Plant hormones in arbuscular mycorrhizal symbioses: an emerging role for gibberellins

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RESEARCH IN CONTEXT

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INTRODUCTION

Arbuscular mycorrhizal (AM) symbioses are common amongst all groups of land plants, with >80% of species forming these associations (Strack et al., 2003). They are of ancient origin, and it is suggested that they may have evolved at the time plants first invaded terrestrial environments (Remy et al., 1994; Barker and Tagu, 2000). AM symbioses are thought to contribute to the acquisition of nutrients, especially phosphorus, in nutrient-poor environments. In return, the fungus receives carbohydrates that are essential for its survival. Whilst the nature of the signals exuded by the plant and the fungus into the rhizosphere has received considerable attention over the last decade and the genetic pathways regulating the symbiosis have been partially determined (Akiyama et al., 2005; Gough and Cullimore, 2011; Maillet et al., 2011), there is still much to learn about the regulation of mycorrhizal development. One area where this applies concerns the role of the known plant hormones. Whilst the effect of fungal colonization on the level of various plant hormones has been examined in some detail and will not be covered in full here (e.g. López-Ráez et al., 2010; Martínez-Medina et al., 2010), the impact of the initial hormonal status of the plant may also be informative, with potential roles for abscisic acid (ABA), ethylene, jasmonic acid (JA), salicylic acid (SA), strigolactones and auxin. A brief overview of our current understanding is given below. Less well understood is the role of the plant hormones, gibberellins (GAs) and brassinosteroids, in mycorrhizal development, and novel information on the roles of GAs and brassinosteroids from pea mutant studies is provided.

Abscisic acid

Strong evidence has emerged for a direct role for ABA in mycorrhizal development. The use of the ABA-deficient mutant sitiens in tomato by Herrera-Medina et al. (2007) allowed the effect of endogenous changes in ABA levels on colonization by Glomus intraradices to be examined for the first time. Their results showed a reduction in the frequency and intensity of colonization in the mutant roots. They also showed that arbuscular morphology was less well developed in the mutants, and this was reflected in reduced fungal alkaline phosphatase activity. Exogenous application of ABA to sitiens plants restored the frequency and intensity of colonization to wild-type levels. These results support the view that ABA is essential for full AM colonization of the roots and for the functionality of the arbuscules. However, part of this effect of ABA deficiency on fungal infection could be due to increased ethylene production in these mutants (Herrera-Medina et al., 2007). Subsequent work using sitiens and another ABA-deficient mutant, notabilis, transgenic plants and ethylene and ABA biosynthesis inhibitors supported the hypothesis that ABA deficiency enhances ethylene levels, which negatively regulates mycorrhizal intensity/hyphal colonization (Martín-Rodríguez et al., 2010, 2011).
However, ABA deficiency appears also to downregulate arbuscular formation directly (Martín-Rodríguez et al., 2011), possibly due to the upregulation of genes related to defence and cell wall modification (García-Garrido et al., 2010).

Ethylene

Zsögön et al. (2008) used the ethylene-overproducing mutant epinastic and the ethylene-insensitive mutant Never ripe in tomato to build on early results suggesting that ethylene is inhibitory to AM colonization (Azcon-Aguilera et al., 1981; Ishii et al., 1996; Geil et al., 2001). However, in both mutants, AM formation was inhibited, creating a paradox. Torres de Los Santos et al. (2011) confirmed that epinastic plants had reduced intensity of mycorrhizal root colonization, and a similar correlation has been found in pea application studies (Morales Vela et al., 2007). Torres de Los Santos et al. (2011) also examined the ripening inhibitor (rin) mutant and showed that it possessed enhanced mycorrhizal colonization, the reverse of the effect of Never ripe. This suggests a role for the RIN pathway in mycorrhizal colonization. RIN is a MADS-box transcription factor that blocks ripening (including climacteric ethylene production; Vrebalov et al., 2002). Therefore, the effects of rin are consistent with an inhibitory effect of ethylene on mycorrhizal colonization. At this stage, it is not clear why the Never ripe mutation reduces AM formation.

