *et al.*, 2012).

It remains to be seen how this CSP is able to discriminate between AMF and rhizobial symbionts, promoting alternate arbuscule or nodule organogenesis programs through the

same signal transduction pathway. This distinction may be due to differential CaM-CCaMK binding required by AMF symbiosis or RNS (Shimoda *et al.*, 2012). Overall though, it is clear that both symbionts are able to induce relatively disparate developmental programs through the same molecular machinery. This CSP model is also supported by phylogenetic evidence, which show that signalling genes are conserved in plant species with intracellular symbionts (Parniske, 2008; Radhakrishnan *et al.*, 2020). This is consistent with the theory that symbiosis with nodule-inducing bacteria was co-opted from the more ancient AMF interaction (Bonfante and Genre, 2008; Parniske, 2008).

1.4. Root endosymbiosis and the plant root developmental program

A consistent theme of both root nodule and AM symbiosis, is the development of novel root organs following endosymbiont detection. In both cases, this process involves a reprogramming of root development in order to influence plant cell division and patterning. As discussed, the perception of both AM and rhizobia results in extensive changes in phytohormone signalling and transport. Perhaps unsurprisingly, these novel strategies are often compared with pre-existing root organ development processes, such as those controlling the formation of lateral roots. Application of both Nod- and Myc-factors are sufficient to induce lateral root formation in the absence of symbionts (Oláh *et al.*, 2005), suggesting an overlap in the machinery involved across these processes. While root nodules may seem closely related to lateral root structures, their evolutionary origin has long been contested, with some suggesting that they represent modified shoots or even existing as a unique root organs (Hirsch, Larue and Doyle, 1997). More recently, studies into some of the major components of the developmental programs required for root nodule organogenesis and lateral root formation have found that, while some elements remain contradictory between the two processes, there is more in common between them than was previously thought.

Development of lateral roots in *M. truncatula* originates exclusively from the pericycle tissue, while nodule development also involves cell division in the cortex and endodermis (Xiao *et al.*, 2014). The site of these lateral root outgrowths are predetermined periodic oscillations in auxin, which produce ‘primed’ founder cells (Dubrovsky *et al.*, 2011; Herrbach *et al.*, 2014). However, unlike during nodulation, these auxin maxima do not require input of cytokinin signalling. In fact, while exogenous cytokinin application to roots is sufficient to induce nodule primordia, it is actually antagonistic to lateral root development (Gonzalez-Rizzo, Crespi and Frugier, 2006; Laplaze *et al.*, 2007). RNAi interference of CRE1 results in plants insensitive to cytokinin, which display increased lateral root density in *M. truncatula* (Gonzalez-Rizzo, Crespi and Frugier, 2006). RNA sequencing analysis comparing gravitropically stimulated *M. truncatula* roots producing lateral roots with others spot inoculated with *S. meliloti* found a significant over-lap in the upregulation of auxin-

associated genes, at early points in each process (Schiessl *et al.*, 2019). Among the included genes was *LATERAL ORGAN BOUNDARIES DOMAIN16* (*LBD16*), a gene shown in *A. thaliana*, to be directly involved in the regulation of cell division in response to auxin (Feng *et al.*, 2012). Here a promoter-β-glucuronidase (GUS) approach showed *LBD16* to be expressed in both nodule and lateral root primordia. This is also reflected in a similar study in *L. japonicus* (Soyano *et al.*, 2019). Overall, these findings suggest that, while the events required to initiate nodule and lateral root development may differ, the processes eventually diverge, making use of the same auxin-mediated pathway to regulate cell divisions within the respective primordia.

Although this may be true of legume-type nodules, the developmental programming involved in the generation of actinorhizal-type nodules can be viewed as more closely related to lateral root formation. Indeed, actinorhizal-nodule primordia are believed to originate from pericycle cell divisions as occurs in lateral root formation, meaning that each of the highly lobed coralloid nodules formed are thought to represent a modified lateral root (Pawlowski and Bisseling, 1996). Actinorhizal and legume nodules differ both ontologically and anatomically, which is most obvious in the patterning of central lateral-root like vasculature seen in the actinorhizal nodules compared with the peripheral vasculature associated with legume-type nodules. However, a recent study by Shen *et al.* has shown that a mutation in the homeotic *NODULE ROOT1* (*NOOT1*) gene was able to partially convert legume-type nodules in *M. truncatula* (Shen *et al.*, 2020). It is important to note that the nodule tissue hosting the nitrogen-fixing symbionts were observed in cells derived from the cortical rather than the pericycle, contradicting earlier reports that early development resembled lateral root formation. While this does mean that this apparent link between lateral root and nodule organogenesis is perhaps more complex than first thought, it is still clear that there is a shared evolutionary lineage between the developmental programmes of actinorhizal and legume-type nodules, with the actinorhizal-type most likely the more ancestral (Shen *et al.*, 2020).

As these endosymbiont-induced changes show a level of conservation across species, it is possible that similar changes may be induced by AM colonisation. The co-opting of the signalling machinery required to stimulate AM symbiosis by nitrogen-fixing bacteria suggests that some of the root development reprogramming seen during nodule formation may find its origin in AM signalling and arbuscule development. Interestingly, the expression of TPLATE, a protein involved in plasma membrane assembly, is prevalent in AM colonised cortical cells (Russo *et al.*, 2019). Russo *et al.* suggest that this protein, with a role in cell division, is upregulated in order to reorganising surplus membrane generated by a diminishing vacuole during AM colonisation of the cortical cells. While this cannot show a direct link between AM induced cortical cell divisions and nodule inductions, it does provide an evolutionary basis by which such a developmental strategy could occur. Moreover, this

demonstrates an AM induced change in root development at the site of infection. In fact, it has been documented that root systems angiosperm trees belonging to the genus *Gymnostoma* are able to form small protuberance or “nodules” colonised by mycorrhizal fungi, in addition to nitrogen-fixing nodules (Duhoux *et al.*, 2001). Ultimately, it is clear that endosymbionts are able to exert a level of influence over the root development program during perception and colonisation, though this may not be wholly surprising as in the absence of such symbiotic relationships, root development is dictated by nutrient availability (López-Bucio, Cruz-Ramírez and Herrera-Estrella, 2003).

1.5. Plant defence and immunity

Unlike in mammalian systems, plants lack mobile immune cells so must instead recognise and respond to potential pathogens on a cell-by-cell basis. Activation of immune responses is costly, so it is vital that immunity is closely regulated to limit unnecessary energy expenditure that would reduce fitness and reproductive capacity. The ‘zigzag model’ proposed by Jones and Dangl in 2006 (Jones and Dangl, 2006), suggests that the plant innate immune system comprises of two distinct branches. The first branch, known as pattern-triggered immunity (PTI) involves the detection of conserved microbial signals (microbe-/pathogen-associated molecular patterns, MAMPs/PAMPs), activating a general defence response. The second branch, known as effector-triggered immunity (ETI), is a response to effector molecules derived from well-adapted pathogens co-evolved to interfere with PTI, resulting in an amplified defensive response.

Much like with symbiosis signalling, recognition of pathogenic microbes through PTI begins at the plant cell surface, where MAMPs/PAMPs are detected by cell-surface receptor kinases known as pattern-recognition receptors (PRRs) (Newman *et al.*, 2013). One such molecular pattern is the highly conserved bacterial peptide-elicitor flagellin 22 (flg22), which is recognised by the plant membrane receptor FLS2, an interaction characterised in the *Arabidopsis thaliana* root system by Millet *et al.* (Millet *et al.*, 2010). PRRs possess an intracellular kinase, transmembrane domain, and specific ligand binding ectodomain, with preferential binding to certain classes of molecular patterns. PRRs containing leucine-rich-repeats (LRRs) bind peptide ligands such as flg22, while those containing lysine motifs (LysM) interact with carbohydrate-based patterns including fungal chitins and bacterial peptidoglycans (Zipfel, 2014; Couto and Zipfel, 2016). Additionally, PRRs interact with co-receptors, to form receptor complexes, which are required to optimal signal transduction (Monaghan and Zipfel, 2012). The flg22 receptor, FLAGELLIN-SENSITIVE 2 (FLS2), forms a complex with BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) in *A. thaliana*. In the absence of BAK1, the early flg22 response is significantly reduced (Heese *et al.*, 2007). Upon pattern binding, the PRR kinase domain phosphorylates its interacting partner (e.g. BAK1), which in turn, phosphorylates down-stream targets,

activating a complex suite of molecular and transcriptomic responses, collectively known as pattern triggered immunity (PTI), which act to limit pathogen infection.

Though PTI involves a range of molecular responses, including rapid production of reactive oxygen species (ROS) and phosphorylation events, one of the earliest observable response to pathogen detection is a transient influx of extracellular calcium into the cytosol (Yuan *et al.*, 2017), This influx can be propagated *via* direct interaction between PRR-complex and a plasma-membrane calcium channel, as in the case of the fungal PRR, CERK1, which interacts with the calcium permeable channel, ANNEXIN1 (Espinoza, Liang and Stacey, 2017). Alternatively, indirect activation of calcium influx has also been demonstrated *via* generation of additional signalling molecules such as ROS. Cytoplasmic calcium signals are relayed by a range of calcium sensor proteins, including CALMODULIN (CaM), CaM-LIKE PROTEINS (CMLs) and CALCIUM-DEPENDENT PROTEIN KINASES (CDPKs). Upon calcium influx, CaMs and CMLs interact with CALMODULIN BINDING PROTEINS (CBPs), inducing conformational change and allowing for downstream activity. Two members of a family of CBPs known as CBP60's in *Arabidopsis*, CBP60g and SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1 (SARD1), positively regulate plant immunity by binding the promoter region of ISOCHORISMATE SYNTHASE (ICS) (Wang *et al.*, 2009, 2011), an important enzyme required in salicylic acid (SA) biosynthesis, and have also been shown to regulate other key immunity genes (T. Sun *et al.*, 2015).

Plant hormones, such as SA, ethylene (ET) and jasmonic acid (JA) are key components in plant responses to biotic stress. Accumulation of SA, which as shown is produced during PTI, is critical for systemic acquired resistance (SAR), a defence mechanism required for long-lasting and whole-plant priming against pathogen attack (Grant and Lamb, 2006).

1.6. Interplay between symbiosis and defence

While symbiosis is seen as a mutually beneficial interaction, the process of symbiont infection parallels pathogenic infections in many ways, which have been well reviewed by Zipfel and Oldroyd (Zipfel and Oldroyd, 2017). Both processes of symbiosis and immune signalling require the plant to sense its immediate surroundings, by perceiving symbiotic or pathogenic signals at the cell surface and responding appropriately. As such, the receptors involved in chitin perception, either for detection of fungal pathogens or recognition of Nod-LCOS or Myc-CO/LCOs, share structural similarity. Indeed, receptors with perceived function for symbiont recognition, such as NFP, can also intervene to detect pathogenic signals in *M. truncatula* (Rey *et al.*, 2013). The receptor kinase CERK1, which is known to activate plant immunity in response to CO's in *A. thaliana* (Liu *et al.*, 2012), also plays a role in establishing AMF symbiosis in *Oryza sativa* (Zhang *et al.*, 2015). Perception of pathogens or symbionts can also be seen to elicit similar cellular responses, including ROS production

and plasma-membrane calcium influx associated with root hair growth (Shaw and Long, 2003). It is clear from these parallel signalling pathways that symbionts and pathogens share an evolutionary history, with some symbionts having adapted pathogenic machinery towards mutualism. For example, the bacterial type III secretion system typically associated with virulence activity in pathogens has been shown to modify host range of the symbiotic rhizobia species NGR234 (*Sinorhizobium fridii*) (Kambara *et al.*, 2009; Gourion *et al.*, 2015). It is generally thought that innate immunity is supressed in root nodules colonized by rhizobia (Berrabah *et al.*, 2014; Berrabah, Ratet and Gourion, 2015), an approach also seen to facilitate the more ancient AMF symbiosis (Zeng *et al.*, 2020). However, a recent study by Benezech *et al.*, shows that root nodules can also act as an entry point for pathogens. Pathogens infecting these nodules are contained and prevented from spreading to the plant proper (Benezech *et al.*, 2020). This suggests that while defences may be lowered to allow access for rhizobia or even pathogens into the nodule organ, there is still some level of immunity that prevents infection of the whole plant. Clearly, there must exist a 'friend-or-foe' mechanism within the symbiosis signalling pathway that is able to modulate immune signalling in response to symbiont perception, but this remains to be described.

In this study, I begin to characterise the CALMODULIN BINDING PROTEIN 60 B (CBP60B) in the model legume *Medicago truncatula,* with putative roles in both symbiosis and defence signalling. CBP60B belongs to a family of transcription factor proteins which have been shown to both positively and negatively regulate immune signalling (Truman *et al.*, 2013; T. Sun *et al.*, 2015), most prominently through regulation of the SA biosynthesis pathway (Wang *et al.*, 2011).

2. Results

2.1. Calmodulin binding protein 60 B interacts with the C-terminal domain of the nuclear localised ion channel

CBP60B was identified as a potential interactor of the nuclear-membrane cation channel, DMI1, *via* a yeast-two-hybrid screening approach, using DMI1 C-terminal as bait (Charpentier, Unpublished). As DMI1 is known to act with the calcium channel CNGC15 to mediate nuclear-localised calcium oscillations, required in early symbiosis signalling, CBP60B was hypothesised as a modulator of oscillatory activity through its interaction with DMI1. Validation of the yeast two hybrid screening result was achieved through GAL4 based yeast two hybrid interaction assay. Full length *CBP60B* fused to the *GAL4 activator domain (AD)* and the *C-terminal DMI1* fused to *GAL4 binding domain (BD)* were co-expressed in the yeast strain AH109. Growth of yeast cells co-transformed with *AD:CBP60B* and *BD:DMI1Ct* on selective medium lacking adenine, histidine (His), Leucine (Leu) and Tryptophan (Trp) for 4 days demonstrates an interaction between CBP60B and DMI1-Ct in

yeast (Figure 2). In addition, the absence of growth in yeast cells expressing *AD:CBP60B* and *BD:CBP60B* suggests that CBP60B do not dimerize (Figure 2). Similarly, no interaction occurs between CBP60B and the human SV40 large T antigen of the murin p53, indicating that the protein is not auto-active.

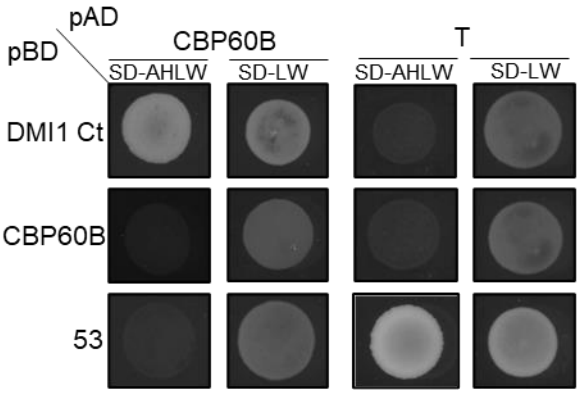

Figure 2 - Confirmation of interaction between CBP60B and DMI1

GAL4 yeast-two-hybrid assays between CBP60B as prey (AD) and DMI1 C-terminal and CBP60B as bait (BD). Murine p53 and its interacting partner SV40 large T antigen (simian virus large tumour antigen) were used as positive a control. SD-AHLW, synthetic dropout medium lacking adenine, His, Leu and Trp; SD-LW, synthetic dropout medium lacking Leu and Trp

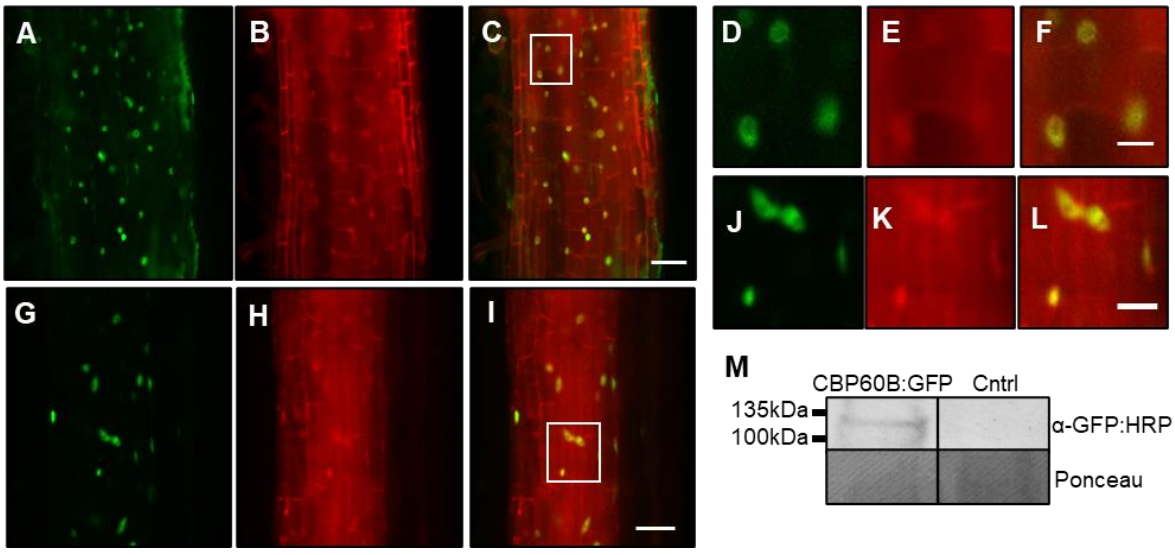

Figure 3 – CBP60B localises to the nucleus

Confocal microscopy of *Agrobacterium* transformed *M. truncatula* roots expressing DsRed with either *CBP60B:GFP* (**A-F**) or *GFP:CBP60B* (**G-L**). The green channel is shown in (**A**), (**D**), (**G**) and (**J**), while the red channel is shown in (**B**), (**E**), (**H**) and (**K**). In (**C**), (**F**), (**I**) and (**L**), the red and green channels are merged. (**D-F**) and (**J-L**) are enlarged from white boxes shown in (**C**) and (**I**) respectively. Scale bars: (**A-C**) and (**G-I**) - 35µm, (**D-F**) and (**J-L**) - 15µm. (**M**) Western blot of total protein extracted from transformed or untransformed *M.* truncatula roots, assessed using α-GFP:HRP antibodies (1:10000).