Strigolactones

The role of strigolactones as rhizosphere signals exuded by the plant to attract AM fungi is well established, through the use of strigolactone-deficient mutants and exogenous strigolactone applications to the rhizosphere in pea and rice (Akiyama et al., 2005; Gomez-Roldan et al., 2008; Umehara et al., 2008). Strigolactones stimulate branching and growth of the hyphae from germinating spores, leading to greater contact with plant roots (Akiyama et al., 2005; Besserer et al., 2006, 2008). However, whether strigolactones also affect early steps of colonization inside the root, either directly or indirectly, is yet to be fully explored. Two recent independent studies, one using rice (Yoshida et al., 2012) and one using peas (Foo et al., 2013a), have provided the first evidence that this may be the case. AM abundance is strongly reduced in the strigolactone-insensitive mutants, d3 in rice (Yoshida et al., 2012) and rms4 in pea (Foo et al., 2013a). These genes are orthologous and encode an F-box protein that is part of an SCF complex that is thought to target proteins for degradation (Ishikawa et al., 2005; Johnson et al., 2006). The receptor systems for both auxin and jasmonic acid include F-box proteins (e.g. Somers and Fujiwara, 2009), and such proteins have been suggested to play a similar role in the strigolactone receptor system (Beveridge and Kyoizuka, 2010). However, the strigolactone-related F-box protein is also involved with responses to other signals, including karrikins, which are plant signalling molecules in smoke (Waters et al., 2012). Therefore, although it cannot be absolutely stated that the phenotype of the d3 and rms4 mutants must be due to their strigolactone insensitivity, this does seem the most likely explanation given that the low mycorrhizal phenotype is similar to that seen in strigolactone-deficient mutant d17 (ccd7) in rice (Yoshida et al., 2012) and rms1 (ccd8) in pea (Foo et al., 2013a). If this is the case, then strigolactones must be influencing AM development through strigolactone perception within the plant, arguing for an endogenous hormonal role in AM development. Defining this role will require a detailed analysis of gene expression changes during AM colonization and after application of mycorrhizal signalling factors in the absence of the fungal partner.

Auxin

While a possible role for auxin in AM formation has been discussed for many years, especially given the increased root branching noted after fungal infection (Gianinazzi-Pearson et al., 1996), there has been little direct evidence. Increases or no change in auxin levels have been noted after infection in different species (e.g. Jentschel et al., 2007; Campanella et al., 2008), while measurements in the fungal hyphae showed that the fungus could not be the source of any changes observed (Jentschel et al., 2007). Zsögön et al. (2008) were the first to use a genetic approach to examine whether the perceived auxin status of the plant altered root colonization by G. clarum. They demonstrated that the auxin-insensitive diageotropica mutant showed no difference in intraradical fungal colonization compared with the wild type. However, a more recent study by Hanlon and Coenen (2011) showed that hyphae would not colonize cultured diageotropica roots, and in fact changed direction and grew away from those roots. There was, however, only a weak inhibition by the mutation in intact seedlings. Hanlon and Coenen (2011) also examined the auxin hyperactive-polar transport polycotyledon mutant and showed that in culture its roots possessed enhanced colonization by G. intraradices. However, this was not observed in intact polycotyledon plants, where reduced colonization occurred (Hanlon and Coenen, 2011). The cause of this paradox is unclear, although it is speculated that supraoptimal levels of auxin may occur due to inputs from the shoot in the intact mutant plants. The development of fungal structures in the roots, including arbuscules, was normal in both mutants, suggesting that while auxin is required for normal AM infection, it is not required for post-infection development (Hanlon and Coenen, 2011).

Independent support for a role for auxin in AM infection came from work on the auxin-deficient (Symons et al., 2002) bushy mutant of pea. Foo (2013) showed that bsh roots had overall reduced mycorrhizal colonization compared with wild-type plants, but that the arbuscules that did develop in the root appeared normal. This mutant also exuded less strigolactones from the roots, probably due to the observed down-regulation of the key strigolactone synthesis gene PsCCD8. Auxin has been reported previously to regulate this gene (Foo et al., 2005; Johnson et al., 2006). Application of the synthetic strigolactone GR24 could partially reverse the effect of the bsh mutant on AM colonization (Foo, 2013). Overall this suggests that auxin may regulate AM formation, at least partially, by controlling strigolactone levels.
Jasmonic acid

Jasmonic acid has long been implicated in the plant’s systemic response to pathogen attack (see review by Ballare, 2011). This has led to questions regarding its involvement in the regulation of AM development, which also involves fungal hyphae penetrating plant cells. A number of approaches by several groups have been taken, but have not yielded a completely coherent picture. Application of JA (or derivatives such as methyl jasmonate) can have a range of effects on mycorrhizal colonization, from positive to inhibitory, depending on species, dose, timing and nutritional conditions (see review by Gutjahr and Paszkowski, 2009). For example, Regvar et al. (1996) showed a clear promotion of AM colonization by low levels of JA, while Ludwig-Muller et al. (2002) showed a reduction with higher levels, although not in the same species. Kiers et al. (2010) clarified the situation to some extent by showing that higher levels of jasmonates can be supraoptimal.

Landgraf et al. (2012) modified endogenous JA levels by repeated wounding, and showed that the resulting plants increased AM colonization, suggesting a positive role for jasmonates in AM development, a view supported by Isayenkov et al. (2005), who downregulated JA levels using antisense expression of the allene oxide cyclase gene and showed delayed AM colonization. Consistent with this result, Tejeda-Sartorius et al. (2008) showed that in the JA-deficient spr2 mutant in tomato (Li et al., 2003), AM colonization was reduced and could be restored by methyl jasmonate application. On the other hand, Herrera-Medina et al. (2008) showed that the tomato JA-insensitive mutant jai-1 possessed increased colonization. This was supported by reduced colonization of the wild type after treatment with methyl jasmonate.

While these results sometimes appear contradictory, most authors agree jasmonate signalling is important for AM colonization and development, and have developed models to explain some of the apparent contradictions (Gutjahr and Paszkowski, 2009), although even this is not universal (Riedel et al., 2008). Clarification will probably need to wait until there is a combined genetic, physiological and molecular approach across the time course of infection and colonization in a single well-defined system. The events involved in early colonization may be quite different from those at later stages and there may be differing roles for JA at different stages.

Salicylic acid

Salicylic acid acts to co-ordinate the plant’s defence against biotrophic pathogens (see review by Lu, 2009) and therefore might be expected to be activated during AM colonization. This indeed appears to be the case, with a short-lived rise in SA levels during the early stages of AM colonization (Bilou et al., 1999). Herrera-Medina et al. (2003) showed that the SA content of the plant affected the rate of AM colonization. They compared wild-type plants with transgenic NahG plants that possessed reduced levels of SA due to enhanced deactivation of SA and transgenic CSA plants with constitutive SA biosynthesis. In time course experiments, they found that the NahG plants possessed more rapid AM colonization while in CSA plants it was retarded, although the final level of colonization was not altered. However, they were cautious and indicated that a causal relationship between SA levels and colonization still needs to be established.

Cytokinins, gibberellins and brassinosteroids

Barker and Tagu (2000) developed a model based on early research that suggested possible roles for both fungal and plant-produced cytokinins in AM development. This early work reported that cytokinin levels were raised in AM-infected plants (e.g. Allen et al., 1980; Shaul-Keinan et al., 2002), although it was not clear whether the cytokinin was of fungal or plant origin (Barker and Tagu, 2000). However, recent studies using the cytokinin-insensitive Medicago truncutula cre1 mutant (Plet et al., 2011) suggest that cytokinins may not play a major role in regulation of mycorrhizal development (F. Frugier and P. Bonfante, pers. comm.).

While early reports suggested that AM fungi produce GAs, evidence for the direct involvement of this class of hormones has remained circumstantial. However, based on evidence that GAs are involved in nodule formation (Ferguson et al., 2005a, 2011), Ortu et al. (2012) examined the expression of a range of GA-related genes in M. truncutula and reported upregulation of some GA biosynthesis genes after contact with the fungus, similar to a report from tomato (Garcia-Garrido et al., 2010). These genes were sensitive to exudates from the symbiotic fungus (Garcia-Garrido et al., 2010). Furthermore, Shaul-Keinan et al. (2002) have shown elevated levels of the biologically active GA4 and its deactivation product GA8 in roots of AM-colonized plants, compared with controls, by gas chromatography–mass spectrometry (GC-MS). These results were, however, contrary to earlier results using bioassays (e.g. Allen et al., 1982), and the differences were only marginally significant. While these results may be suggestive of a role for GAs during AM colonization, they leave as many questions unanswered as answered. The time is ripe to use the well-defined GA synthesis and GA response mutants in an AM-susceptible species such as pea (e.g. Ross et al., 2011) to explore further the role of plant-derived GAs in AM development.