2.2. Calmodulin binding protein 60 B is localised to the nucleus in *Medicago truncatula* roots

The molecular machinery required for generation of symbiotic calcium oscillations are specifically localised to the nuclear membrane (Capoen *et al.*, 2011; Charpentier *et al.*, 2016), including the ion channel, DMI1. In order to assess a likely function for interaction between CBP60B and DMI1 *in planta*, it was first important to confirm that the putative interacting partners are similarly localised within the cell. Constructs containing *CBP60B* fused to GFP in both N-terminal (*GFP:CBP60B*) and C-terminal positions (*CBP60B:GFP*) were constitutively expressed in *Medicago truncatula* roots, transformed *via Agrobacterium rhizogenes* mediated transformation. After 7 days of growth, roots expressing the plant transformation marker DsRed were selected for confocal microscopy analysis. Confocal microscopy analysis revealed that GFP fluorescence was present in the nucleus for both *GFP:CBP60B* and *CBP60B:GFP* expressing roots (Figure 3A-L). A western blot of protein extracted from these transformed roots, using α-GFP:HRP antibodies, confirmed that the

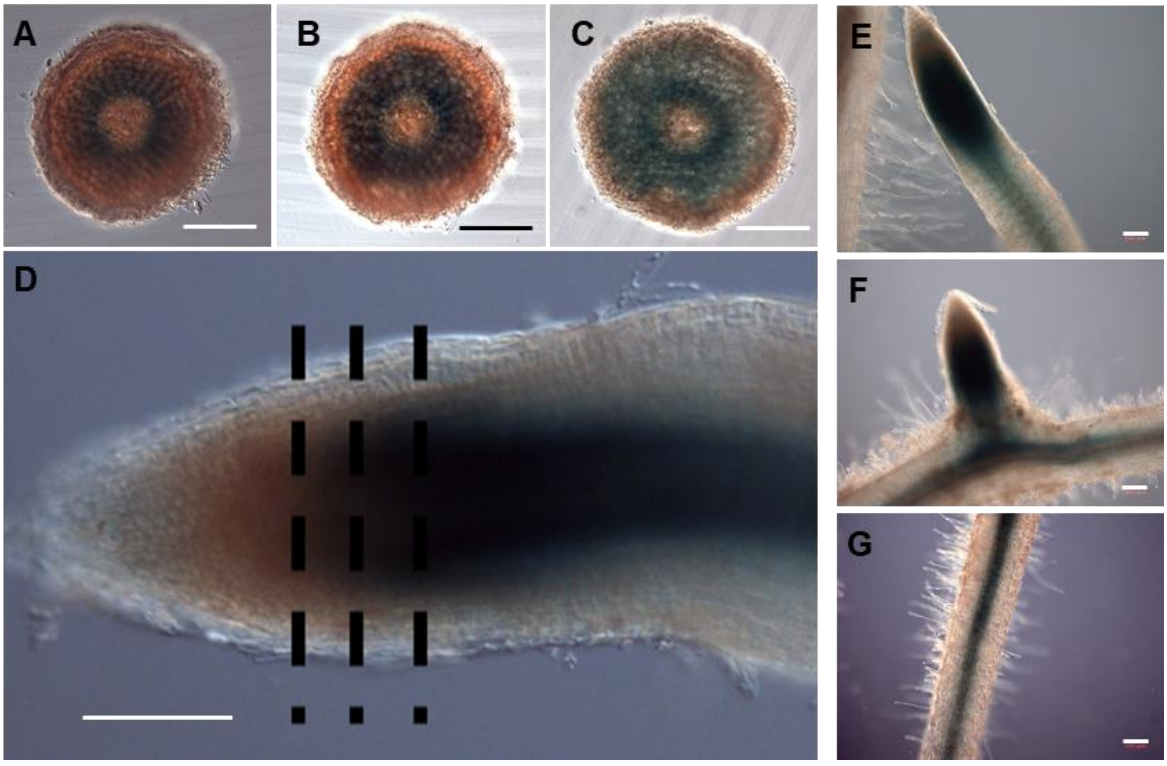

Figure 4 - Expression pattern of *pCBP60B:GUS* in non-colonised *M. truncatula* roots

Light microscopy of *A. rhizogenes* transformed *M. truncatula* roots expressing *pCBP60B::GUS*. The expression pattern in primary and lateral roots is shown by histochemical GUS staining (blue). (**A-B**) Root tip transverse sections, dissected as shown in (**D**). (**E**) GUS stained primary root tip (**F**) GUS stained lateral root tip (**G**) GUS stained root, scale bars = 100µm

full length CBP60B:GFP is expressed (Figure 3M). However, the full length GFP:CBP60B was not detected through this method, perhaps owing to lower levels of expression in the root selected. The demonstration that CBP60B is localised in the nucleus in *M. truncatula*

roots is consistent with predicted nuclear localisation signal (NLS) in the N-terminus of CBP60B (Figure 7E).

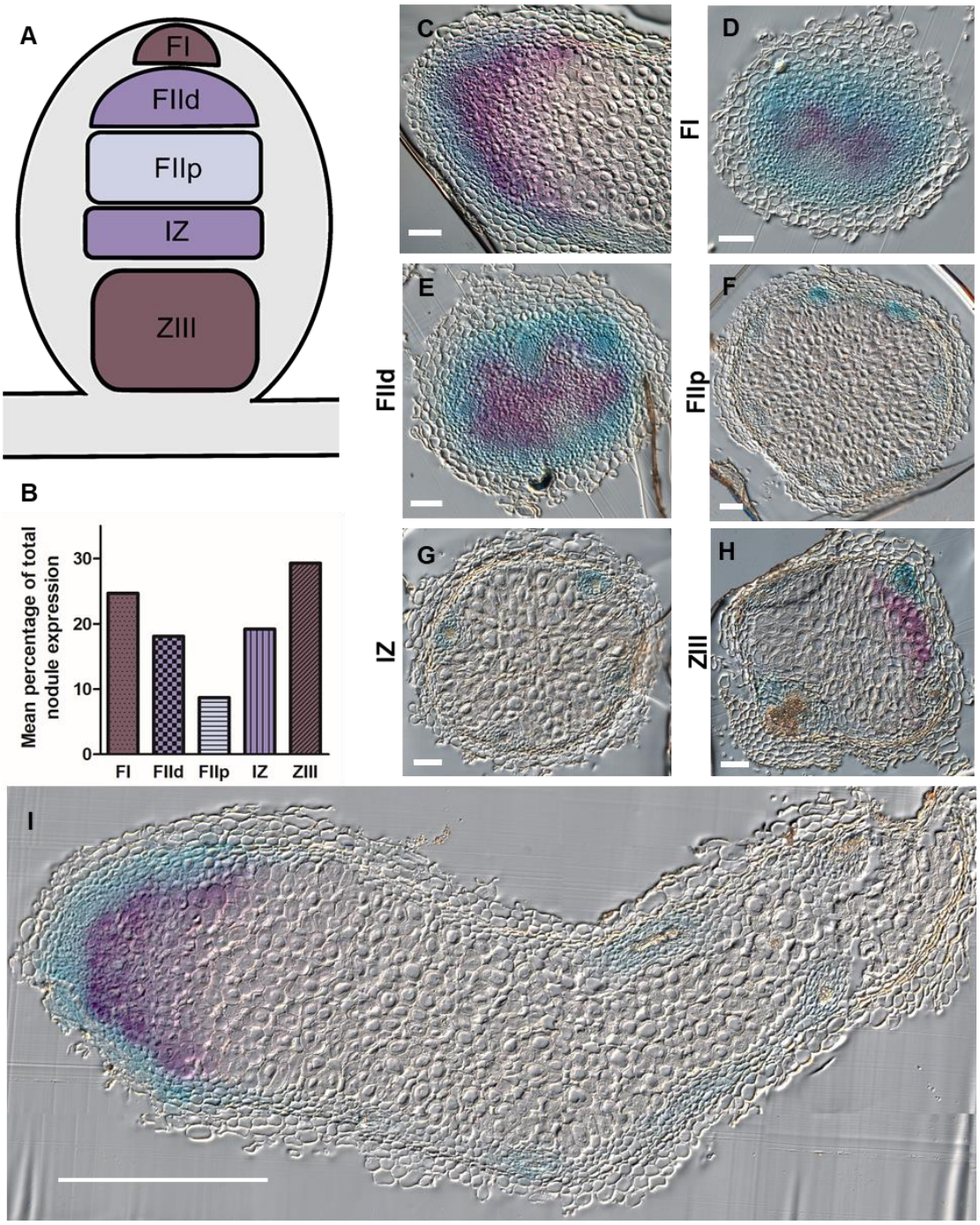

Figure 5 - Expression pattern of *pCBP60B:GUS* in *M. truncatula* nodules colonised by *S. meliloti 2011/LacZ*

(**A**) Diagram of fractions found in mature indeterminate nodule, as described by Roux *et al.* Fractions are termed Fraction I (FI), Fraction II distal (FIId), Fraction II proximal (FIIp), interzone (IZ) and the nitrogen fixation zone (ZIII) *(**B**) MtCBP60B expression in nodule fractions.* Highest relative expression is shown in zones FI and ZIII for three biological replicates. Light microscopy of mature nodule sections from *A. rhizogenes* transformed *M. truncatula* roots expressing *pCBP60B::GUS,* 29 days after inoculation with *S. meliloti* strain 2011/LacZ. The expression pattern in nodules is shown by histochemical GUS staining (blue), while *S. meliloti* infection is visualised via Magenta-GAL staining (purple). (**C**) Nodule tip cross section, scale bar = 100µm (**D-H**) Nodule transverse sections for each nodule zone (as in **A**), scale bars = 100µm (**I**) Whole nodule cross section, scale bar = 500µm

2.3. The expression pattern of *pCBP60B:GUS* in colonised and non-colonised roots suggests a diverse role in root developmental programs

To gain an insight into CBP60B function and how it is influenced by symbiont perception and interaction, it is important to determine its expression pattern both in symbiont-colonised and non-colonised roots. GUS staining of non-colonised *M. truncatula* roots expressing the β-glucuronidase reporter gene, driven by the *CBP60B* promoter, demonstrates that *CBP60B* is highly expressed in the root apical meristem of both primary and lateral roots (Figure 4). Transverse sectioning of the root tip (Figure 4A-D) reveals that it is expressed in the pericycle, endodermis and cortex of the root tip (Figure 4C). Additionally, *CBP60B* is expressed in the pericycle of the root axis (Figure 4G).

Gene expression patterns of indeterminate *M*. *truncatula* nodule zones were previously determined *via* laser-capture microdissection (LCM) coupled with RNA sequencing by Roux *et al.* (Roux *et al.*, 2014). The indeterminate nodule structure is typically divided into four zones including the meristem, the infection zone, the nitrogen fixation zone and the senescent zone. The fractions analysed by Roux and co-workers represent a further subdivision of the typical nodule structure zonation (Maróti and Kondorosi, 2014) and are shown in Figure 5A, where FI corresponds to the meristem, FIId and FIIp represent the

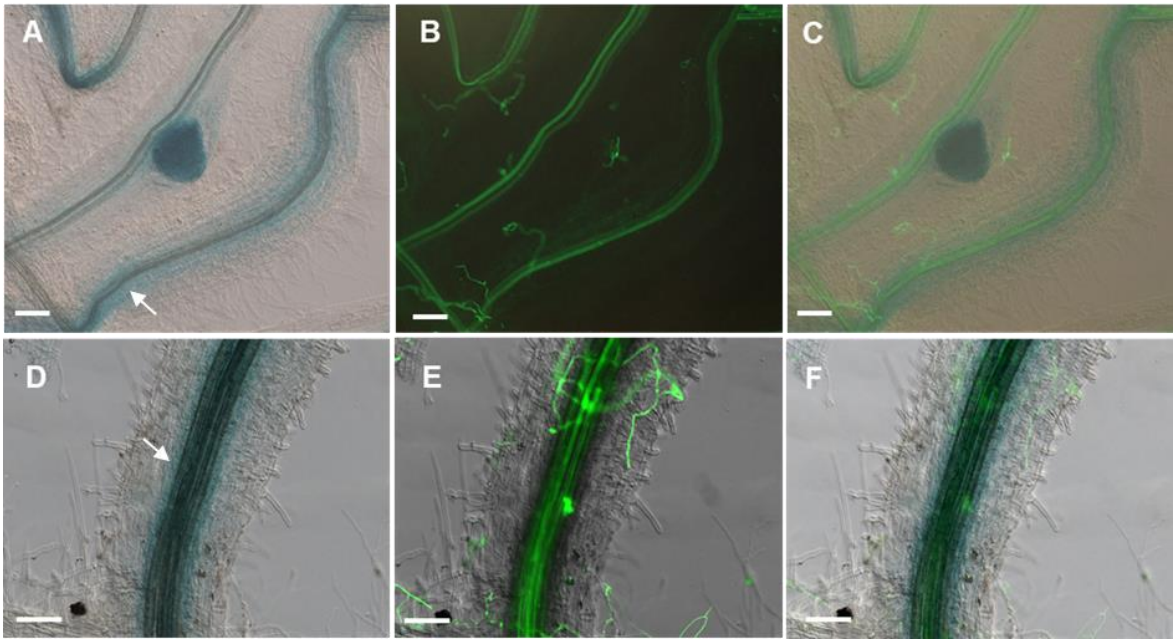

Figure 6 - Expression pattern of *pCBP60B:GUS* in *M. truncatula* roots colonised by *Rhizophagus irregularis*

Light and fluorescence microscopy of *A. rhizogenes* transformed *M. truncatula* roots expressing *pCBP60B::GUS*. The expression pattern in roots is shown by histochemical GUS staining (blue), infection by *R. irregularis* is shown through WGA staining (green). (**A** and **D**) light microscopy showing GUS stained roots, white arrows indicating GUS staining in the cortical cell layers, (**B** and **E**) fluorescence microscopy showing fungal colonisation, (**C** and **F**) light and fluorescence microscopy overlaid. Scale bars = 100µm.

distal and proximal regions of the infection zone, the nitrogen fixing zone (ZIII) and an interzone (IZ) separating FIIp and ZIII (Roux *et al.*, 2014). Analysis of *CBP60B* expression in this data set indicates that, relative to whole-nodule expression, *CBP60B* is most highly expressed in the meristematic apex (FI) and the nitrogen-fixing ZIII (Figure 4B). These data correspond, in part, to the observations of transformed roots, exhibiting mature nodules, 29 days after inoculated with *S. meliloti 2011*/LacZ. It is clear from light microscopy of nodule sections that the strongest expression of p*CBP60B:GUS* is exhibited at the nodule meristem (FI, Figure 5A and I), the region of active cell division responsible for the elongated shape seen in indeterminate nodules. Importantly, there appears to be an overlap between this region of high GUS activity and the region colonised with *S. meliloti*, as shown in purple (Figure 5C-E). However, the expression p*CBP60B:GUS* in zones FIId, FIIp, IZ and ZIII is not consistent with the expression patterning published by Roux *et al..* This is most explicitly demonstrated by the transverse nodule sections corresponding to each of the nodule fractions (Figure 5D-H). While expression is relatively high in zones ZI and FIId, zones FIIp, IZ and ZIII show very low expression, limited to the nodule vasculature. Notably, little GUS staining is seen in the ZIII zone (Figure 5H), despite the suggestion that this region has the highest level of expression overall (Roux *et al.*, 2014). However, it is possible that this is the result of the LCM process itself, as this approach may incorporate tissue from the root at the base of the nodule where *CBP60B* is shown to be expressed.

GUS staining of similarly transformed roots inoculated with the AMF, *Rhizophagus irregularis*, exhibited an expression pattern of *pCBP60B:GUS* more similar to the non-colonised roots (Figure 6), whereby GUS staining is associated mostly with the pericycle and endodermis. Nevertheless, at the sites of colonisation, GUS activity is spread further into the cortex (white arrows shown in Figure 6A and D), especially compared with non-colonised roots (Figure 4G). This is interesting as the cortex is the root layer that hosts developed arbuscule containing cells. The structure of the fungal endosymbiont within the transformed roots was visualised using WGA-Alexa488, revealing the hyphae *via* fluorescence microscopy (green, Figure 6B and E). Despite strong staining of the root vasculature, fungal penetration sites and intraradical hyphae are visible, confirming *R. irregularis* colonisation (~30% colonisation). However, as it was difficult to identify arbuscule containing cells using WGA-Alexa488 staining without cutting the root tissue, it is hard to determine expression of *pCBP60B:GUS* within these cells.

Taken together, though, these results suggest a role of CBP60B in root nodule development as well as potentially in AM colonization. Interestingly, *CBP60B* is expressed in the pericycle and in the lateral and primary root meristem suggesting a link between CBP60B and root development.

2.4. Symbiont interaction has a varied effect on *CBP60B* expression

To assess possible modulation of *CBP60B* expression during symbiosis interactions, the relative expression of *CBP60B* was determined *via* quantitative reverse transcription (RT)

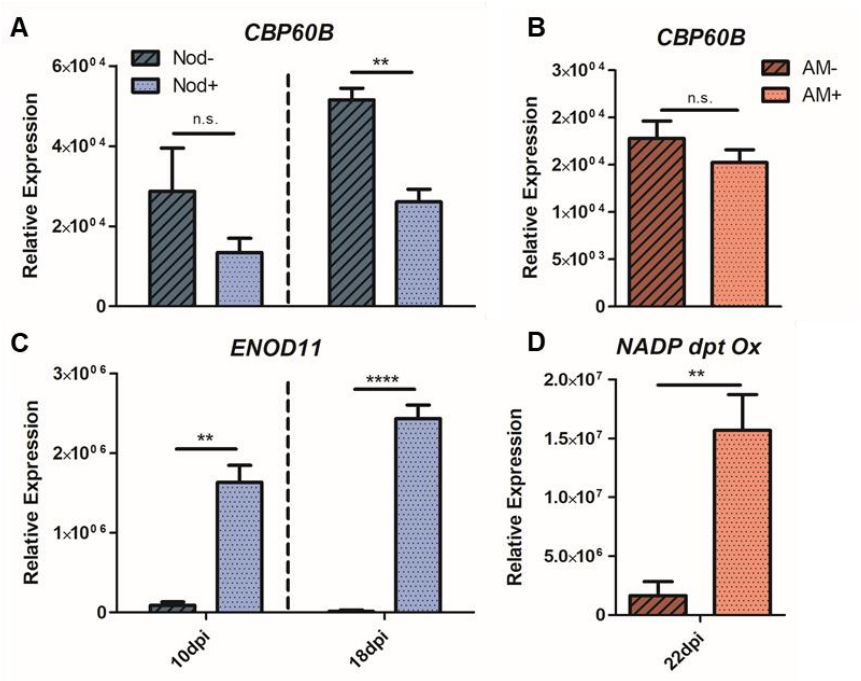

Figure 7 - Quantitative expression of *CBP60B* following symbiont inoculation

(**A** and **C)** Quantitative RT-PCR monitoring (**A**) *CBP60B* and (**C**) *ENOD11* expression in *M. truncatula* roots 10 and 18 days post inoculation with *S. meliloti 2011* (Nod+) or mock treatment (Nod-), relative to the expression of the *UBIQUITIN-CONJUGATING ENZYME* (*UBC9).* (**B** and **C**) Quantitative RT-PCR monitoring (**B**) *CBP60B* and (**D**) *NADP dpt ox* (nicotinamide adenine dinucleotide phosphate–dependent oxidoreductase) in *M. truncatula* roots 22 days post inoculation with *R. irregularis* (AM+) or mock treatment (AM-). (**A-B**) Values shown are sample means for n=3 (each with 2 technical replicates) ± S.E., **P≤0.01, ****P ≤0.0001 (One-tailed Student's independent T-test)

PCR of *M. truncatula* roots inoculated with either the nitrogen-fixing rhizobacteria, *Sinorhizobium meliloti 2011,* or the arbuscular mycorrhizal fungi, *Rhizophagus irregularis*. Root samples from plants inoculated by *S. meliloti 2011* showed no difference in *CBP60B* expression compared with non-inoculated roots after 10 days of inoculation (Figure 7A; Student's independent T-test, $t(4)=0.353$, p=0.1237). However, samples collected after 18 days inoculation showed significantly reduced levels of *CBP60B* expression in inoculated roots compared with the water control (Figure 7A; Student's independent T-test, $t(4)= 5.901$, p= 0.0021). At both time points, colonisation of the roots by *S. meliloti 2011* is confirmed by significantly higher levels of *ENOD11* in the inoculated samples, a marker gene characteristic of Nod factor induced endosymbiosis signalling (Student's independent T-test, 10dpi; $t(4)=7.136$,p=0.001, 18dpi; $t(4)=14.29$, p<0.0001). In contrast, no difference in *CBP60B* expression was seen between uninoculated *M. truncatula* roots or roots inoculated with *R. irregularis* at an early time point of AMF symbiosis; 22dpi (Figure 7B; Student's independent T-test, $t(4)=1.111$, p= 0.1644). Colonisation and activation of symbiotic

signalling was confirmed by expression of nicotinamide adenine dinucleotide phosphate–dependent oxidoreductase (NADP dpt Ox), a non-sulfated-LCO induced gene upregulated early in AM symbiosis signalling (Maillet *et al.*, 2011), upregulated highly in *R. irregularis* treated roots (Figure 7D; Student's independent T-test, $t(4)=4.295$, $p=0.0063$). All together, the absence of *CBP60B* expression regulation at early stage of colonization and the observation that CBP60B is downregulated after 18 days inoculation with Sm2011 suggest that the expression of *CBP60B* may be suppressed at a late stage of symbiosis interaction. It will be important to assess levels of *CBP60B* expression at later stages of AM infection, to determine if similar changes are seen when roots are more heavily colonised with *R. irregularis.* The functional significance of these changes in *CBP60B* expression remain to be seen, but it is clear, particularly from the expression patterns in the RAM and nodule apex, that CBP60B is likely to be involved in organogenesis in *M. truncatula* roots.