Even less is known about the involvement of brassinosteroids in AM development since no brassinosteroid-deficient mutants appear to have been examined for AM abundance and BR levels have not been examined in AM-colonized plants compared with non-colonized plants. Well-defined BR synthesis mutants are also available in pea.

In this study the GA synthesis mutant na-1 and the GA-response mutants la and cry-s of pea, were used to explore the influence of GA levels and signalling on AM colonization. The na-1 mutant has a null mutation in the kaurenoic acid oxidase gene that is expressed in vegetative parts of the plant (Ingram and Reid, 1987; Davidson et al., 2004). The oxidation of kaurenoic acid is an early step in the biosynthesis of active GAs. Alleles la and cry-s are thought to be null mutations in the two DELLA genes of pea (Potts et al., 1985; Weston et al., 2008, 2009). DELLA proteins are negative regulators of GA signalling and are degraded in the presence of bioactive GAs (Harberd et al., 2009). In addition, AM colonization in the brassinosteroid-deficient lkb mutant was examined. The lkb mutant results from a leaky mutation in the
gene involved in campesterol production during brassinosteroid biosynthesis (Nomura et al., 1999; Schultz et al., 2001).

MATERIALS AND METHODS

Plant material, growth conditions and fungal inoculum

The wild-type (tall) line used was line 107, derived from Pisum sativum cv. ‘Torsdag’, with genotype NA LA CRY. Genotypes na-1 LA CRY (nana phenotype), NA la cry-s (slender) and na-1 la cry-s (slender), on a line 107 background, were obtained by backcrossing six times to the line 107 parent after an initial cross between line 107 and line 188 (genotype na-1 la cry-s; Weston et al., 2008). The occurrence of slender segregates in the progeny of plants that obviously carried na-1 facilitated the identification of the triple mutant.

Plants were grown under glasshouse conditions and received modified Long Ashton solution (Hewitt, 1966) with 0-05 mm NaH2PO4 weekly as described by Foo et al. (2013a). Before planting, dolerite chips and vermiculite were mixed with G. intraradices fungal spores (approx. 8000 spores per pot; Premier Tech Pty. Ltd, Quebec, Canada) and one-fifth volume of leek or corn G. intraradices pot cultures. This was topped with vermiculite, and seeds were planted. At the time of scoring, roots were rinsed and the whole root system was cut into approx. 1 cm segments, cleared using boiling 5% KOH and stained using the ink and vinegar method (Vierheilig et al., 1998). Mycorrhizal colonization of roots was scored according to McGonigle et al. (1990), where 150 intersects were observed from 25 root segments per plant and scored for the presence of arbuscules, vesicles and/or intraradical hyphae. Root colonization was calculated from the percentage of intersects that contained any internal fungal structures, and arbuscule frequency was calculated from the percentage of intersects that contained arbuscules, both relative to the number of cell files. Due to growth under glasshouse conditions in Tasmania, Australia, where summer and winter average temperatures vary significantly, the absolute colonization rates between experiments varied somewhat depending on the time of year the experiment was carried out. However, replicate experiments always showed the same relative differences between genotypes and/or treatments (data not shown).

GA3 application

Wild-type (line 107) and na-1 mutant plants were planted with fungal inoculum as described above. When plants were 2 and 4 weeks old, 10 µg of GA3 was applied to the uppermost expanded leaf in ethanol, and a similar volume of 100% ethanol was applied to control plants. Eight weeks after planting, shoot length was scored, shoot and root fresh weight were measured and roots were harvested for mycorrhizal scoring and/or gene expression studies. The roots of GA3-treated na-1 plants were divided into roots that formed before GA3 treatment and displayed the typical thick na-1 root phenotype (typically the primary root and the 2–3 cm of secondary root closest to attachment to the primary root) and roots that formed after GA3 treatment that were much thinner and similar in the number of cell files to wild-type roots (typically the remainder of the secondary root including the root tip; see Fig. 2D).