2.5. A *Tnt1* insertion in *MtCBP60B* results in a deletion of 171 bp located centrally in the coding sequence

In order to determine the function of CBP60B, a *Tnt1* insertion mutant (*cbp60b*-1) was obtained from the Nobel Foundation. PCR was used to establish the position of the *Tnt1* insertion, which was found to be placed at the 3' end of the 7th exon (position 6014, Figure 8A-B, PCR primers found in Table 1). Reverse transcription (RT)-PCR of cDNA synthesised from both wild-type (WT, R108) and *cbp60b*-1 roots revealed a deletion of 171bp across the site of *Tnt1* insertion (Figure 8C) between positions 1161 and 1911 of the coding sequence. Quantitative RT-PCR demonstrates that this deletion did not have a significant effect on the amount of the truncated *CBP60B* transcript being produced in the plant roots compared to the full-length transcript in the WT (Figure 8D). WT and *CBP60B-1* sequences were used to predict their corresponding protein structures (Figure 8E). The WT structure includes an N-terminal nuclear localisation signal (NLS), a highly conserved calmodulin (CaM) binding domain and a putative C-terminal DNA binding domain (DBD1). Also, based on the original yeast-two-hybrid screen between CBP60B and DMI1 C-terminal, the DMI1 binding domain is located centrally, overlapping with the CaM binding domain. Importantly, though this highly conserved CaM binding domain, identified in the CBP60 family in *A. thaliana*, is described as the site of CaM interaction, the actual CaM binding domain exists outside of this conserved region (Reddy, Ali and Reddy, 2002; Zheng, Majsec and Katagiri, 2020). In fact, the CBP60B homolog in *A thaliana* is thought to bind CaM at the C-terminal end of the protein (Reddy, Ali and Reddy, 2002; Zheng, Majsec and Katagiri, 2020). Determination of the true CaM binding in MtCBP60B is yet to be fully characterised, *via* functional interaction with CaM. The deletion in the gene transcript in the *cbp60b-1* mutant is predicted to lead to a deletion of 57 amino acids from position 387 to 445, leaving the NLS, CaM binding domain and DBD1 intact. Importantly, this deletion could disrupt the

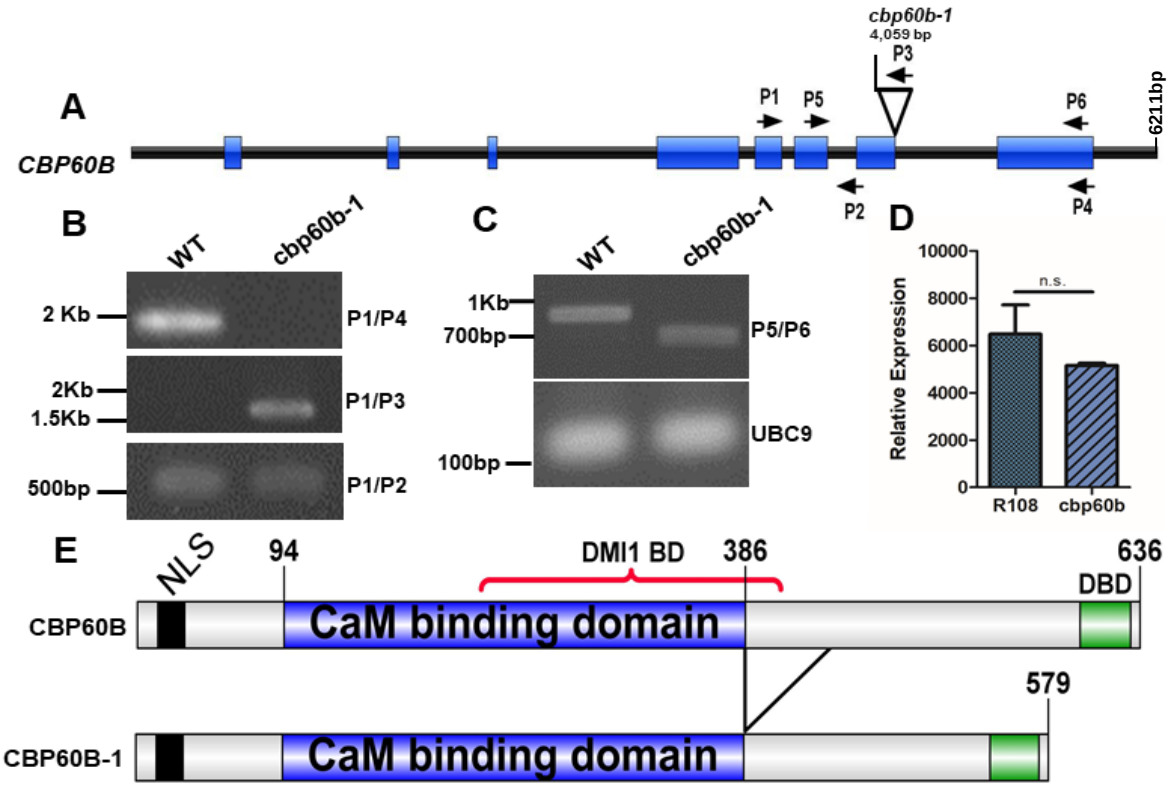

Figure 8 – Characterization of *MtCBP60B tnt1* insertion line

(**A**) The exon (blue boxes) and intron (black lines) structure of the genomic sequence of CBP60B, showing the predicted position of Tnt1 insertion, as determined by PCR and sequencing **(B)** The site of *Tnt1* insertion was confirmed by comparing PCR products generated from R108 (WT) and *cbp60b-1* line. The PCR product of primers P1 and P2 is used as a control. (**C)** Reverse transcription (RT)-PCR of *CBP60B* using P5 and P6 primers. The expression of the ubiquitin-conjugating enzyme gene *UBC9* is used as control. (**D**) Quantitative RT-PCR monitoring expression of *CBP60B* in WT (R108) and *cbp60b-1* roots, relative to the expression of *UBC9* (n=3, with 2 technical replicants) (**E**) The predicted structure of the CBP60B wild type amino acid structure, including N-terminal Nuclear Localisation Signal (NLS) and predicted C-term DNA-binding domain (DBD1). The *Tnt1* insertion in the *MtCBP60B-1* lead to a deletion of 57 a.a. from the position 387 to 445.

putative site of DMI1 interaction, which may be crucial in differentiating the roles of CBP60B as a transcription factor and an interactor of DMI1. Overall, I have confirmed the presence of a *Tnt1* insertion in the *CBP60B* sequences in the *cbp60b-1* line and used this to predict the protein structure generated by the truncated gene. This will prove useful in future analyses of this mutant line, and the characterization of the CBP60B function.

2.6. MtCBP60B acts as a putative regulator of defence signalling in response to the pathogenic ascomycete fungus *Fusarium graminearum*

Members of the CBP60 family have previously been shown to demonstrate a role in both positive and negative regulation of immunity in *Arabidopsis* (Truman *et al.*, 2013). A phylogenetic tree was generated using related CBP60 amino acid sequences from 7 plant species (Figure 9A) found that MtCBP60B is relatively distinct from AtCBP60G, AtCBP60A

and *At*SARD1 that have been implicated in plant immunity (Wang *et al.*, 2011; Truman *et al.*, 2013; T. Sun *et al.*, 2015). This suggests that *MtCBP60B* is not closely related to *CBP60* genes known to modulate plant immune responses. Additionally, expression analysis of established *M. truncatula* nodules co-infected with the pathogenic bacteria *Ralstonia solanacearum,* published by Benezech *et al*. (Benezech *et al.*, 2020), showed no significant

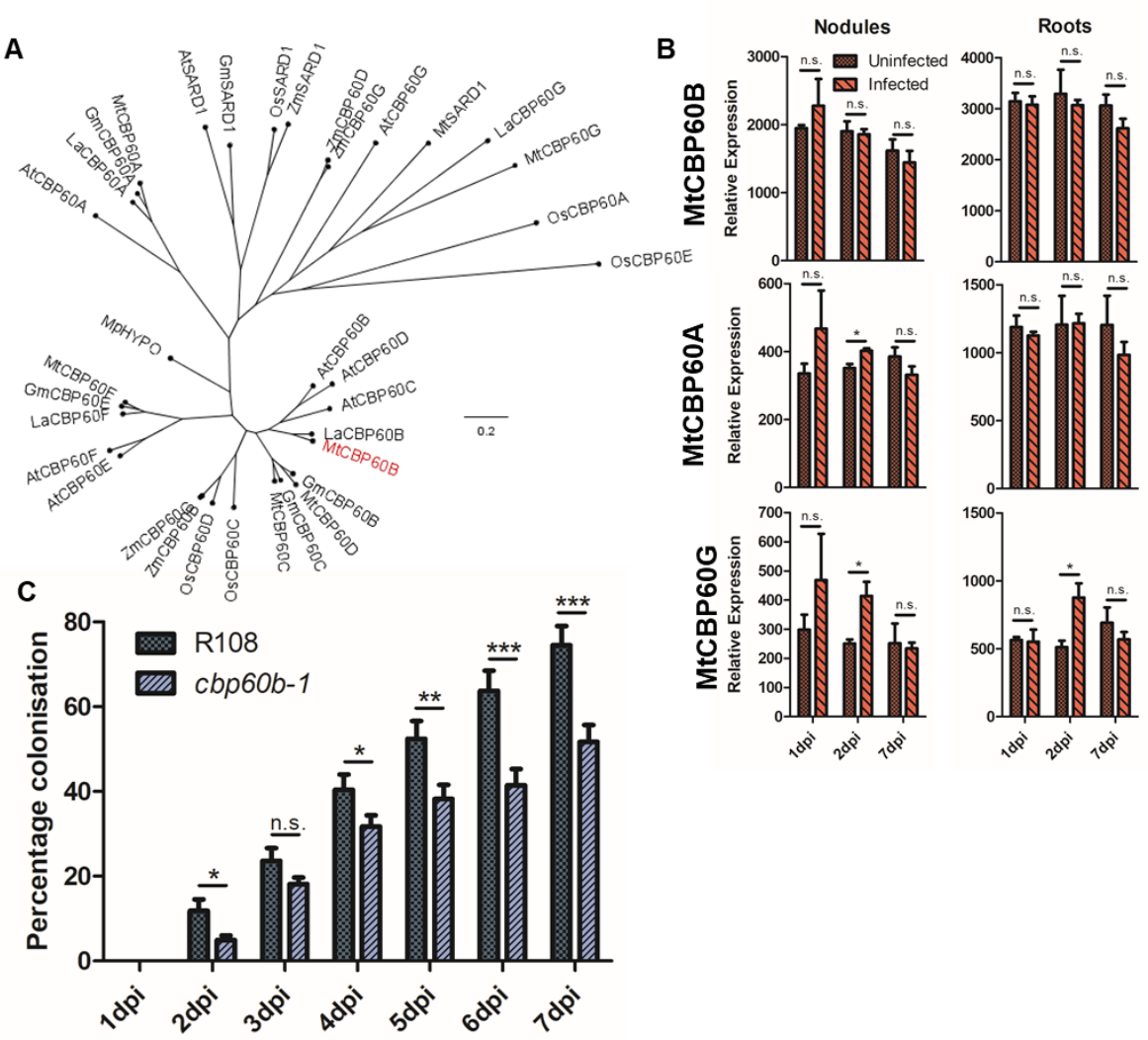

Figure 9 – The role of CBP60B as a putative regulator of defence

(**A**) Phylogenetic tree showing the evolutionary relationship between proteins belonging to the CBP60 family and related calmodulin binding proteins from *Medicago truncatula* (*Mt*), *Arabidopsis thaliana* (*At*), *Lupinus angustifolius* (*La*), *Oryza sativa* (*Os*), *Glycine max* (*Gm*), *Marchantia polymorpha* (*Mp*) and *Zea mays* (*Zm*). The protein of interest MtCBP60B, is shown in red. The tree was generated from 35 amino acid sequences using the Maximum Likelihood Method. Branch length represents the number of substitutions per site, with scale shown at the bottom. (**B**) Differential expression analysis of *MtCBP60* genes in nodules or roots uninfected and infected by the root pathogenic gram-negative bacteria *Ralstonia solanacearum*, 1, 2 and 7 days post inoculation. The data refer to transcriptomic analysis carried out by Benezech *et al*.. Values shown are mean ± SE (n=3). (**C**) Preliminary infection data comparing the percentage colonisation of WT (R108) and *cbp60b-1* roots by the pathogenic ascomycete fungus *Fusarium graminearum,* after 1 to 7 days inoculation at the root tip. Values shown are mean ± SE (R108 1-5 & 7dpi, n=16, R108 6dpi, n=13 *cambp60b-1*, n=18). *P≤0.05, **P≤0.01, ***≤P0.001 (One-tailed Student's independent T-test).

difference in *CBP60B* expression when compared with uninfected nodules, at 1, 2 and 7dpi (Figure 9B; Welch's unequal variances t-test; 1dpi, t(2)=0.829, p=0.247 Student's independent t-test; 2dpi, t(4)=0.268, p=0.401; 7dpi, t(4)=0.735, p=0.251). Expression analysis in co-infected roots prompted similar results (Figure9B, Student's independent T-test; 1dpi, *t*(4)=0.280, p=0.397; 2dpi, *t*(4)=0.464, p=0.333; 7dpi, *t*(4)=1.563, p=0.097). Alternatively, expression of *CBP60A* was significantly increased at 2dpi in nodules (Figure 9B: Student's independent t-test; 2dpi, *t*(4)=3.704, p=0.01), though no differences were seen at other nodule time-points (Figure 9B, Student's independent t-test; 1dpi, *t*(4)=1.132, p=0.160; 7dpi or at any time-point in root tissue (Figure 9B, Student's independent t-test, 1dpi, *t*(4)=0.720, p=0.256; 2dpi, *t*(4)=0.048, p=0.482; 7dpi, *t*(4)=0.937, p=0.201). Expression of *CBP60G* was significantly upregulated in nodules at 2dpi (Figure 9B, Student's independent t-test; 2dpi, *t*(4)=3.230, p=0.016), but not at 1 or 7dpi (Figure 9B, Student's independent t-test; 1dpi, *t*(4)=1.018, p=0.183; 7dpi, *t*(4)=0.261, p= 0.404). Similarly, *CBP60G* expression was significantly higher in roots after 2dpi (Figure 9B, Student's independent t-test; 2dpi, *t*(4)=3.193, p=0.015) while no difference in expression was seen at 1 or 7dpi (Figure 9B, Student's independent t-test; 1dpi, *t*(4)= 0.147, p= 0.445; 7dpi, *t*(4)=0.983, p= 0.191). Together, these transcriptomic data show that CBP60A and CBP60G, CBP60 family members known to act in pathogen defence responses in *A. thaliana*, are upregulated in either roots or nodule tissues 2 days after inoculation with the bacterial pathogen *R. solanacearum*. It is also shown that CBP60B shows no change in regulation in nodule or root tissues in response to the same treatment. While CBP60B does not appear to be induced by *R. solanacearum*, it does not rule out a role in defence signalling. Notably, the level of *CBP60B* expression was consistently higher than either *CBP60A* or *CBP60G* in both nodule and root tissues, regardless of pathogen treatment (Figure 9B), which could be important for an underlying role in defensive signalling. It is also possible that CBP60B function is linked to plant immunity in a pathogen specific manner, whereby upregulation of *CBP60B* will only be observed in response to specific pathogens. Indeed, a slurry containing the pathogenic ascomycete fungus *Fusarium graminearum*, was applied to root tip of both WT and *cbp60b-1* mutant. The percentage of root colonised by *F. graminearum,* from the site of inoculation towards the aerial parts of the plant, was found to be significantly lower in *cbp60b-1* compared to the WT, on all but one day over the first 7 day period (Figure 9C). Although this is only a preliminary assay, the results are very exciting as they show a promising improved defence phenotype in *M. truncatula* roots expressing a truncated CBP60B and suggest that CBP60B could function in plant immune response.

2.7. Root nodule and AM symbiosis is impaired in *cbp60b-1* roots

Nodulation was assessed in the mutant line relative to the WT at 10, 18 and 28 days post inoculation with *S. meliloti2011.* At each time-point, the number of nodules per dry-weight

of total root tissue was significantly lower in *cbp60b-1* (Figure 10A; 10dpi, Student's independent T-test, *t*(18)= 1.978, p=0.0317; 18dpi, Student's independent T-test, *t*(18)= 1.801, p=0.0443; 18dpi, *t*(18)= 1.798, p=0.0445). This phenotype was detected across two independent repetitions. Critically, mutant nodules were found to be normally colonised when sectioned nodules stained with X-GAL were viewed under a light microscopy (Figure 10B), indicating that this mutation does not prevent the rhizobia from inhabiting the nodule organ. Similarly, *cbp60b-1* roots exhibited a reduced percentage colonisation with AM structures compared with the WT. This was most obvious at the early time-point, 20 days after inoculation with *R. irregularis*, where significantly fewer internal AM structures, such as intraradical hyphae and arbuscule containing cells, were observed (Figure 10C; Intraradical hyphae, Welch's unequal variances T-test, *t*(11)=2.238, p=0.0234; Arbusculated cells, Welch's unequal variances T-test, *t*(10)=2.606, p= 0.0131). At this early

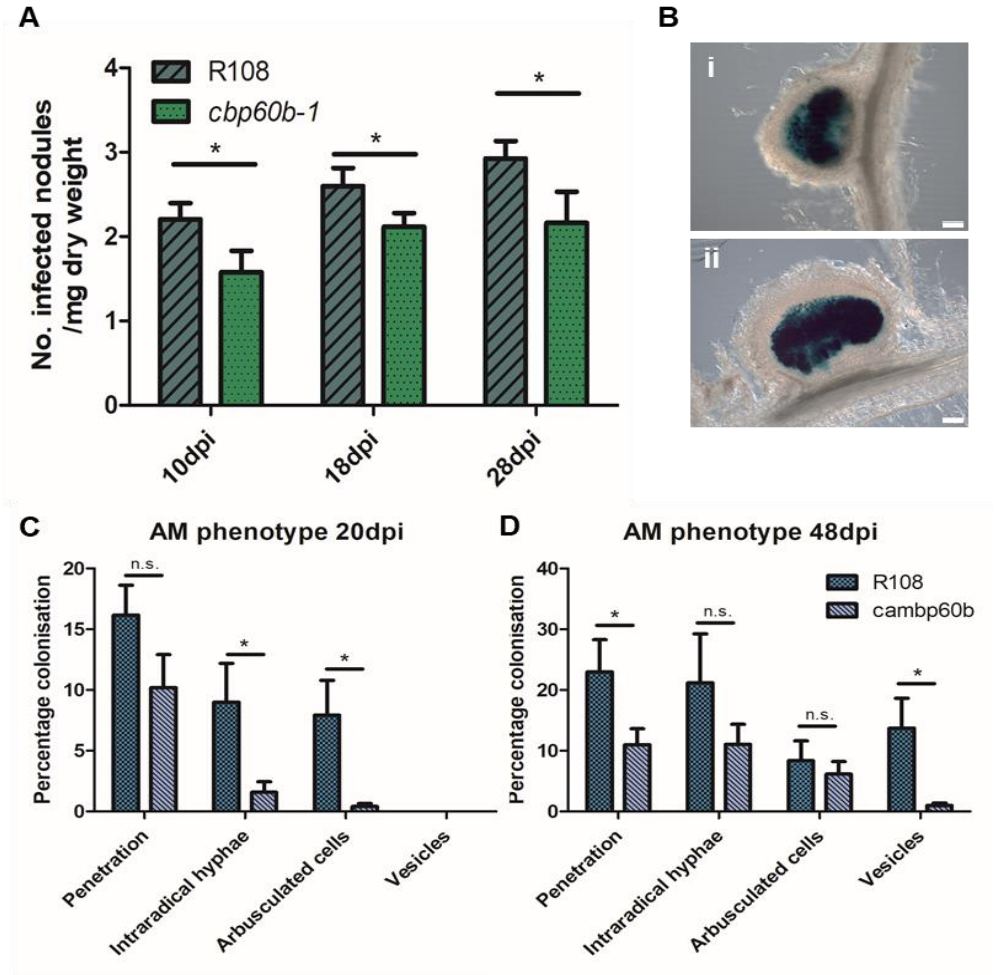

Figure 10 - *cbp60b-1* is impaired in root nodule and AM symbioses

(**A**) Nodule number per root dry weight of WT (R108) and *cambp60b-1* at 10, 18 and 28 days post inoculation with *S. meliltoi 2011/LacZ* (n=10), data presented represents the second independent nodulation assay (**B**) DIC pictures of (**i**) WT and (**ii**) *cbp60b-1* nodules colonised by *S. meliloti 2011/LacZ,* as visualised by X-GAL staining. Scale bar = 100µm. (**C** and **D**) Percentage AM structures in WT (R108) and *cbp60b-1* root, (**C**) 20 and (**D**) 48 days after inoculation with *R. irregularis* (n=12). *P ≤0.05 (One-tailed Student's independent T-test/ Welch's unequal variances T-test).

stage of root colonisation, these data suggest that *cbp60b-1* is impaired in the formation of secondary AM structures, despite similar levels of AM penetration into the root (Penetration, Student's independent T-test, *t*(21)=1.616, p= 0.0605). However, 48 days after inoculation, this observed phenotype is no longer seen. Indeed, at this stage *cbp60b-1* roots have significantly fewer AM penetration events throughout the root (Figure 10D; Penetration, Student's independent T-test, *t*(19)=2.195, p=0.0204) but similar percentage coverage of intraradical hyphae and arbusculated cells (Figure 10D; Intraradical hyphae, Welch's unequal variances T-test, *t*(10)=1.165, p=0.1356; Arbusculated cells, Student's independent T-test, *t(*19)=0.5910, p=0.2807). Also, the mutant exhibited significantly fewer AM vesicle structures at this time-point, compared with the WT (Figure 10D; Vesicles, Welch's unequal variances T-test, *t*(8)=2.556, p=0.0169), a structure usually associated with a late stage in AM colonisation. These data hint that the mutant line *cbp60b-1* may be delayed in AM symbiosis, as it appears that the presence of internal AM structure in the mutant "catches up" with that of the WT from 20 to 48 days post inoculation. It is important to note that these AM observations are based only on a single assay, though it will be important to see if similar results are reproducible, and if this phenotype differs after a longer time period. Overall, it is clear that the truncation of CBP60B in the mutant line *cbp60b-1* is detrimental to the plants ability to form symbiotic interactions, most obviously in the interaction with *S. meliloti*, but also in the delayed development of internal AM structures when interacting with *R. irregularis.*

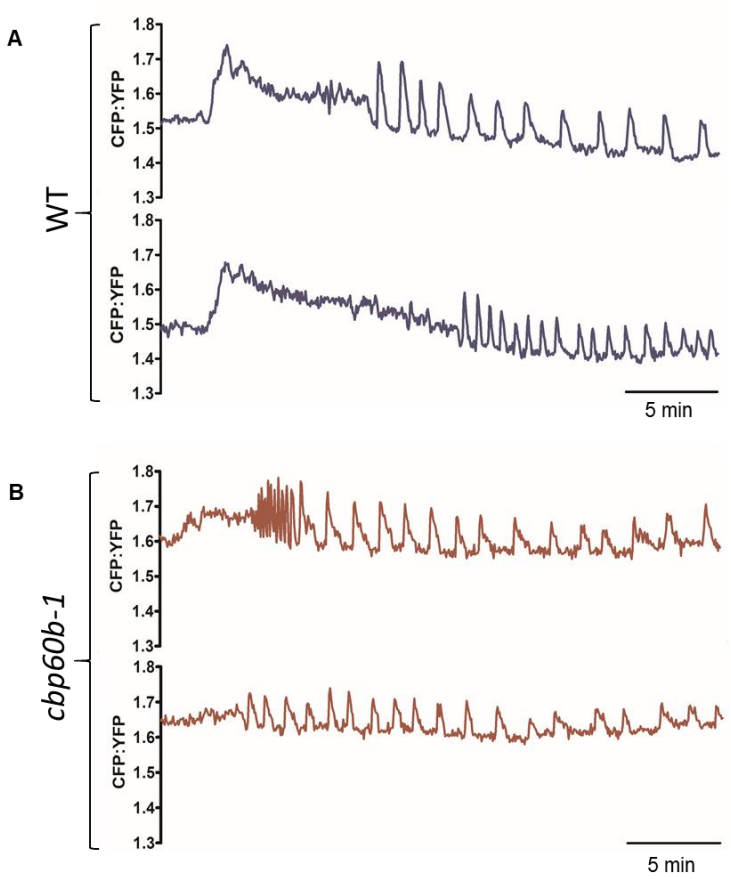

Figure 11 - Nuclear Calcium spiking in the *cbp60b-1* root hair.

Nod factor-induced nuclear calcium spiking in the backcrossed *cbp60b-1/YC3.6* line (B) and WT YC3.6 (A), measured by the ratio of CFP and YFP fluorescence emitted (CFP:YFP). 10^{-8}M Nod factor was applied at t=0. Sale bar = 5 minutes

2.8. Nuclear calcium spiking is not impaired in *cbp60b-1* root hairs following NF application

Nuclear-associated oscillations in calcium concentration are indicative of early symbiosis signalling, elicited in response to both rhizobial Nod factors and AM derived Myc factors. WT plants stably transformed to express the yellow cameleon Ca^{2+} sensor (Krebs *et al.*, 2012), YC3.6, localised to the nucleus, were used to determine the WT nuclear calcium response to the application of 10^{-8}M Nod factor (Figure 11A). This sensor, which constitutes a CFP and YFP connected by a central CaM binding peptide of the myosin light-chain kinase, changes conformation upon Ca^{2+} binding to the CaM domain, allowing for Förster resonant energy transfer (FRET) between the CFP and YFP domains. Therefore, the ratio of CFP/YFP fluorescence is measured overtime as a proxy for Ca^{2+} changes. As this calcium spiking is known to be regulated by a number of nuclear-associated ion channels, including the CBP60B interactor, DMI1, it was important to assess the effects of the *cbp60b-1* mutation. The *cbp60b-1* line was backcrossed into the stably transformed YC3.6 line and NF was also applied to 1-day old *cbp60b-1/YC3.6* seedling roots (Figure 11B). No defect in nod factor-induced calcium spiking was observed in the root hairs of *cbp60b-1* mutants, however, this result was expected as *CBP60B* is not expressed in root hair. In the future this backcrossed line will be a great tool to investigate the calcium response in *cbp60b-1* during nodule development.

3. Discussion

In this study, I have begun to characterise the CALMODULIN BINDING PROTEIN 60B (CBP60B) in *Medicago truncatula*. While the predominate aim is to elucidate function and activity of this CBP60B protein with regards to AM and root nodule symbiosis, I was also able to propose additional putative roles in plant defence and organogenesis. However, though the results collected here are exciting, pointing to a variety of modes of action for CBP60B, it is clear that future work will be required to determine the precise function of CBP60B.

3.1. MtCBP60B is required for AM and root nodule symbioses

Extensive research into signalling in AM and root nodule symbiosis over the last 30 years has identified a suite of shared components required to initiate and regulate both distinct symbiosis processes. Central to this shared symbiosis pathway is the induction of nuclear calcium oscillations, which function to induce downstream changes in gene expression. Necessary to the generation of these calcium spikes are a number of ion channels localised to the nuclear membrane, including DMI1 (Ané *et al.*, 2004), CNGC15 (Charpentier *et al.*, 2016) and MCA8 (Capoen *et al.*, 2011). Modulation of DMI1 activity, which is thought to work in tandem with the CNGC15 to coordinate nuclear calcium influx (Charpentier *et al.*,

2016; Charpentier, 2018), could be important in regulation of downstream symbiosis signalling. CBP60B was identified as a strong interactor of DMI1 *via* a yeast-two-hybrid screen, using the DMI1 C-terminal as bait. Interaction between DMI1 C-term and CBP60B in yeast was confirmed using a GAL4 based interaction assay (Figure 2). Though this result does not demonstrate an *in planta* interaction, it is a good indication that CBP60B and DMI1 share binding affinity, suggesting that CBP60B could represent one such modulating factor in DMI1 activity. To demonstrate that these proteins are closely associated in the *M. truncatula* system (<10nm between interacting partners; Miller *et al.*, 2015), a bimolecular fluorescence complementation could be carried out by transforming roots to produce DMI1 and CBP60B fusion to opposing N- or C-terminal regions of a split YFP molecule. Critically, fluorescent confocal microscopy of transformed roots expressing CBP60B-GFP fusion constructs localised CBP60B to the nucleus (Figure 3A-L). While this localisation is perhaps unsurprising, owing to known members of the CBP60 family functioning as transcription factors (Zhang *et al.*, 2010), placement of CBP60B in a similar subcellular location to DMI1 supports a functional significance to DMI1-CBP60B binding.

In order to determine a function of CBP60B during symbiotic interactions, it was important to demonstrate its pattern of expression in root tissue, both in non-symbiont-colonised tissues or those infected with rhizobia and AMF. By expressing the β-glucuronidase (GUS) reporter gene, driven by the 2kB *CBP60B* promoter, in nodule forming roots, it was shown that *CBP60B* is most highly expressed at the nodule meristem. Expression was also seen through the length of the nodule to a lesser degree, limited to the peripheral vasculature tissue (Figure 5). This reflected the patterning seen in the non-colonised roots, where the highest GUS staining was observed at the root apical meristem and along the axis of the root in the vasculature-associated pericycle tissues (Figure 4). While these findings were partly consistent with a broad transcriptomic analysis of distinct nodule regions using LCM, which indicated that *CBP60B* was highly expressed in the nodule apex (Figure 5A and B, Roux *et al.*, 2014), it was also suggested that expression was highest in the nitrogen-fixing Zone III region. This result was not reproducible using this GUS analysis method. Furthermore, the relative proportions of expression suggested for each region does not correspond well to the GUS staining here either, as the nodule apex shows a far stronger staining than the weak vasculature staining observed throughout the rest of the nodule (Figure 5D-H). As postulated previously, this could represent an issue with the LCM technique used to excise specific regions. For example, there is a chance that this method resulted in the inclusion of root pericycle tissue, where *CBP60B* is also shown to be highly expressed. Alternatively, the expression pattern could be sensitive to factors such as nodule age. GUS staining roots transformed with the *pCBP60B:GUS* construct over a time-course of nodule development will help to elucidate if any such phenotype was missed in this experiment. Importantly, Magenta-GAL revealed that the expression of *pCBP60B:GUS*

overlapped with the nodule zone containing the rhizobacteria *S. meliloti* (Sm2011, Figure 5C), suggesting that induction of *CBP60B* could be regulated by symbiont proximity. Indeed, *pCBP60B:GUS* expression in roots colonised by *R. irregularis* appeared to extend from the pericycle into the cortical cell layers, where arbuscules are known to form (Figure 6), especially when compared with the restricted staining seen in the non-colonised root axis (Figure 4G). It is important to note that WGA staining of the internalised AM tissue was weak relative to staining of the root vasculature, so it is difficult to distinguish the arbuscule containing cells in these samples. Improved resolution of these arbuscule structures, by cutting roots longitudinally prior to staining, is required to confirm *CBP60B* expression in AM colonised cells, but it is clear from the presence of hyphal penetration and intraradical growth that *CBP60B* is being expressed in these infected tissues. Together, these results suggest that pattern of *CBP60B* expression in the roots is influenced by the presence of an internalised symbiont, especially in sites actively interacting with the symbiont itself. This is a strong indication that CBP60B activity is downstream of symbiont perception, which is not surprising given its binding to DMI1. However, the proposed *CBP60B* promoter used throughout these experiments is yet to be confirmed by successful complementation the symbiosis deficient phenotype demonstrated in the *cbp60b-1* line *via* root transformation with a *pCBP60B:CBP60B* construct.

The observed changes in the pattern of *CBP60B* expression upon symbiont infection, indicate that root wide *CBP60B* expression levels are also altered during this interaction. This was assessed by qRT-PCR, using cDNA extracted from whole root system inoculated with either *S. meliloti* or *R. irregularis*, including appropriate non-inoculated controls. At an early time-point, 10 days after inoculation with rhizobia, the expression of *CBP60B* in inoculated and non-inoculated roots was not significantly different (Figure 7A). Importantly, these early colonisation time-points were taken prior to the full symbiosis organ development, showing that the level of *CBP60B* expression is not modulated before symbiont progression through the root tissue. Similar results were also found early in AM colonisation (Figure 7B). This early time point is confirmed by the absence of *PT4* (marker of arbuscule development) upregulation, and the induction of the early signalling reporter gene *NADP dpt ox.* Based on the GUS analysis, *CBP60B* is not seen to be expressed in the root epidermis in non-colonised roots (Figure 4), so if changes in expression levels are required for its function, there would likely be a time delay as the signal was transduced from the site of symbiont perception at the root surface to the pericycle layer. *CBP60B* expression at a later point in rhizobia inoculation was shown to be significantly lower compared with non-inoculated samples (Figure 7A). Given that CBP60B was found to be expressed in mature nodules, this result was somewhat surprising, as transcript levels might be expected to increase owing to the presence of more tissue shown to specifically express *CBP60B*. It is possible that, following nodule formation, expression of *CBP60B* along the

root axis is limited, but this was not obvious in the GUS stained root samples. It would be interesting to compare *CBP60B* expression in nodule and root tissues in the same plant, to determine the extent to which each tissue contributes to this overall phenotype. Additionally, ascertaining if *CBP60B* expression is also reduced at a later AM infection time point is critical to assess a shared functional significance between symbiosis interactions. Conversely, the related *Arabidopsis thaliana CBP60* gene, *AtCBP60g*, is actually upregulated in response to root microbes (Wang *et al.*, 2009). In *A. thaliana*, CBP60g acts as a positive regulator of plant immunity by inducing key SA biosynthesis genes, so it makes sense that upon detection of a root pathogen, *AtCBP60g* expression is increased in order to heighten the defence response. As microbe detection appears to reduce *CBP60B* expression, it is possible that the CBP60B protein could ubiquitously promote interaction with, or even penetration symbionts at the root-soil interface. Furthermore, this observed reduction in expression could be a mechanism which regulates the number of these symbiont interactions occurring. The true impact of this reduction in *CBP60B* expression remains to be seen but, owing to its hypothesised transcription factor activity, downregulation could have a large knock-on effect on downstream gene expression. The full effect of this could be assessed through an RNAseq of CRISPRCAS9 *CBP60B*, which should also give an indication of the major function(s) of CBP60B.

The clearest indication of a gene's role in root nodule or AM symbiosis is in the direct influence over symbiotic organ development in a mutant line. Here, a *Tnt1* insertion mutant line acquired from the Nobel Foundation, named *cbp60b-1*, was used to assess both root nodule and AM symbiosis phenotypes. PCR based characterisation of *cbp60b-1* showed that the *Tnt1* insertion was situated at the end of Exon 7 (Figure8A and B) and produced an alternative splicing event in transcribed CBP60B mRNA, resulting in the splicing of 171bp of the coding sequence (Figure 8C). Regardless, RT-PCR analysis found that there was no difference in expression of the WT and mutant forms of *CBP60B* (Figure 8D), indicating that this deletion had not affected the regulation of gene expression itself. The predicted effect on protein sequence was a 57 amino acid deletion following the conserved CaM-binding domain, theoretically leaving the C-terminal DNA binding domain intact (Figure 8E). Critically, this deletion is predicted to disrupt part of the DMI1 binding domain, determined through the yeast-two-hybrid screen. Whether this shortened CBP60B protein is actually manifested in the mutant line remains to be demonstrated; it is possible that this large deletion results in misfolding and degradation *in planta.* The expression of *CBP60B-1* fused to *GFP* in *M. trunctual* roots will be helpful to assess if this truncated CBP60B-1 is stable in *planta* as well as its localization. Conversely, if the shortened form is expressed as predicted, it is essential that the effect of the deletion on DMI1 binding is determined, primarily *via* a yeast-two-hybrid interaction with the DMI1 C-term bait used previously and *in planta* via BiFC. Assuming that this binding is impaired, the c*bp60b-1* mutant could prove

a very useful tool in elucidating the respective roles of DMI1 binding and transcription factor activity in the overall protein function, as it is possible that they have independent functions.