Gene expression

The expression of three genetic markers for mycorrhizal development in pea, PsPT4, PsGST and PsTi, previously described in Grunwald et al. (2004) and Kuznetsova et al. (2010), were monitored in roots grown with fungal inoculum for 6–8 weeks. Two whole secondary roots were harvested per plant, and roots from 3–4 plants were pooled into a single replicate and frozen in liquid nitrogen. The remaining root tissue was harvested and scored for mycorrhizal colonization as stated above. Between two and four biological replicates were harvested. RNA was extracted from approx. 100 mg of frozen tissue with an RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 1 µg of RNA with Superscript III (Invitrogen, USA) according to the manufacturer’s instructions. cDNA was diluted and duplicate, real-time PCRs were performed using SensiMix SYBR Master Mix (Bioline, Australia) in a Rotor Gene 2000 (Corbett, USA). Real-time PCR was performed using 100–200 pmol of actin (Foo et al., 2005), PsTi (Grunwald et al., 2004) and PsPT4 and PsGST (Kuznetsova et al., 2010) primers. A standard curve for each gene was generated using serially diluted plasmids containing the cloned fragment of each amplicon, and the average concentration of duplicate samples was calculated. Relative gene expression of each biological replicate was achieved by comparing the concentration of the gene of interest with the actin concentration for that sample.

Western analysis

Young internode tissue was ground in liquid nitrogen and extracted with (3:1 v/w) 100 mM phosphate pH 7-4, 400 mM NaCl, 0-5 % dodecy-β-maltoside, 50 µM proteasome inhibitor MG132 and 1/1000 dilution of Sigma protease inhibitors for plants. The extracts were centrifuged at 16 000 rpm (Sorvall SS34) for 45 min. DELLA proteins, from 1 mL of extract, were precipitated by addition of an equal volume of ice-cold 40% saturated ammonium sulfate and kept on ice for 2 h, followed by centrifugation as described above. The pellets were dissolved in 150 µL of SDS sample buffer (Laemmli, 1970). Proteins were subjected to SDS–polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride (PVDF) membranes and analysed by western blotting (Towbin et al., 1979).

The membrane was blocked with 0-5 % IBlock (Tropix Ltd) in phosphate-buffered saline containing 0-1 % Tween-20 (PBST). Murine monoclonal antibody (MAb) BC9 (Sun et al., 2010), which recognizes the highly conserved residues Val-Xaa-Xaa-Tyr-Xaa-Val-Arg immediately downstream of the Asp-Glu-Leu-Leu residues within the DELLA motif and which can inhibit the binding of GID1 to DELLA proteins (Sheerin et al., 2011), was used to identify pea DELLA proteins. Briefly, the blocked membrane was incubated with MAb BC9 (500 ng mL−1 in blocking buffer) for 1 h at room temperature, washed three times in PBST containing 0-1 % I-Block and incubated in 1/40 000 dilution of peroxidase-
labelled goat antimouse IgG FC specific (Sigma A9309) in blocking buffer for 1 h. The membrane was washed as above and developed with chemiluminesence reagent Western Lightning Plus (PerkinElmer LAS Inc. NEL104). Chemiluminesence was measured on an LAS 3000 (FujiFilm Inc).

Statistical analysis

All data were analysed using the statistics package JMP (SAS). For pair-wise comparisons, t-tests were performed and for multifactorial experiments two-way factorial analyses of variance (ANOVAs) were performed. Values were log-transformed where appropriate.

RESULTS

Gibberellins and AM colonization

The roots of GA-deficient na-1 plants have a significantly altered structure due to the important role that GAs play in root development (Yaxley et al., 2001). In particular, na-1 roots contain significantly more cortical cell files, resulting in much thicker roots (Fig. 1C; average number of cortical cell files: wild type, 10.5 ± 0.3; na-1, 13.6 ± 0.7). The sampling method used to measure fungal colonization of the roots counts the number of intersects that contain arbuscules or any other fungal structure (McGonigle et al., 1990), and an increase in the number of cortical cell files that host these structures would result in an artificial increase in root colonization. To account for this, the percentage of the root containing arbuscules or any fungal structure was calculated relative to the number of cell files. Using this measure, roots of na-1 plants contain approx. 40 % more arbuscules than wild-type roots (P, 0.05; Fig. 1A). While the total number of fungal structures is not significantly increased in the mutant (Fig. 1A), the ratio of arbuscules to hyphae is significantly higher in the na-1 roots (Fig. 1B). This is clearly seen in the photograph of na-1 roots (Fig. 1C).