C*bp60b-1* plants inoculated with *S. meliloti* exhibited significantly fewer nodules per mg root dry-weight when compared with the WT across 3 time-points (Figure 10A). While nodules were still able to grow in the mutant line and were shown to be colonised by rhizobia normally (Figure 10B), there is an obvious defect in the number of nodules developing. This defect is not so striking in AM inoculated roots but is still discernible to an extent. Very early in AM colonisation (20dpi) *cbp60b-1* mutants displayed a similar percentage of hyphal penetration events throughout the roots compared with the WT but also significantly reduced internal structures such as intraradical hyphae and arbuscule containing cells (Figure 10C). At this stage, vesicles were not identified in either line. This phenotype is comparable to the reduction in nodules seen in the rhizobia inoculated roots, suggesting that the CBP60B mutant is impairing development of symbiotic structures. However, at a later stage (48dpi), this disparity in the percentage of roots colonised by internal structures is largely negated, instead the mutant exhibits lower percentage coverage of penetration events and vesicle structures (Figure 10D). These large, spherical vesicles accumulate storage products for the AMF, but only appear later in the symbiont lifecycle. To further assess this putative delayed symbiotic phenotype in the *cbp60b-1* mutant line, additional time-points in both rhizobia and AMF inoculated roots will be required. If this mutation is delayed rather than simply impaired, the mutant line would be expected to “catch-up” with the WT phenotype given enough time. Overall, it is clear that this *Tnt1* mutant has resulted in a symbiotic phenotype, particularly with regards to root nodule symbiosis, providing strong evidence that CBP60B plays an active role in the symbiosis signalling process. In order to confirm that this observed phenotype is the result of the *Tnt1* insertion into the *CBP60B* gene, rather than the effects of another insertion site, it will be critical to complement this mutant line with the full length *CBP60B*. Furthermore, to reinforce the observed phenotype, CRISPR-Cas9 based gene editing will be used to produce a CBP60B knock-out (KO) mutant. Assessing the symbiotic phenotype of this KO line may also indicate how disruption of DMI1 binding has resulted in the phenotype seen in *cbp60b-1*. Alternatively, if the symbiosis phenotype of the KO mutant is identical to *cbp60b-1*, it will indicate that the effect caused by the deletion is sufficient to impaire the function of CBP60B, assuming that the CBP60B-1 is stable.

Nuclear calcium oscillations are central to the symbiosis signalling pathway induced by rhizobia and AMF perception and are decoded to prompt changes in gene expression associated with symbiont internalisation and symbiotic organ development. NF induced nuclear calcium oscillations recorded in *cbp60b-1/YC3.6* calcium reporter lines showed no impairment of calcium spiking in root hair cells (Figure 11B). As CBP60B is not shown to be expressed in the root hair cells, even under symbiont inoculated conditions (Figure 4, 5 and

6), this WT calcium phenotype was expected. This result indicates that CBP60B, as a DMI1 interactor, does not play a role to modulate nuclear calcium influx in response to root surface symbiont recognition. However, nuclear calcium oscillations have also been observed in the cortex cell layer, associated with the symbiont progression into the root tissue, with high frequency spiking seen in colonised cells and lower frequency spiking in adjacent cells which are subsequently colonised (Sieberer *et al.*, 2012). In this way, CBP60B, which we have shown to be expressed in the cortex following AM inoculation (Figure 6), could play a role in modulating the calcium machinery during symbiosis, perhaps priming cortical cells for future infection. Using the already generated *cbp60b-1/YC3.6* calcium reporter line, this hypothesis could be assessed by measuring changes in calcium spiking in the cortical cell layers at an early point in symbiont entry into the root.

3.2. *CBP60B* expression patterns suggest a role in root organogenesis

Regulation of root architecture, the spatial configuration of the root in the soil, is essential to support the plant structurally and to ensure that it is able to uptake water and nutrients competitively, especially in soils with an uneven distribution of resources (Lynch, 1995). Development and maintenance of these structures is achieved through growth of the primary and lateral roots, driven by coordinated cell division and elongation at the root apical meristem (RAM) (Lynch, 1995). In *A. thaliana,* organisation and regulation of the RAM is dependent on the auxin gradient generated by polar transport (Motte, Vanneste and Beeckman, 2019), while lateral roots emerge from the meristematic pericycle of the primary root, also in an auxin dependent manner (Jing and Strader, 2019). Control of these processes requires the plant to interpret signals from an array of external stimuli and environmental cues. Notably, the availability of nutrients, such as nitrogen, is able to drastically affect root architecture. For example, increased root branching is triggered under high ammonia conditions (Lima *et al.*, 2010), but when nitrogen is scarce lateral root development is impeded. This is regulated by the activation of a N-responsive signalling pathway, including the generation of C-TERMINALLY ENCODED PEPTIDEs (CEPs) (Ohkubo *et al.*, 2017), and subsequent inhibition of auxin biosynthesis (Zhu *et al.*, 2020). Interestingly, this nitrogen-starvation signalling response is also responsible for the antagonistic regulation of nitrogen-fixing root nodule or lateral root developmental programs in *M. truncatula* (Zhu *et al.*, 2020). Universally, the growth and development of all of these root organs is dependent on meristematic activity and regulation. Here we demonstrate, through staining of *pCBP60B:GUS* transformed roots, that *CBP60B* is likely expressed at the sites of rapid cell division; the RAM (Figure 4D-F) and the pericycle (Figure 4A-C), as well as the in the nodule apex (Figure 5C,D and I). These findings strongly suggest that CBP60B plays a regulatory role in root organogenesis. It is feasible that CBP60B present in these tissues is able to regulate organogenesis associated genes through its transcription factor activity, downstream of phytohormone or other plant signals. Indeed, a recent study

in *A. thaliana* has demonstrated that DMI1-mediated nuclear calcium spiking has been linked to primary root development, functioning in the root meristem cells (Leitão *et al.*, 2019). Defective calcium spiking in roots deficient in AtDMI1 was shown to result in altered auxin distribution at the meristem, implying that nuclear calcium oscillations can influence auxin homeostasis and therefore root development. As a DMI1 interactor, CBP60B is therefore well placed to modulate calcium signalling, which may be important for controlling developmental programs. This could help to explain the reduced nodule phenotype observed in *cbp60b-1* mutants, especially given the predicted disruption of DMI1 binding. Assuming that CBP60B was able to play this overarching role in the development of root architecture, a striking root developmental phenotype might also be expected in the *cbp60b-1* line. While some individuals of this mutant line did display noticeably shorter roots than the WT, this phenotype was not consistent throughout the population, though it will be important to quantify this phenotype. It is possible that these observations were the result of additional *Tnt1* insertion sites in the mutant population, rather than the CBP60B mutation itself. These background effects should be negated in the backcrossed line *cbp60b-1/YC3.6* which will be investigated in the future.

3.3. A reduction in *Fusarium* infection in the *cbp60b-1* implicates CBP60B in defence signalling

At the root-soil interface, plants come into contact with a variety of different microbial pathogens, with a myriad of mechanisms for infection of root tissues. Rapid and effective immune signalling is essential for plants to overcome or mitigate the effects of harmful pathogens in the environment, so a suite of defensive responses is activated by pathogen perception. Notably, accumulation of the signal molecule SA is associated with SAR, which helps to improve whole-plant resistance to future pathogen attack (Grant and Lamb, 2006). In *A. thaliana*, transcription factors belonging to the CBP60 protein family regulate SA signalling by influencing expression of key SA biosynthesis genes (Wang *et al.*, 2009, 2011; Truman *et al.*, 2013), and more recently have been shown to function more broadly in immunity signalling (Sun *et al.*, 2015). Of the eight members of the CBP60 family in *Arabidopsis* (AtCBP60a-g and AtSARD1), only three have been identified as regulators of defence: the partially redundant positive regulators SARD1 and CBP60g (Wang *et al.*, 2011; T. Sun *et al.*, 2015; Sun *et al.*, 2018) and the negative regulator CBP60a (Truman *et al.*, 2013). Phylogenetic analysis was carried out, using related CBP60 protein sequences across 7 species, aiming to determine the relatedness of MtCBP60B with the defence signalling associated proteins identified in *Arabidopsis.* It was found that MtCBP60B was relatively distinct from the immunity associated cluster of CBP60's, instead appearing to be more related to CBP60C's and CBP60D's (Figure 9A). This is consistent with previous phylogenetic analyses, which placed CBP60a, CBP60g and SARD1 in a separate clade to

CBP60b, CBP60c and CBP60d (Wang *et al.*, 2011; Zheng, Majsec and Katagiri, 2020). It has been suggested that this immunity-related clade diversified from the prototypical clade (CBP60b-f) during the emergence of the Angiosperms, and display a faster rate of evolutionary change than "non-immune-related" CBP60's (Zheng, Majsec and Katagiri, 2020). Overall, these data suggest that CBP60B may not play an active role in defence signalling. Indeed, Truman *et al.* deemed that the defence phenotypes exhibited in T-DNA insertion mutants by the remaining members (AtCBP60b-f), in response to inoculation with the bacterial pathogen *Pseudomonas syringae* pv *maculicola* ES4326, were not sufficient to warrant further investigation, despite a significantly increased growth of the pathogen observed in *cbp60c* and *cbp60d* (Truman *et al.*, 2013). However, as these non-immune-related CBP60's are yet to be properly characterised, it is still possible that they are able to function in the defence response in an as-yet undetermined way. Interestingly, these tests of defence phenotype in the *AtCBP60* T-DNA mutant lines was exclusively assessed by leaf infiltration. As we have shown, in *M. truncatula CBP60B* is expressed in root tissues, playing a potential role in root endosymbiosis processes. As such, it is possible that MtCBP60B is as critical for immunity signalling as AtCBP60a,g and AtSARD1, but its function is specific to root infection.

Transcriptomic analysis of *M. truncatula* nodules and roots co-inoculated with *S. meliloti* and *R. solanacearum*, a gram-negative plant pathogen bacterium, found that expression of *CBP60B* was not significantly induced at 1, 2 and 7 days after bacterial treatment (Figure 9B). Expression of both *CBP60A* and *CBP60G* was upregulated in nodules after 2 days, while *CBP60G* was also induced in root tissue 2 days after the treatment. Induction of expression *CBP60A* and *CBP60G* is unsurprising, given the active role they have been shown to play in defence and expression *AtCBP60g* has been shown to be significantly increased following the application of *Pseudomonas* pathogenic strains and MAMPs (Wang *et al.*, 2009). However, it is clear that no such change occurs in the regulation of *CBP60B* expression. Again, this hints that CBP60B does not play a role in this defensive response to *R. solanacearum*, though it should be noted that the level of *CBP60B* is consistently higher in both nodule and root tissues than *CBP60A* or *CBP60G*, regardless of the pathogen treatment. Previously, we demonstrated a strong link between CBP60B and RNS, so this could relate to the interaction with *S. meliloti.* It is possible that underlying high levels of expression in the root contribute to defence signalling; for example, CBP60B present in the root may function at the moment of pathogen perception or infection, rather than being induced afterwards.

Alternatively, CBP60B may have a more pathogen-specific role in defence signalling. The model plant pathogens discussed so far, *P. syringae* and *R. solanacearum*, are both gram-negative bacteria that infect plant tissues *via* wound or natural openings such as the stomata (Vailleau *et al.*, 2007; Gimenez-Ibanez and Rathjen, 2010). Other pathogen

species are known to employ different strategies to penetrate and infect root tissues. The *Fusarium* genus of filamentous, soil-associated fungi is host to a number of broad-range and highly virulent plant pathogens. Studies into the mode of infection using fluorescently *Fusarium spp.* strains suggest that invasive hyphae are able to actively penetrate root tissues (Skadsen and Hohn, 2004; Olivain *et al.*, 2006; Wang *et al.*, 2015), in some cases forming an appressorium-like enlarged structure to force entry (Wang *et al.*, 2015). One such *Fusarium* species, *Fusarium graminearum*, is a causal agent for two of the most agronomically important diseases in wheat and other small-grain cereal crops, *Fusarium* head blight (FHB, Goswami and Kistler, 2004) and *Fusarium* root rot (FRR, Wang *et al.*, 2015). Here, inoculation of WT and *cbp60b-1* mutant *M. truncatula* seedlings with *F. graminearum* mycelia at the primary root tip showed that colonisation up the root axis was significantly reduced in the mutant line (Figure 9C), suggesting that functional CBP60B may be required to increase susceptibility for infection. Interestingly, *F. graminearum* is thought to colonise the plant from the site of infection by penetrating the vascular tissue, before spreading through the uninfected plant tissue (Wang *et al.*, 2015). As the GUS pattern of *pCBP60B* expression in the pericycle tissues indicated (Figure 4A-C, G), hyphal penetration into the vasculature requires passage through cells where levels of *CBP60B* expression are relatively high. It is important to note that the measurements of *F. graminearum* colonisation was based on the presence of necrotic tissue generated by the infection, but the development of these were not continuous from the root tip upwards. Often, concentrated spots of highly necrotic tissue developed separate from previous sites. As *F. graminearum* is known to grow extensively on the root surface prior to hyphal entry (Wang *et al.*, 2015), it is possible that each of these sites represent an individual point of infection rather than a progression through the root tissue from a point of entry at the root tip. Ultimately, this would mean that the reduced infection phenotype observed in the *cbp60b-1* line is associated with changes to external fungal progression or penetration rather than the rate of growth within the root itself. In order to elucidate this, it would be useful to generate a fluorescently tagged *F. graminearum* transformant, so that the site of infection and progression through the root tissue could be compared in the WT and mutant lines. To confirm this *Fusarium* resistant phenotype, the infection assay should be repeated, ensuring that a nutrient poor medium is used (e.g. BNM) as opposed to the rich ModFP medium. Starvation of the *M. truncatula* roots may result in a more pronounced infection phenotype. Overall, this observed phenotype intimates that either CBP60B plays a role in general plant defence, or if this response is specific to *F. graminearum* infection, independent of defence signalling. Though CBP60B elicits no obvious changes to gene expression following bacterial infections, it may be required for the infection process of a range of root penetrating pathogens. Therefore, it will be essential to test the colonisation phenotype of other root penetrating pathogens in the *cbp60b-1* line.

The reduced *F. graminearum* root colonisation in the *cbp60b-1* line reflects the phenotype observed in symbiont inoculated plants, especially in *S. meliloti* treated roots (Figure 10). It is possible that *F. graminearum* is able to utilises similar mechanisms to symbionts during the infection process. Indeed, the related beneficial endophyte, *Fusarium solani* strain K (FsK), which was isolated from *Solanum lycopersicum*, has been shown to induce responses usually associated with the CSP, including nuclear calcium oscillations, in legume plants (Skiada *et al.*, 2020). While generations of nuclear calcium signals might be explained by the similarities between *Fusarium* derived chitin-based molecules, the activation of CSP components and the impaired FsK colonisation in *CCaMK* and *CYCLOPS* mutants (Skiada *et al.*, 2020) seems to suggest that the legume is treating FsK as a symbiont. The pathogenic *F. graminearum* is known to be a hemibiotroph, meaning that for part of its lifecycle, while the fungi are invading the host, it is acting biotrophically before switching to a necrotrophic growth program. During this first stage, it is beneficial for fungal pathogen to remain asymptomatic, so that the plant defensive response is not activated. As such, it is possible that *F. graminearum* is able to interact with the symbiosis signalling pathway, as occurs in FsK, to facilitate entry into the root tissue. In this way, the plant may perceive the *F. graminearum* as a symbiotic partner, therefore allowing entry with reduced induction of an immune response. In the *cbp60b-1* mutant line, the progression of fungal infection is reduced, supporting the role of CBP60B as a promoter of symbiont colonisation, perhaps required to prime cells for infection. With the induction of nuclear calcium oscillations shown in FsK infection, and the role of CBP60B as a DMI1 interactor, it will be interesting to see the effects of *F. graminearum* infection on *dmi1* mutant roots. If the infection process requires these symbiotic-like calcium responses, root colonisation might be expected to be severely diminished compared with the WT. Overall, this study indicated that CBP60B is able to modulate both symbiosis and specific defence responses. As a DMI1 interactor, CBP60B is well positioned to modulate calcium signalling in response to either symbiont or pathogen, so could act as an important player in distinguishing “friend” from “foe”.

In conclusion, this study has so far assembled strong evidence to suggest that CBP60B plays a direct role in root nodule and AM symbiosis. It has been shown to interact with and localise in proximity to, DMI1, a key player in the CSP. GUS analysis suggests that *CBP60B* is specifically expressed in symbiont colonised tissues and symbiosis organs, while symbiont interaction is able to modulate levels of expression. Critically, mutants in *CBP60B* gene (*cbp60b-1)* displayed a reduced nodule phenotype as well as a potential delayed AM colonisation phenotype. These results support CBP60B as a promoter of root colonisation, possibly by regulating calcium signalling in a DMI1 dependent way in the root cortex, though this remains to be seen. CBP60B was also implicated in root development as GUS analysis showed that the pattern of *CBP60B* expression was closely associated with mitotically

active regions. It is possible that CBP60B is also involved in the control of organogenesis in the root. This would make sense, given its role in symbiosis colonisation in the pericycle, as these tissues are known to undergo rapid cell division upon symbiont colonisation. In previous studies in *A. thaliana*, the CBP60B homolog had not been thought to play a role in plant defence like the key immunity regulators CBP60A, CBP60G and SARD1. However, (cbp60b-1) mutants were found to be significantly less susceptible to infection by *F. graminearum* compared to the WT. This helps to support the theory that CBP60B positively regulates colonisation of the root but may also indicate that *F. graminearum* is able to induce symbiosis signalling in order to facilitate root infection. However, further research is needed to unravel the exact connection here between symbiosis and defence signalling, and the role that CBP60B plays within this.

4. Materials and Methodology

4.1. Plant material and growth conditions

Wildtype (WT) or *cbp60b-1 Medicago truncatula* seeds were scarified and sterilised with 10% bleach for 3 min, washed and plated on Water Agar (WA) media before stratification at 4°C for 5 days. Germinated seeds were transferred to Modified FP media plates and plants were grown in controlled-environment rooms (22°C, 80% humidity, 16h photoperiod, 300µmol m^{-2} s^{-1} light intensity). For analysis of gene expression and symbiosis phenotyping of *M. truncatula* roots under symbiosis conditions, one week old plants were grown in Terragreen/Sand (Oil-Dri Company, Wisbech, UK) and inoculated with either 5ml *Sinorhizobium meliloti* (Sm2011, ~OD_{600}=0.001) or *Rhizophagus irregularis* (Endorize; Agrauxine, France) at a ratio of 5:5:1 (Terragreen/Sand/Spores).

4.2. Arbuscular mycorrhizal colonisation and nodule counting for symbiosis phenotyping

Symbiont inoculated *Medicago truncatula* plants were grown in terragreen/sand as described above. *R. irregularis* inoculated roots were dug up at 20 and 48dpi and cleared by submerging in fresh 10% KOH for 15 min at 95°C. Roots were immediately washed 3 times in water and subsequently fungal structures were stained in acidic ink (5% ink, 5% acetic acid) for 5 min at 95°C. Using a binocular light microscope, percentage colonisation of specific AM structures in the stained roots was determined using the grid intersect method (Giovannetti and Mosse, 1980). *S. meliloti* inoculated plants were dug up at 10, 18and 28dpi before the roots were fixed in in 100 mM sodium phosphate pH 7.0, 10 mM KCl, 1 mM MgCl2 and 2.5% glutaraldehyde for 30 mins in a vacuum chamber, followed by 1h at room temperature. Sm2011 β-galactosidase activity was determined by staining overnight in 0.1 M sodium phosphate (pH 7.2), 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6 and 0.02 M 5-bromo-

4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL) at 28°C. The number of nodules per plant was assessed using a binocular light microscope. The roots were dried at 37°C overnight and the dry weight of each root measure to calculate the number of nodules/mg dry weight for each plant.

4.3. Genotyping

The CBP60B *Tnt1* insertion mutant (*cbp60b*-1/NF14708) was obtained from the Noble foundation *Tnt1* mutant collection (https://medicago-mutant.noble.org/mutant/). The *Tnt1* insertion line was genotyped to identify homozygous individuals in a segregating population *via* PCR using gene specific primers (Table 2) and DNA extracted with the DNeasy 96 Plant Kit (Qiagen). The *Tnt1* insertion was identified using primers P1/P3, the wild type using P1/P4.

4.4. Hairy root transformation of *Medicago* roots

Transformation of young *Medicago* roots was achieved through an *Agrobacterium rhizogenes* mediated method as previously described (M. Charpentier *et al.*, 2014) using the *A. rhizogenes* strain AR1193. Constructs contained a red florescent protein (DsRed) plant marker to facilitate selection of fully transformed roots by fluorescence microscopy, which were used for further analysis.

4.5. Gene expression analysis

RNAs were extracted from root tissue using the Plant RNAEasy Kit (Qiagen), treated with TURBO DNA-*free* (Invitrogen) and 1µg RNA was reverse transcribed using SuperscriptII reverse transcriptase (Invitrogen). Quantitative gene expression was determined by SYBR® Green based qPCR on a Bio-rad thermocycler. For each treatment condition, three biological replicates were used, each with a technical replicate. Gene specific primers used are detailed in Table 2, and expression was normalised for *UBC9*, using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

4.6. Localisation *via* confocal microscopy

Constructs containing *CBP60B* fused to GFP in both the N-terminal and C-terminal positions by sub-cloning *CBP60B* into the vectors *pK7WGF2 pLjUB-DsRed* and *pK7FWG2 RR* respectively *via* Gateway cloning (Invitrogen). Young *M*. *truncatula* roots were transformed using *A. rhizogenes* mediated transformation and successfully transformed roots were determined by the presence of the DsRed plant marker. Localisation of *GFP:CBP60B* and *CBP60B:GFP* demonstrated using a Zeis LSM780 confocal microscope equipped with a x40/1.2 water objective. GFP was excited at 488 nm line by an Argon ion laser and emitted fluorescence was collected between 500 to 550 nm. DsRed was excited

at 561 nm and emitted florescence was collected at 592-638 nm. Optical sections were taken through the sample, with a step size of 1 micrometre. ImageJ was used to process microscopy images and add scale bars.

4.7. Histochemical GUS staining and visualisation of CBP60B expression patterning in symbiont colonised and non-colonised roots

The *CBP60B* promoter region, the 2kB sequence upstream of the *CBP60B* gene, was cloned using primers P13 and P14, and used to produce the *pCBP60B:GUS via* Gateway cloning. Roots expressing *pCBP60B:GUS* were generated *via A. rhizogenes*-mediated transformation as described. Roots were fixed in cold 90% methanol for at 30 min at -20 °C, after which time the methanol was removed and the roots washed with the rinse solution (0.5 M Na_2HPO_4, 0.5 M NaH_2PO_4, 0.1 M $K_3Fe(CN)_6$, 0.1 M $K_4Fe(CN)_6$). The rinse solution was removed, before the roots were stained with the stain solution (rinse solution with 2 mM X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronide). Roots were vacuum-infiltrated for 30 min and then incubated at 37 °C, in the dark, for 24 h. Nodule forming root samples were also stained for the presence of Sm2011 using 0.02M 5-Bromo-6-chloro-3-indolyl β-D-galactopyranoside (Magenta-GAL). AM structures were stained with fluorescent Alexa Fluor 488 WGA. GUS expression patterns and symbiont colonisation was visualised using a DM 6000 microscope (Leica).

4.8. WGA fluorescent staining of AM structures

AM inoculated roots were washed with sterile dH2O, before overnight incubation at room temperature in 50%, followed by 2 days in 20% KOH for 2 days at room temperature. Roots were washed again with steril dH2O, incubated in 0.1M HCL for 3 h, before rinsing first in sterile dH2O, and then in 1x phosphate-buffered saline (PBS). Root samples were stained overnight with Wheat Germ Agglutinin (WGA) labelled with Alexa Fluor 488 by incubation in a 1 x PBS solution containing 0.4 µg/ml Alexa Fluor 488 WGA in darkness at 23°C.

4.9. Root sample embedding and sectioning

LacZ stained nodules and non-colonised GUS stained root were embedded in 6% agarose before sectioning at 30µm with a Vibratome. GUS stained nodules were embedded using the Technovit® 7100 plastic embedding kit (Kulzer) and sections of 15µm were made using a Microtome.

4.10. Yeast-two-hybrid interaction

Gal4-based yeast two-hybrid assays were carried out using the yeast strain AH109 in order to assess interactions between CBP60B and DMI1. Gateway cloning (Invitrogen) was used to sub-clone C-terminal domain of DMI1 (S343 to E882) and *CBP60B* into the vector pBD-GAL4-GW or pAD-GAL-GW to produce fusions with the Gal4 binding domain (BD) of activating domain (AD). The murine p53 and its interacting partner the SV40 large T-antigen were used as controls (CLONTECH PT30241). Yeast co-transformed *via* the lithium acetate method (Charpentier *et al.*, 2016) were grown on double dropout synthetic medium lacking Leu and Trp (-LW) for 3 days at 28°C. Interaction was assessed by growth on a quadruple dropout synthetic medium lacking adenine, His, Leu and Trp (-AHLW) for 4 days at 28°C.

4.11. Western blot

15µl of whole cell protein extract was fractioned by SDS-PAGE using 4-20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-rad) before transfer onto a polyvinylidene difluoride membrane. After 1hr incubation in blocking buffer (5% milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20)), membranes were washed with TBST 3 times for 15mins before incubation with antibodies. For identification of localisation GFP fused CBP60B, membranes were incubated with a primary α-GFP-N-terminal antibody (1:500) in 1% milk TBST solution for 1hr. Following a further 3 washing steps with TBST (15mins each), membranes were incubated with using rabbit polyclonal α-HRP (1:1000). Blots were developed via ECL detection kit (Amersham) and visualised on film (FujiFilm) after exposure over-night. Membranes were stained using Ponceau Staining Solution to detect protein binding.

4.12. *Fusarium graminearum-Medicago truncatula* pathogenesis assay

Fusarium graminearum isolate was cultured on a 25ml potato dextrose agar media (PDA) by applying a 30µl droplet of spore suspension to the centre of the plate. The culture was allowed to grow for 1 week in controlled-environment rooms (22°C, 80% humidity, 16h photoperiod, 300µmol m^{-2} s^{-1} light intensity) before homogenisation with 50ml of sterile dH_2O. A 50µl droplet of the mixture was placed on the root tip of week-old *M.* truncatula plants grown on Modified FP media (1.8%). Plants were grown on filter paper, with three plants per 10cm square plate and root tips at approximately the same level (Figure 8A). Upon *Fusarium* inoculation, plates were stacked at a 70° angle in a box, with lids slightly open. The box was lined with blue roll and filled with sterile water to a level just below the inoculum. Plates were photographed each day for 7 days. The progression of *Fusarium* infection from the root tip using ImageJ software with the NeuronJ plugin. At each time point, the full root length was measured, as well as the distance between the inoculated root tip and the furthest signs of necrotic tissue from the site of inoculation. These values were used to calculate the percentage colonisation of the roots.

4.13. Phylogenetic analysis

To identify the evolutionary relationship between the CBP60 family of proteins, predicted protein sequences from genomes of *Medicago truncatula*, *Arabidopsis thaliana, Lupinus angustifolius*, *Oryza sativa*, *Glycine max*, *Marchantia polymorpha* and *Zea mays* were downloaded using Phytozome. Sequences were confirmed as members of the CBP60 family using protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned using MEGA10 (https://www.megasoftware.net/), utilising the MUSCLE alignment algorithm. A phylogenetic tree was generated through the Maximum Likelihood Method based on the JTT matrix-based model using MEGA10, with a bootstrap of 250. Branch length Branch length represents the number of substitutions per site.

4.14. Statistical analysis

Quantitative statistical analyses were performed using either a Student's independent T-test or Welch's unequal variance T-test, depending on variance within the data set. Variance was assessed by an F-test for equality of variances. All tests were carried out using GraphPad version 5.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

5. Appendix

Table 1 – Primers used in this study

Name	Figure	Function	Sequence annealed	Sequence 5'-3'	Direction
P1	8	Genotyping	*CBP60B (WT)*	GCATTATCCTCCTGCATTGG	fwd
P2	8	Genotyping	*CBP60B (WT)*	GCTTCAAAGGAAACTACAAACTCTG	rev
P3	8	Genotyping	*CBP60B (cbp60b-1)*	CAGTGAACGAGCAGAACCTGTG	rev
P4	8	Genotyping	*CBP60B (WT)*	CTATTCATCTAACTCCTCTATCTGTGC	rev
P5	7/8	RT/QRT-PCR	*CBP60B (WT)*	AGGATCGCTCTCGTCCTGGCA	fwd
P6	7/8	RT/QRT-PCR	*CBP60B (WT)*	GCAGCAATGAGGTGGGGCTTC	rev
P7	7	QRT-PCR	*NADP dpt ox*	GGTTGATTGCTCTTCTCTCCCC	fwd
P8	7	QRT-PCR	*NADP dpt ox*	AAGTGATTGCTCGTCCAACCC	rev
P9	7	QRT-PCR	*ENOD11*	CCATTGCCTAAACCACCTGTTA	fwd
P10	7	QRT-PCR	*ENOD11*	CTTGTTGCTTGCAAATACGCTT	rev
P11	7/8	QRT-PCR	*UBC9*	GAAGACAGGTCGTTGGGTGT	fwd
P12	7/8	QRT-PCR	*UBC9*	AGTTCCTGAGGCAACAGTGG	rev
P13	4/5/6	Promoter cloning	*proCBP60B*	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCTGATACATCGATGATTAAATGACAA	fwd
P14	4/5/6	Promoter cloning	*proCBP60B*	GGGGACCACTTTGTACAAGAAAGCTGGGT CTATACACTCTCTAACAGTAAAACCCT	rev

Table 2 – Protein sequences used for the phylogenetic analysis

Protein Name	Amino Acid Sequence

AtCBP60A	MRIPTYDFGSKFSVVQEVMRLQTVKHFLEPVLEPLIRKVVKEEVELALGKHLAGIKWI CEKETHPLESRNLQLKFLNNLSLPVFTSARIEGDEGQAIRVGLIDPSTGQIFSSGPASS AKLEVFVVEGDFNSVSDWTDEDIRNNIVREREGKKPLLNGNVFAVLNDGIGVMDEIS FTDNSSWTRSRKFRLGVRIVDQFDYVKIREAITESFVVRDHRGELYKKHHPPSLFDE VWRLEKIGKDGAFHRRLNLSNINTVKDFLTHFHLNSSKLRQVLGTGMSSKMWEITLD HARSCVLDSSVHVYQAPGFQKKTAVVFNVVAQVLGLLVDFQYIPAEKLSEIEKAQAE VMVIDALSHLNEVISYDDEVSMMRNVLNAPASQGSVAGIDYSGLSLTSLDGYGFVSS LHNTAECSGKHSDDVDMEVTPHGLYEDYDNLWNCSHILGLEEPQSELQSALDDFMS QKNASVGGKAHSKRWTKLFSVSRWLSVFKYVKLGKIX
AtCBP60B	RAKRNLDGNDDDQPERKRPAFASVIVEALKVDSLQKLCSSLEPILRRVVSEELERALA KLGPARLTGSSGSSPKRIEGPDGRKLQLHFKSRLSLPLFTGGKVEGEQGAVIHVVLID ANTGRAVVYGPEASAKLHIVVLEGDFNTEDDEDWTQEEFESHVVKERSGKRPLLTG EVYVTLKEGVGTLGELVFTDNSSWIRSRKFRLGLRVVSGCCDGMRIREAKTEAFVVK DHRGELYKKHYPPALNDDVWRLDKIGKDGAFHKKLTAEGINTVEDFLRVMVKDSPKL RTILGSGMSNKMWDALVEHAKTCVQSSKLYIYYAEDSRNVGVVFNNIYELSGLISGD QYFSADSLTDSQKVYVEGLVKKAYENWNLVIEYDGKSLLDLKQPQRLSITHTNLENY STAAIDHPMQMVAGHSSSMPPNQSPVLSDFAIGGYDQTLATRYHSHPQLLNSNPRA QFEVASCSTSQDQFMGNLHQTQSTINNQHMNGLALGPSQSSTSGYQNINPSSVHQ ADLNHLEDWSNPRERGPDDFFSEEEIRLRSHEMLESEDMQQFLRLFSMGGGGNGS ATHLPEDGYTFPSFLHTPMQGYDEDRGRSGRAVVGWLKIKAAMRWGFFIRRKAAE RRAQIVELDD
AtCBP60C	REKRKLEEDDNQQQQQQPERKRPALASVIVEALKMDSLQRLCSSLEPILRRVVSEEV ERALAKLGPARLSERSSPKRIEGIGGRNLQLQFRSRLSVPLFTGGKIEGEQGAAIHVV LLDMTTGHVLTVGPEASAKLDVVVLDGDFNTEDDDGWSGEEFEGHLVKERQGKRP LLTGDVQVTLKEGVGTLGELIFTDNSSWIRCRKFRLGLRVSSGYCEGMRVREAKTEA FTVKDHRGELYKKHYPPALDDEVWRLEKIGKDGAFHKKLNKAGIYNVKEFLRLMVKD SQKLRTILGSGMSNRMWETLAEHSKTCVLSEMLYVYYPEDSVGVVFNNIYEFSGLIS GKQYYPADSLSDNQKGYVDGLVRKAYENWEQVIEYDSKSLMNFNQVSKTDDIDYS MPVSVPSQPSTSYSDVTVEAYNQSPASSFPGQSQLADTTYMHFGNSSFAPQDQLV NNTHESQSMINSNGGVRLALGPATGSQNQEQLVHPPPEINSYNDWSNTCNRGVDG FLSEEEIRARSNEMLENDDMQQLLRLFSMNGGDQQTPLNMGEDSFGFHSFGQTSM ADYEEDRSNSGKAVVGWLKIKAAMRWGFFIRRKAAQRRAQIVQLDE
AtCBP60D	MKRNFERNDDDKPERKRPALASVIVEALKVDSLQKLCSSLEPILRRVVSEEVERALAK LVPTRLTTSSVFSPKRIGGPDGRNLQLHFKSRLSLPLFTGGRVEGEQGATIHVVLIDA NTGRPVTVGPEASLKLEVVVLGGDFNNEDDEDWTQEEFESHVVKEREGKRPLLTG DLFVVLKEGVGTLGEIVFTDNSSWIRSRKFRLGLRVPSGYCDGIRIREAKTEAFSVKD HRGELYKKHYPPALNDEVWRLEKIGKDGAFHKRLTAAGIVTVEGFLRQLVRDSTKLR AILGSGMSNKMWDLLVEHAKTCVLSGKLYIYYTEDSRSVGVVFNNIYELSGLITEDQY LSADSLSESQKVYVDGLVKKAYENWNQVVEYEGESLLNLNQPERLDISQTDPVTALA SYSTVPLSQFPEFAIEGYNQTLTTALPHNPQAQFDFVPQQDQFIGIQQPQTQTNIENE NVTRLVLGPPQSSTGGYQDIKSSADQENLNPFEDWTNLSENDFFSEEEIRQTSHDLL ANEDMQQLLFSMGGGKGEDGFTFPSFMQNTPMMQGYDEEGRGRSGKAVVGWLK VKAAMRWGFFIRRKAAERRAQIVELHD