In order to test further if the mycorrhizal phenotype of na-1 mutants was due to the altered root structure in the mutant, or to a more direct effect of reduced bioactive GA levels, the effect of applying the synthetic GA3 on mycorrhizal colonization of na-1 plants was observed. This bioactive GA restores the elongation of newly formed shoot and root tissue of na-1 roots, but cannot alter the structure of roots or shoots that are already formed. This can be seen at the whole root level where GA3 application resulted in a larger and more branched root system compared with the untreated na-1 plants after 8 weeks growth (Fig. 2C). It is also visible at the level of individual secondary roots, with the roots becoming thinner after treatment (Fig. 2D) and containing fewer cell files, similar to wild-type roots. It was possible to monitor events in roots that had completed elongation but not arbuscule formation at the time of GA application (termed ‘na roots’ in Fig 2; ‘WT-like roots’ refer to those formed after GA application). Treatment with GA3 significantly reduced the number of arbuscules compared with the untreated na-1 plants (P < 0.01; Fig. 2A), to levels similar to those found in wild-type plants. Treatment with GA3 also reduced the ratio of arbuscules to hyphae (Fig. 2B; P < 0.05) and caused a smaller decrease in the total number of fungal structures (P < 0.05). It is important to note that the thick sections of na-1 roots that formed before GA3 treatment (‘na roots’) and the thinner wild-type-like roots that formed after GA3 treatment (‘WT-like roots’) both contained fewer arbuscules and fungal structures than untreated na-1 roots (Fig. 2A, B). Clearly, the differences seen in AM structures in response to GA3 application are not a consequence of altered root structures in the na-1 plants but a direct result of elevating bioactive GA levels. However, no significant differences in AM
colonization (data not shown) were observed in the leaky GA-deficient mutant *ls-1* (Yaxley *et al.*, 2001), suggesting that substantial (greater than that found in the *ls-1* mutant) changes in GA levels are required to affect AM colonization.

**DELLAs and AM colonization**

The *la* and *cry-s* mutations are both thought to be null mutations because of a 190 bp insertion and a frameshift deletion, respectively, which are predicted to result in non-functional proteins because of out-of-frame stop codons (Weston *et al.*, 2008). Southern analysis also suggested that pea only contains two DELLA proteins (Weston *et al.*, 2008). This was confirmed for vegetative tissue by a western analysis, which showed that two distinct proteins were observed in plants containing the genes *LA* and *CRY*, while no bands were present in slender (*la cry-s*) plants (Fig. 3).

![Fig. 2. Mycorrhizal colonization in wild-type and *na-1* mutant (severely gibberellin-deficient) plants with or without GA3 treatment. (A) Percentage of the root colonized by arbuscules and all fungal structures; (B) ratio of arbuscules to hyphae; and (C–E) photographs of colonized roots. (C) Whole roots (scale bar = 5 cm), (D) individual secondary roots, with arrow indicating transition from the ‘*na root*’ phenotype to the ‘WT-like root’ phenotype (scale bar = 1 cm), and (E) mycorrhizal structures (scale bar = 100 μm). For (A, B) *n* = 6–7 and values are means ± s.e.](https://academic.oup.com/aob/article-abstract/111/5/769/193343)

![Fig. 3. Western analysis of DELLA proteins from wild-type and slender (*la cry-s*) pea seedlings, showing two DELLA proteins in the wild type, LA and CRY, and no DELLA proteins in slender (*la cry-s*) mutants.](https://academic.oup.com/aob/article-abstract/111/5/769/193343)
also significantly reduced ($P < 0.001$; Fig. 4B). Indeed, few arbuscules developed in the DELLA-deficient mutant roots (Fig. 4C). This suggests that the role of GAs in AM colonization is internal to cells in the plant root, as the action of DELLA proteins is thought to involve the expression of genes within cells, rather than the efflux of compounds into the rhizosphere. If the role of GAs is indeed internal, *la cry-s* should be epistatic to the effect of *na-1* on arbuscule number and the ratio of arbuscules to hyphae. This is indeed the case in the triple mutant *na-1 la cry-s* where the level of arbuscules and the ratio of arbuscules to hyphae are reduced compared with wild-type plants (Fig. 5; $P < 0.05$ and $P < 0.01$, respectively).