AtCBP60E	MNKRGYECSQEDTDKLPESKRQKVPALASVIVEAVKVDSLQRLCSSLEPLFRRIVSE EVERALSRLGNAKLTSRSPEPKRIQDRNGRNLQLHFRTRMPPHLFTGGKVEGERGS AIHVVLIDANTGNVVQTGEESASKLNVVVLEGDFNDEDDEDWTREHFESFEVKERE GKRPILTGDTQIVLKEGVGTLGELTFTDNSSWIRSRKFRLGVKPASGYGDSFCIREAK TEPFAVKDHRGELYKKHYPPAVHDEVWRLDRIAKDGVLHKKLLKANIVTVEDFLRLLV KDPQKLRNLLGSGMSNRMWENTVEHAKTCVLGGKLYVFYTDQTHATGVVFNHIYEF RGLITNGQFLSLESLNHDQKISADILVKLAYENWHKAIEYDGKLLNCLPVAEKEIKSLL EPKMVSAQTAPNHQQLHNQNNRQTVQGHQNAITYSPVPQPIDYPQFAQQHCNQLL PSFPCNVQDYNRSMESSNDSSSYNGEDWCPPRAAGQGLEDIFSEEIRLRSSEMLET DDMQRLLKTFGIGVNTVGTQGGFGQTDESCYGYSIPYQAQIDNTYRRERNRGSGKA VVGWLKLKAALRWGIFIRKKAAERRPQIVEIDX
AtCBP60F	MNNRGHGHNQEHADNLPESKRQKLPALASVIVEAVKVDSLQRLCSSLEPLFRRIVSE EVERAISRLENSKSTSRSTEPNKIQGLDGRNLQLRFRTRMPPHLFTGGKVEGEQGS AIHVVLIDANTGNVIQTGEESMTKLNIVVLDGDFNDEDDKDWTREHFESFEVKEREG KRPILTGDRHVIIKEGVGTLGKLTFTDNSSWIRSRKFRLGVKPATGFHIREAKTEPFAV KDHRGELYKKHYPPVLHDEVWRLDKIAKDGALHKKLLKSNIVTVEDFLQILMKDPQKL RSLLGSGMSNRMWDNTVEHAKTCVLGGKLYAYYTDQTHQTAVVFNHIYEFQGLIAN GHFLSSESLNHDQKISADTLVKTAYENWHKVVEYGGKLLNCLPVAKKGIKSLPEPKM AQNHQTQQKALQCQQTVNGYSSDPQHLIEYPFVQQPCYQLRDYTSMESSSVSGSY NGGLEDIFTEEIRARSSEMLETDNMQRLLKTFGISGGFGNRDESIYGFSDQYEAQIDK GYMREGGRGAGKAVVGWLKLKAALRWGIFIRKKAAERRPQIVEIDX
AtCBP60G	RNSPSFHGGSGYSVFRARNLTFKKVVKKVMRDQSNNQFMIQMENMIRRIVREEIQR SLQPFLSSSCVSMERSRSETPSSRSRLKLCFINSPPSSIFTGSKIEAEDGSPLVIELVD ATTNTLVSTGPFSSSRVELVPLNADFTEESWTVEGFNRNILTQREGKRPLLTGDLTV MLKNGVGVITGDIAFSDNSSWTRSRKFRLGAKLTGDGAVEARSEAFGCRDQRGESY KKHHPPCPSDEVWRLEKIAKDGVSATRLAERKILTVKDFRRLYTVNRNELHNIIGAGV SKKTWNTIVSHAMDCVLDETECYIYNANTPGVTLLFNSVYELIRVSFNGNDIQNLDQP ILDQLKAEAYQNLNRITAVNDRTFVGHPQRSLQCPQDPGFVVTCSGSQHIDFQGSLD PSSSSMALCHKASSSTVHPDVLMSFDNSSTARFHIDKKFLPTFGNSFKVSELDQVHG KSQTVVTKGCIENNEEDENAFSYHHHDDMTSSWSPGTHQAVETMFLTVSETEEAG MFDVHFANVNLGSPRARWCKVKAAFKVRAAFKEVRRHTTARNPREGLX
AtSARD1	AGKRLFQDLDSDQENKSEKRIKSVLPSLASPISSVFGALISENTLRSVLEPVIRKVVRQ EVEYGISKRFRLSRSSSFRIEAPEATTPTLKLIFRKNLMTPIFTGSKISDVDNNPLEIILV DDSNKPVNLNRPIKLDIVALHGDFPSGDKWTSDEFESNIIKERDGKRPLLAGEVSVTV RNGVATIGEIVFTDNSSWIRSRKFRIGAKVAKGSSGQGVVVCEAMTEAIVVRDHRGE LYKKHHPPMLEDEVWRLEKIGKDGAFHKKLSSRHINTVQDFLKLSVVDVDELRQILG PGMSDRKWEVTLKHARECILGNKLYISRGPNFFMILNPICEVMKALIDGHVLSSQESL NQPYVKNLVRDAYSKGNFLEVGERTANEAALLTQGDDLDQQYAASHYQNIEIDKSY QQNGYVQERSTNNLEIVNEGYITTPAEFNICFTGSSSQNHINPFX
GmCBP60A	SLKRRPDDGKTPDDKRRKPPPFSSVVRDVMKLQSLGHLLEPILEPLVRKVVKEEVEA ALKRHLTSMKQTCGKEFHTTELRNLQLQFENSICLPVFTGARIEGEDGSNLRIGLVDA LTGKVVSTGPESSAKVEIVVLEGDFEEESETWMPEEFKSNIVREREGKKPLLTGDVIL YLKDGIGMVSEISYTDNSSWTRSRRFRLGARVVDNFDGVRIREAKTESFIVRDHRGE LYKKHHPPGLSDEVWRLEKIGKDGAFHKRLSREKIVTVREFLTLLNLDPAKLRSILGT GMSAKMWEVTVEHARTCVLDTTRHVYFPSNSQEPGVVFNAVGQVTGLLSECDYVT VDKLTETEKADAQNAVTAALRQGEKYATFEDEDSLMDGSSHLTNVLYSPSSPKTEG

	SSANKILAPQKTGGFNYPPANASSPDIMSSIYSVGGTSSLDDYCLPNFDSMGLRYDQ TLSFPVQVSNSLICDTDSMAHAFSDEDHLQFFDTDLQSHVQADLQSAIDSFMLARPT ANGGAQRRWRKVCNVLKWFMVRKRGNQIQVRLX
GmCBP60B	REKRGLDSASAEEGQPDRKRPALASVIVEALKVDSLQKLCSSLEPILRRVVSEEVER ALAKLGPAKLNTGRSSPKRIEGPDGKNLQLHFKTRLSLPLFTGGKVEGEQGTAIHIVLI DANSGHIVTSGPESCVRLDVIVLEGDFNNEDDDNWDEEEFDSHIVKEREGKRPLLTG DLQVTLKEGVGTLGELTFTDNSSWIRSRKFRLGLKVASGCCEEMRIREAKSEPFTVK DHRGELYKKHYPPALNDEVWRLEKIGKDGSFHKRLNKAGIYMVEDFLRLVVRDPQR LRNILGSGMSNKMWDILVEHAKTCVLSGKLYVYYPEDARNVGVVFNNIYELSGLIAND QYYSADSLSENQKVYVDTLVKKAYDNWMHVIEYDGKSLINDNEDKALDTTHPQAPM TSHEYSNSLQQISIPALPLPVHPGQPSMDSGVTVGGYHDGTASRFSLQPHPNLNSSI QFDDNAFPLQNQLMSASHHAQLPRNENGQTIGPPQSSTHGFEPVSISNPTYRGVEE YFPEEEIRIRSNEMLENEDMQHLLRIFNMGGQPHPTFNAQDDGYPSSSTYISANPMG YNFDDEPNRSSGKAVVGWLKLKAALRWGIFIRKQAAERRAQLVELDD
GmCBP60C	REKRALDSGSADEDQPQRKRPALASVIVEALKVDSLQKLCSSLEPILRRVVSEEVEC ALAKLVPAKLSGRSSPKGIEGPDDSSLQLQFRTRLSLPLFTGGKVEGEHGSAIHIVLID TTTGHVVTCGPASCVKLDVIVLEGDFNNEDDDNWSEEYFDSHVVKEREGKRPLLTG DLQVTLKEGVGTLGELTFTDNSSWIRSRKFRMGLKVSPGCYEGMRIREAKTEAFTVK DHRGELYKKHYPPALNDEVWRLEKIGKDGSFHKRLNKAGIYTVEDVVQLVVRDPQR LRNILGSGMSNKMWDVLVEHAKTCVLSGKLYVYYPDDARNVGVVFNNIYELSGLITN DQYYSADSLSDGQKVYVDTLVKKAYENWMHVIEYDGESLLNYNQNKTLGTSQPLAP VGSHDYSISNSLDQQTSIPSLPVPLTTGQPSMNPAVTVGGYHNVTTTRCSMQPQND NLHSSIQFDNTAFPLQNQLMSASHHSQFPRNENGLTLGTRQPATPGFQNVSISNPN YRGLEDYFPEDEIRTRSHEMLENEDMQHLLRIFNMGGGQSHAPFNTQEDAYPYSSA YMPAASMSSNLDDEQNRSSGKAVVGWLKLKAALRWGIFIRKRAAERRAQLVELDD
GmCBP60E	VEKRGYELVEEGDDAQHHLTQSKKPKLPGLASVIVEALKVDSMQRLCSSLEPLLRKI VSEEVERALAKLGHAKLTERSPPPRLEGPAAKNLQLQFRTRMPPHLFTGGKVEGEQ GSAIHVMLMDPNTGSVVQVGPESVAKLNVVVLEGDFNEEVDDDWTKEHFESHEVK EREGKRPLLTGDLQVSLKEGVGTFGDLTFTDNSSWIRSRKFRLGVKVAPGYCEEIRI REGKTEAFAVKDHRGELYKKHYPPALHDEVWRLDRIAKDGALHKKLIQAKIVTVEDFL RLLVREPQKLRSILGSGMSNRMWENTVEHAKTCVLGGKLFVYYTDETNSAGIVFNNI YELRGLISDGQFFSLESLAPNQKMSVDSLVKKAYENWDQVVEYDGKVLNSLANSKK GSRAVATQIMHHNSFQEQQYASAKNKVSYVSSEPNQHLQITNNYSSGPGLTDYPFG RSDNQMVGTSLTDSQIALPGSMNYMSGENHEIGSTYFSGDWSRPRNGQGLDDIVA EELRLRSSEMLESDDMQRLLKTINAEVNRSANLGHSNEGCYTYSLQYEPQMYQTFN EDQGKSSGKAVVGWLKLKAALRWGIFIRKKAAERRALLTELNX
GmSARD1	SAKRFFNDSDQDPENPGGKRMRNTRPSFASVIGEVVMVKNLENLFSAMEPLLKRVV GEEVDQAMRQWSRSFARSPSLRLQAMDQQQPSTLQLCFSKRLSLPIFTGSRILDVD GNPINIVLMDKSNGQGVPTSLSNAIKLEIVVVDGDFPLNDNDEDWTSEEFNRHIVKER NGKRPLLAGELNVIMRDGIAPTGDIEFTDNSSWIRCRKFRVAVRVVPGTNPGGVRIR EAMTEAFVVKDHRGELYKKHHPPMLHDEVWRLEKIGKDGAFHRKLSSEGIKTVQDF LKLAVIDALKLRNILGMGMSDKMWEVTIKHAMTCDIGSKMYIYRGPEFTIFLDPVCKLI RADVNGHTFSNRDPMSHLNKAYIDKLVKEAYARWSNLEEIDEVLNDNIALLTQGDQT VEQFANNQPAAASVETTYDQNQYYSDKSGSYVANNNTQMGCCEWSLNQAYSPAP FANGFPFSFSVRQSDGDITASGSSSVDVDGATRHNX

LaCBP60A	SNKRRPEEGKPKYDFADDKRRKAPSFSSVVREVMKLQSVRNLLEPILEPLLRRVVRE EVELALRKHLTSMKQTSGKEMHIIESRSLQLQFENSISLPVFTGARIEGEDGSNLRIGL VDAITGKVVRSGPESVAKVEIIVLEGDFEDESDNWMPGEFKSNIIREREGKKALLTGD VILYLKDGTGMVGEISFTDNSSWTRSRRFRLGARVVGNFNGIIIREAKTGSFVVRDHR GELYKKHHPPSLSDEVWRLEKIGKDGAFHKRLSREKIRTVEDFLTLLNRDPAKLRSIL GTGMSTKMWEVTVEHARTCVLESTRHLYFPPHSQQKTGIIFNAVGQLTGLLSECEH VPVDKLSETEKAEAQSSVISAFKQGENVSTFEDEVSLMDVSSNLTNALYSPRSLKTE VSSGNKLFVPQKAGVGFNYAQASASSPDIMSSIYSIGGISSFDDYVLPNFDSMGLRY DQTLSFPDQLTNSLACETDSTAPAFTDEDHMQFFDNDIHFQCHIQADLQSAIDGFML GRTSNATGKAQKRWRKIFNVLKWFMVWKGVKCR
LaCBP60B	MGKRSLEGGGEDDQPERKRPTLANVIVEALKVDSLQKLCSSLEPILRRVVSEEVERA LAKLGPARLSGRSSPKMIEGLDGRNLRLRFRSRLSLPLFTGGKVEGEQGAPVHVVLV DADTENVVITGPEACVKLDVVVLEGDFNNEDDEDWTQEEFESHVVKEREGKRPLLT GELQVTLKEGIGTLGELTFTDNSSWIRSRKFRLGLKVASGFSESIRIREAKTEAFTVKD HRGELYKKHYPPALTDEVWRLEKIGKDGSFHKKLNSAGIFTVEQFLRLVVKDSQKLR NILGSGMSNKMWEALLDHAKTCVLTGKLYVYYPEDTRNVGVIFNNIYELCGLITGEQF FSADSLTDSQKVYVDSLVKKAYENWDQVIDYDGKSLVSVPENNVANSEIQIESIDYTI DLDHQLHLPILPASVPSEHQMNSGMSVGGYNDHMATRCPGQSLIEHSNLRNHFDSS LYLSNDQLINSAHQTQTTRNGRGAVGLALGPPQSSTSGFLAGSPSIQPSTINPFDDW SHNRDKGVDEFFSEDEIRLRSHEMLENEDMQHLLRLFSMGGNSSMNAGDGYSIPSF MPTPMPNFDEDRSRSGRAVVGWLKIKAAMRWGFFIRKIAAEKRAQIVELDE
LaCBP60F	VEKRGYDLVEKGDDDEDAQHHVTQSKKPKLPGLASVIVEALKVDSLQRLCSSLEPLL RKIVSEEVERALAKLGPAQLAERSPPRIEGPGAKNLQLHFRTRMPPHLFTGGKVEGE QGAAIHVVLQDPNTGNVVQVGPQSVAKLNVVVLEGDFNEEADDDWTKEHFESHEV KEREGKRPLLTGDLQVSLKEGVGTVSDLTFTDNSSWIRSRKFRLGVKVAPGSCEGIR VREGKTEAFAVKDHRGELYKKHYPPALHDEVWRLDRIAKDGALHKKLIQAKISTVED FLRLLVRDPQKLRSILGSGMSNRMWENTVEHAKTCVLGGKLFVYYTDESGSTGIVFN NIYELRGLIADGQFFSLDSLTLDQKMSVDSLVKKAYDNWNQVIEYDGKVFNSKKGSR SLVTPMLLHNNVQEQHYTYPKNRLPYVPSEPNQHLQITNNYSSGPELSDYPFGRSD NQMVGTSSNNSQIALSGSMNYIPDEHQEVGGTYFQGEWSKQRSGQGLEDIVAEEL RLRSSEMLGSDDMQRLLKTINYGHSNEACYTYSLQYEPQMYDSFSEDHGKSSGKA VVGWLKLKAALRWGIFIRKKAAERRAQLTELN
LaCBP60G	QSKSSFCMPRSFQQKGGEGDFQVLAQITKRRCTENSNLEELCSVIQNVRSLLQMKP KIEQHLTHLVRRIVQEELESRILLLRPIINQTETSFKLVFKNEVPPIFFTQSKINAKDDKPI EIALYDTISKSIVTEGPLSSIRIEICVLNGEFGSNGSEDWNSDEFNAKILSQRKGKEHLL KGDRVITLKNGVGIIKNISFTDISRWIRSGRFRLGAKVFQRTLNEANIKEGRSKPFMVR HYRNEARMKNQCSSLNDEVWCLKMIKRNGKIHQQLCSNEINTVKDLLQLYTTNQVSL QKIIGKNSWDSIIKQAKACHIDNDKWYIYHSSAAEQSISLVFNCIYEVVEVSFNGQNPC SIESLNLKDKFLVERVKQQAYTNVKDNLIPLETTTDGLLENFASVQPVQHSAMGQALE QGQLEMLPNLCEQGTSTSHVGEADAHNYDHQGTEQLLEFGEMSFNWEEMIASCLS PLNGDGL
MpHYPO	REKRPLDKDTSSDGMPEEKRQRVPALASVIVEAVKMDSLQKLCSTLEPLLRRVVGEE VERVLAKLTPAKVGFRSSPKRIQGSDSRSLRLQFRNKLALPLFTGSKVEGEQGSAIH VVLQDASTGQVVAVGAESSAKLEVVVLEGDFSADDEEDWPQEDFENHEVRERDGK RPLLTGELFVTLKDGVGTLGELTFTDNSSWIRSRKFRLGVKISSGYCEGLRIREAKTE PFTVKDHRGELYKKHYPPALHDEVWRLDKIGKDGAFHKRLNQSGIQTVEDFLRLVV

	MDPQKLRNILGNGMSNKMWEGTVEHAKTCVLSGKLHVYYADDKQNIGVIFNNISQL MGLIADGQYMSVDSLSDSEKVYVDKLVKVAYENWENVVEYDGEALIGVKPYKLRGID AHTEDPIVGQGSSGHQSLTSASNQLAVSSTQIMTSQRPQSGGQTYTTPEQKVHRKY LNQVNSMAEHHNSQGFLNSGQYPSQPSSISLTHQTYLHGVNPPVTGLALGLPHASIT TPPGPSIAVLNGTTMLPGTVNSPNDWPRYKELRGQDGLSRTMQMDELLSEDELRVK SMELLENEDMHLQIQQLLRMFNAASSEVPSNPYSGGQGEDNFSFGAFTPPPDLNVG MDRARVHGRANVGWLKLKAALRWGIFIRKRAAARRAQLEEVED
MtCBP60A	SLKRGPDDNKTPDDKRRKPPPFSSVVREVMKLQSVRNLMEPILEPLVRRVVREEVE LALKKHLSSIKQTCGKEMNTSESRTLQLQFENSISLPVFTGARIEGEDGSNLRIRLVDA LTGKVVCTGPESSAKVEIVVLEGDFEEESDIWMPEDFKNNIVRERDGKKPLLTGDVIL YLKDGLCMVGEISYTDNSSWTRSRRFRLGVRVVDNFDGIRIREAKTDSFIVRDHRGE LYKKHHPPSLSDEVWRLEKIGKDGAFHRRLSREKIRTVKDFLTLLNLDPAKLRTILGT GMSAKMWEVTVEHARTCVLDTTRHVSFASHSQQPHVVFNAVGEVTGLLAESEYVA VDKLSETEKADAQISVISALNQCDFASFEDEVSLMDGYSHLTNVHYSPSSPRTEGSS ANKLLALQKTGGFNYTQESASSTDIMPSIYSVGGTSSLDDYGLPNFESLGLRYDQHL GFPVQVSNSLICDMDSIVHAFGDEDHLQFFDADLQSQCHIEADLHSAVDSFMPVSST SMTKGKAQRRWRKVVNVLKWFMVKKRRNQLYRX
MtCBP60B	MGKRALEGGGDDDQPERKRPALASVIVEALKVDSLQKLCSSLEPILRRVVSEEVERA LAKLGPARISGRSSPKRIEGPDGRNLRLQFRSRLALPLFTGGKVEGEQGAPIHVVLV DANSGNVVTSGPESCIKLDVVVLEGDFNNEDDEDWSQEEFESHVVKERQGKRPLLN GELQVTLKEGVGTLGELIFTDNSSWIRSRKFRLGMKVASGFGESIRIREAKTVAFTVK DHRGELYKKHYPPALGDDVWRLEKIGKDGSFHKKLNNAGIFTVEDFLRLVVKDQQKL RNILGSGMSNKMWEALLDHAKTCVLSGKLYVYYPEDTRNVGVIFNHVYELRGLITGE QFFSADSLSDNQKVYVDSLVKKAYDNWEQVVEYDGKSLVDAEQNNNTVESENELH VESIDYDGGLDHQLLMPSLPMSVASEQQINSAMPVGGFNNSMVTRYPSQALIGNSS SRSHFDDSLYLSNDHLLGNAHQSQSSRNDHSTVGLALGPPQSSTSGFHAGSSSMQ PPAPNPFDDWSNNRDKGVDDFFSEDEIRVRSNEILENEDMQHLLRLFSMGGHPSM NTEDGYSFPSFMPSPMPNFDEDRSRPGKAVVGWLKIKAAMRWGFFIRKIAAEKRAQ IEELDE
MtCBP60C	REKRTLDSTSTDEDQPDRKRPALASVIVEALKVDSLQKLCSSLEPILRRVVSEEVERA LAKLAPTNLSGRSSPKRIENPDGGNLQLKFRTRLSLPLFTGGKVEGEQGTAIHIVLIDA NTGHVVTSGPASCVRLDVIVLEGDFNNEDDDTWSQEEFDSHIVKEREGKRPLLTGD LQVTLKDGVGTLADLTFTDNSSWIRSRKFRLGLKVSSGCCEGMRIREAKTEAFTVKD HRGELYKKHYPPALTDEVWRLEKIGKDGSFHKRLNKAGICSVEDVLQLVVRDPQRLR NILGSGMSNKMWEVLVEHAKTCVLSGKLYVYYPDDARNVGVVFNNIYELSGLITNDQ YYSADSLSDSQKVHVDTLVKKAYENWMHVIEYDGKSLLNYNQNRTLGMSQHQVPV SSHDYSISNSLDQQISTPSLPVHVPTGQHSMDPGATVGGYHHGTATRFSMQPQNAD VNSALQFGNTAFPLQNLLTSVSHQSQLPRNENELSLGPPQSATPGFQTVGLSDPTY RGFEDFFPEDDIRIRSHEMLENEDMQHLLRIFNMGGQPHTSFNAPEDEYPYSSAYM PATSTNYNVDDERNRSSGKAVVGWLKLKAALRWGIFIRKKAAERRAQLVELDD
MtCBP60D	AEKRSLDSTSAEDGQPDRKRPALASVIVEALKVDSMQKLCSSLEPILRRVVSEEVER ALAKLGPTKLNGRSSPKRIEGPDDNNLQLHFKTRLSLPLFTGGKVEGEQGAAIHIVLV DANTGHIVTSGPESCAKLDVFVLEGDFNNEDDENWSEEEFESHIVKEREGKRPLLSG DLQVILKDGVGTLGEISFTDNSSWIRSRKFRLGLKVSSGFCDGMRIREAKSEAFTVKD HRGELYKKHYPPALHDEVWRLEKIGKDGSFHKRLNKAGVFNVEDFLRLVIRDPQRLR NILGSGMSNKMWDILVEHAKTCVLSGKLYVYYPEDARNVGVVFNHIYELSGLIANDQ

	YHTADSLSESQKVYVDTLVKKAYDNWMHVIEYDGKSLLNYNQDKNLEPAHPQALMG SHEYSNLIQQTSIHSLPHPVNTGQPSMDTGATVGGYHDGTTTSFSMQSQNTNLNSSI QFDDNAFSLQSQLMSVPHQAQLQRSENGMMLGMPQTVTHGFQTASISNSTYRVED FFPEEEIRIRSHEMLENEDMQQLLRMFNMGSHAHASFNAHEDGYPNPSAYMPANN MSYNFDDEPKSSSGKAVVGWLKLKAALRWGIFVRKKAADRRAQLIELDD
MtCBP60F	VEKRSYDDEDAQNHLTQSKKPKLPGLASVIVEALKVDSLQRLCSSLEPLLRKIVSEEV ERALAKLDHAKLGDRSSPARIEAPGEKNLQLHFRTRMPPHLFTGGKVEGEQGAVIHV VLLDPNTGNVVQVGPESVAKLNVVVLEGDFNEEIDDDWTKEHFESHEVKEREGKRP LLTGDLQVSLKEGVGTLGDLSFTDNSSWIRSRKFRLGVKVAPGYCDGIRVREGKTEA FAVKDHRGELYKKHYPPALHDEVWRLDRIAKDGALHKKLIQSRIVTVEDFLRLLVREP QKLRSVLGSGMSNRMWENTVEHAKTCVLGTKLFVYYTDKTHSTGIMFNNIYELRGLI ADGQFFSLESLTSNQKMSVDSLVKKAYDNWDQVIEYDGEVLNSLTNSKRGSKSVAA HTMHQNNFQEQQYASAKGRASYVSSDQNQHLQITNNYSSSSDYQMVGTSQIALPG TMNYNMSGDNNPEIGGNYYHGDWSRQRNGQGLEDIVAEELRLRSSQMLEGDDMQ RLLKTINEGANFGHSNENCYTYRLQYEPQMYHSFGEDNVKCSGKAVVGWLKLKAAL RWGIFIRKKAAERRAQLTELNX
MtCBP60G	ASKRTSHTSDSCDDNVTSTSTKRIKQHHIHGNLEEEQQKEIQHLLFPTISASALALRLE PLLRKLIQEAIHLHLNPSCGLTLMSNTFGRRRLQFCFMNKLPNRIFTKSTIKAEGDEPL QIELRDVENQQRVVMEEGSSMKIQLCVLYGDFEKEDWTAEEFNTQIAPPREGKEELL KGNEFITLRNGVADIDKEIEFTDISKGRNGQFRLGVKIVQSNSIGVCIREGRSEPFKVL DVRGKNYEKHDRPSLNDEVWRLKGIRKNGPLDKLLASDGIHTVKDFLRLYITNEASL REKIGKIARNSWNTIVAHAKDCDVDNDDERYIYYSTEQPISFLVFNAIYKVVEVTFHNE QNARSIQSLNQQEKRLVERVKQHAYKYFNDWNPLPIDTTTLGLEETLTGVQNAQYD GQDQALQQSDFLVCQQGQKEIGQSYVQPCISTSYVNEGMDNYQIYVDPMPDIREIP QNNHVEGEMYIDGDGYGSHFPVVEGRYSMENLMNDYPIYTTCEPENYNLYGFSDV AECSTHVNFLDSSMDISSSDKSKAVWCKVGIAIKWVISIRRVAAAAKRNANLFYFN
MtSARD1	SDRKSLKNHKSTSHGVTKQGPISGLRYVINTLRTNCHDSILLESFIRGVVRDVVESKF QERLLSSEKANEAGKSGARPLELCFINNNNNKLSGPFFSQSNIIAKDEPPLQVALFDV GSKSIVNVGPFSSTKIEICALDGGFGSEDWTEIEFKANILRERDGKQPLLVGERFITLK NGVASISKTIFTDNSRWLRSKMFRLGVKAMQNGDIIKEGRSQPFRVKDNRGQPNEK HFPPFLNDFVWRLEKIAKDGRFHKRLSSNGIHTVKDLLQLLIINESSLHGIFEKIQRKS WLAIIEHAKSCVLDDHKLYSYGTIGQPILLFNAIYKLVGVTFDVQKFYLPETLTPNLKHS VEIVKQDAYKDVCNLKPVDETFLNSISLGACIQSAGQFGAPVQGQTDIGQGYVQPCM STSYVNEGMHDYQINPEPVPDIRDIPQNNHVGAEMYIEGDSHGSRFPVTQGGHSIEN LTNFPPLDTTWELDFLDVEFLNSAMEILSSGKSKAVWCKIRAVIKWGISVRKVAAARR IYX
OsCBP60A	LERRGSEKRLRVTVPVAAAAAVAAVGVGTAALASPATRMLRKIVLVLLFLLRMSERVT VVESISQIGRMVQRLHNAQGVIIKKLENIQENMLERMENMQERMEDISHEVKQLKHL HSNRHADQHPGLEPNTNVQLRFLDNLKTPVYTEKNITAESNEAIRIGIFEGDNMITDG PLSKVKVEIVVLRGDFSNDGRVSWTEEQFNNHIVQGRNGQGFVLGGDCGVWLKKG ENRLGKIRFKEGSSRTRSRMFILGARVCKSENTGVRVQEAVMKPVTVLDRRNEANE KRHPPMLDDEVFRLEEICKDGTYHKRLQKAKIFTVHDFLKALNTNAKKLREEVLQMK KKTNSWDKMVGHARECCLRDQHELKAYQSEEENATLFFNGVHQIVGAKFGGDYVIY ENFDPAQKTKVNKLKDRAHAKLDDIPSDFVMKNNIPEPISPTSAAAAGPSNRSDHQM PNQGTIGAENLCNGVAFYSNAICDCSTSNPNDVSTHDYPDQAPTPFPDWQQDLQRL

	MSSSDTIDWPSFERIVLGGTSEESSSAQHQVHQLHESMPPATSPWVAAPQSRAQH GEEPSRLPFPG
OsCBP60C	MKEKRGLEAAAAGGDGHPEAKRARPPALASVIVEALKVDSLQRLCSSLEPILRRVVS EEVERALGRLGPATITGRSSPKRIEGPDGRNLQLQFRTRLSLPLFTGGKVEGEQGAA IHVVLLDAGTGCVVSSGPESCAKLDIVVLEGDFNNEDEEGWSGEEFESHVVKEREG KRPLLTGDVQVTLKEGVGTVGELTFTDNSSWIRSRKFRLGLKISSGFCEGIRIREAKT EAFMVKDHRGELYKKHYPPALKDEVWRLEKIGKDGSFHKRLNKAGISTVEDFLRLVV RDPQKLRSILGSGMSNKMWDILVEHAKTCVLSGKYYIYYSDENRSIGAIFNNIYAFCG LISGEQFYSSESLDDSQKLFADALVKKAYDNWMYAIEYDGKALLNSKPKKKAAPTGH VETHPPLSQPASYEQRISSASMTGPSPAVSFISGGSGTGTDSIGYDGNQAATQPSQL QSTSANVPVPYDDTFSFLPPSMLMGSDNQETGNDGMGLELGQLQQAISQSQSIQPA NVGYDDWTRSQNGQFADDFTEDIRMKSHQMLESEDMQQLLRVFSMGGASTSLQE DAFGFPTYMPSPLPNLGFEGERTRSSGKAVVGWLKIKAAMRWGIFVRKKAAERRAQ LVELDD
OsCBP60D	GSKRALDAGGGGGDDDDRAPKRPRVPALASVIVEALKVDSLQKLCSSLEPILRRVVS EEVERALAKLGPAATPARIQGRSSPKRIEGPSGINLQLQFRSRLSLPLFTGGKVEGEQ GAAIHVVLLDANTGRVVTSGPESFAKLDVLVLEGDFNKEQDEDWTEEEFENHIVKER EGKRPLLTGDLQVTLKEGVGTIGELIFTDNSSWIRSRKFRLGLRVSSGFCEGVRVKE AKTEAFTVKDHRGELYKKHYPPALKDDVWRLEKIGKDGAFHKKLNSNGIYTVEHFLQ LLVRDQQKLRTILGSNMSNKMWESLVEHAKTCVLSGKHYIYYSSDARSVGAIFNNIYE FTGLIADDQYISAENLSENQRLFADTLVKQAYDDWINVVEYDGKELLRFKQKKKSVTT RSDTAKASTSYPSSYGSTHSHKQLTGGPVNIEQSSMSSMSEDGTRNMSNGSQAAR YAANPQDISQSITMPYDMSSLRPEEQFAGSSIQTQASRSSNMLALGPTQQQNFEFS ALGQSMQPSPLNPFDDWSRLQENRGGVDDYLMEEIRVRSHEILENEEDMQQMLRIL SMGGSSANMNHGDGFSPFMPSPAPAFNYEDDRARPSGKAVVGWLKIKAAMRWGIF VRKKAAERRAQLVELED
OsCBP60E	QDREEKDGKGDEGSRGSPPAKRPRSSCGFDDKQTYMLQEILRMTQQQNERMDYL FREIQELVEKVSSLTAVRSGFGGYHQQIAAPSRMLSDDSDQNCTVPLRLQFVNSCS NDKYSTHKIEADDENPLQVAIYDHNDKIVTMEPFSSMRVHIVAIDGDFDDDNKGQWT KEYFHSKIVPGRPHKGHLLSGKLYFRLQNGVGYLNSAKFQDNSSFVPSRKFKLGVM AADERISVRIQEGVTESFAVKDVRGYLTKKNPNPSPRDAVYKLSKIAKNGDRHKLLE QNGIKTVEDFLSFYNNSEYDLRKILGKISDQDWDLIIAHAQKCRPGVYSSCLKESSVS HEHEALSIRNDSYCLQESFSMQPSHTLQEQLDVQGMHPQISSTYDGSVGLSDIGVP YRFQPDTLDKNLMHHGQLEGIQVVIDPHVPSVESVDMPVSSMDNSTLEVFSSQQQH SFKCNNTPELGNGLSQVNSFDWNLDCLDGDVDVNALWGSKEDFPSENMGQGGHV YTETPGPGGSYSAAEQNLGHLSVSEAGSISYNEISAVNEAGSWSHRSLSTPPPVRG AASMRNRGISFSSPRGTGGRRGTGRWRRAEGSGDYGTDNISNTSTYFTGEEQP
OsSARD1	AAKRLHDGYGQEDQPDDKRVRRMPSFSTVIREALMVKQMQTLFVALEPLLRRVVQE ELQAGLVRSPRYIERMSPETPPAQPPMWKLAFRFKPQLPIFTGSKIEDVNGNPLEIILV DVDTGAPATISQPLRVEVVPVLGDFPPDDREHWTAEEFQQRGIVKERSGKRPLLTG DVSLTMRDGCVVVNELQFTDNSSWVRCRRFRIGVRVVPGSYDGPRIGEAMTEPFVV RDHRGELYRKHYPPVLGDDVWRLEKIGKEGAFHRKLTQHNVRNVQEFLRLLTVKPD ELRAIMGDGMTDRMWEVTTSHAKKCVPGDKVYMYSTQHGTVYVNSIFELVKVELAG

	VEYQLHQLNRGQKAFVHQLLLAAYEQRNNLQEADAMALHCNDVPLLQNAAEITIPAL GDTQLWIQNSLNSQEIDFQVDEIPQANFALQWTGQMYNISGX
ZmCBP60B	GSKRGLDPTGGGGGGDNDDHAPKRPRIPALASVIVEALKMDSLQKLCSSLEPILRRV VSEEVERALAKLGPARIQGRLLIYKTNIHENRSSPKRIEGPDGRNLQLQFRSQLALPIF TGGKVEGEQGAAIHVVLLDANTGCVVTSGPESFAKLDILVLEGDFNKDEDEDWTEEE FESNIVKEREGKRPLLTGDLQVTLKEGVGTIGELTFTDNSSWIRSRKFILGLRIAPGFY EGIRVREAKTEAFPVKDHRGEFASMLILYLLVYKKHYPPTLKDDVWRLEKIGKDGAFH KKLNASGIYTVEDFLQLLVRDQQRLRSILGSGMSNKMWDSLVDHAKTCVLSGKHYV YYARDSRNVGAIFNNIYEFTGLIADDQFISAENLTDNQKGYADALVKKAYEDWMQVV EYDGKALLSFKQKKKSVMTRSDAAVASTNHPASNGSAISQKQLSLPAKAGQTSSAGI MNEDGTRNAYNANGNQSARYAATTQNIPANVAMQYDRSAVSPESQFSGSSLQSQA SRGSNTLALGPPQQHQSFAFPALGQSMRPTGLNPFEEWPQQQENCGGVDDYLME EIRMRSHEILENEEMQQMLRLLSTGGAGTNLTEDGFSFPSYMPAPSPKLSYEDDRT RAPGKAVVGWLKIKAAMRWGIFVRKKAAERRAQLVELDD
ZmCBP60C	MLILYLLVYKKHYPPTLKDDVWRLEKIGKDGAFHKKLNASGIYTVEDFLQLLVRDQQR LRSILGSGMSNKMWDSLVDHAKTCVLSGKHYVYYARDSRNVGAIFNNIYEFTGLIAD DQFISAENLTDNQKGYADALVKKAYEDWMQVVEYDGKALLSFKQKKKSVMTRSDAA VASTNHPASNGSAISQKQLSLPAKAGQTSSAGIMNEDGTRNAYNANGNQSARYAAT TQNIPANVAMQYDRSAVSPESQFSGSSLQSQASRGSNTLALGPPQQHQSFAFPALG QSMRPTGLNPFEEWPQQQENCGGVDDYLMEEIRMRSHEILENEEMQQMLRLLSTG GAGTNLTEDGFSFPSYMPAPSPKLSYEDDRTRAPGKAVVGWLKIKAAMRWGIFVRK KAAERRAQLVELDD
ZmCBP60D	DLKRALDVEEEVVDGDEEELAGCCPDAKRRRTSVNSSMQEAIGAQYMQRHLPKLEP FLRRVVQEEVHNVLVRHIDSAHRLPLQLKTSSKRYRLQFQGNLPQTLFTGNRVEAEN RHPLRLVLTDAATSQTVSSGPLSSAKVELLVLDGDFNADERLEHTEREFGESVVFER EGKRPLLSGEVVVVLDRGTASVRDISFTDNSSWIRSRRFRLGARMSRASSIEERVQE AVSNPFLVKDHRGEVYKKHHPPALADDVWRLEKIGKDGVFHKKLADFGIHTVQDFLR NLVMDQYGLRSLLGSGMSNKMWESTVEHARECVLDDRLYSYCSGHGIVLLFNCVY EVVGVVVGSHCFTLAALTPTQKALVAKLQQDAYKFPDRIAEFKVQSQSSTEQQAPAA VIQAPPAPAPVPATQMLGLPHGVVQPCAAGSHDSLLLSPQLLQHQQQPLSEAQLED VLQSASAAEPWFPPFGAAGGFDARDPFDVQFSGSQPCGLLLSSTGARLX
ZmCBP60G	DLKRTLDVEEEVVDGDEDELACCPDAKRRRTFVDSSMQEAIGAQYMQRHLPKLEPF LRRVVQEEVHNVLIRHIDSAHRLPLQLKTSSKRYKLQFQGNLPQTLFTGNRVEAESK QPLRLVLTDATTGQTVASGPMSSMKVELLVLDGDFNADERLVEHSEKEFSESVVFE REGKRPLLSGEVVVVLEKGAASIRDISFTDNSSWIRSRKFRLGARMSRASSIEERVLE AVSNPFLVKDHRGEVYKKHHPPALADDVWRLEKIGKDGVFHKKLADFGIHTVQDFLR NLVMDQYGLRSLLGSGMSNKMWESTVEHARECVLDDRLYSYCSGHGTVLLFNCVY EVVGVLVGGHCFTLSALTPTQKALVGQLQQDAYKFPDRIAEFKVQSQSAGVEQPPA AVESPPSVPGAQVLGLPHGVVQVQPSAGAPSAHDLLLSPRLLQHQQQPLSEAALED VLQSASAAHQLDAAEPWFPSFGAGGFDARDPFDVQFGGGSQPCGLLLSSTGARL
ZmSARD1	AAKRLYNGYEQDGDQPNDKRMRRLPSFSTVIREAMMQKHMQSLFRCLEPLLRRVV KEELHAGLTLMQSPRYIERLPAERAAWRLAFRTPPQLPIFTGSKIEDEAGNPLEVILVD ADTGSPAALPQALRVELVPVYGDFPQDGREDWSDDEFQRNVVKERAGKRPLLTGD VSLALRDGRATVGELQFTDNSSWVRCRKFRIAARVVPGAWDGARVQEAMTEAFIVR DHRGELYRKHYPPVLADDVWRLEKIGKEGAFHRKLRRSNVGTVQEFVRMLMVKPD

	ELRAILGDGMTDRMWEATTNHARTCATDDKVYAHSTPHGTIYVDSVFNVVRVDIGG VEWPLHQLNRGQTMVVQQMLQDAYEHRHSLQEAEPFMAHGHAASDAPLLQNAVP ALVDTPPLWFPNAAEMDFPVDDVVPVPQANSSFGYQWTGQAFHMPGX

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