**AM gene expression markers**

Two genetic markers of AM development were examined in the GA-deficient *na-1* mutant and three in the DELLA-deficient *la cry-s* mutant, to explore further the role of bioactive GAs via the DELLA signalling pathway in AM function. The genes monitored were *PsPT4*, which encodes a putative inorganic phosphate transporter essential for arbuscule function in *M. truncutula* (Javot et al., 2007; Kuznetsova et al., 2010), *PsTI*, which encodes a putative trypsin inhibitor specifically associated with arbuscule development (Grunwald et al., 2004), and *PsGST*, which encodes a putative glutathione S-transferase; members of the latter gene family have been proposed to play roles in biodefence (Kuznetsova et al., 2010; Hao et al., 2012). These genes are specifically upregulated in wild-type mycorrhizal root tissue and not in mycorrhizal pea *sym36* mutants that fail to develop arbuscules (Duc et al., 1989; Kuznetsova et al., 2010). Therefore, these genes may play roles during arbuscule development and function. In the *la cry-s* plants, the

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**Fig. 4.** Mycorrhizal colonization in wild-type and slender (*la cry-s*; DELLA-deficient) mutant plants. (A) Percentage of the root colonized by arbuscules and all fungal structures; (B) ratio of arbuscules to hyphae; and (C) photograph of a colonized root (scale bar = 100 μm). For (A, B) $n = 5$ and values are means ± s.e.

**Fig. 5.** Mycorrhizal colonization in wild-type and triple mutant *na la cry-s* (gibberellin-deficient and DELLA-deficient) plants. (A) Percentage of the root colonized by arbuscules and (B) ratio of arbuscules to hyphae. Values are means ± s.e., $n = 6–8$. 

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expression of all three genes was strongly downregulated compared with the wild-type plants (Fig. 6A; \( P < 0.001 \)). The reverse trend was the case in the GA-deficient na-1 plants for PT4 and GST genes, although the changes were not significant. As expected, application of GA3 to na-1 mutants significantly reduced the expression of these two genes compared with untreated na-1 plants (\( P < 0.05 \); Fig. 6B).

**Brassinosteroids and AM colonization**

The brassinosteroid-deficient mutant lkb was used to examine whether endogenous brassinosteroid levels influence AM colonization of pea roots. The lkb mutation results from a single base substitution in the pea homologue of the Arabidopsis DIMINUTO gene that catalyses the conversion of 24-methylenecholesterol to campesterol (Schultz et al., 2001). It is a leaky mutation that results in a reduction in brassinosteroid levels in all vegetative tissues by about 5-fold (Symons and Reid, 2004). There was no difference in either arbuscular formation or AM colonization between roots of lkb plants and comparable wild-type plants (Fig. 7). While the lkb mutation is leaky and therefore care must be taken in interpreting these results to mean that brassinosteroid levels have no impact on AM colonization, they do suggest that if they are involved, very large changes in their level are required before an effect is observed.
DISCUSSION

Endogenous GA levels and GA signalling influence the formation of arbuscules in mycorrhizal pea roots (Figs 1, 2, 4 and 5). GA appears to suppress arbuscule formation in pea, as a biosynthesis mutant with severe reductions in GA had elevated arbuscule development (Fig. 1), and a completely DELLA-deficient mutant (Fig. 3) that is unable to de-repress GA response exhibited significant reductions in arbuscule formation (Fig. 4). Importantly, our results suggest that the role of GAs in AM colonization is internal in the plant root cells, and this is consistent with the fact that la cry-s is epistatic to the effect of na-1 on arbuscule number and the ratio of arbuscules to hyphae (Fig. 5). The conclusions drawn from our mutant studies are consistent with previous reports that application of exogenous GA inhibits arbuscule formation in pea (El Ghachtouli et al., 1996; Slezak et al., 2000). However, exogenous application does not permit a distinction to be made between events in the rhizosphere and those occurring within the root cells.

Mutant studies indicate that GAs are likely to influence mainly the later stages of mycorrhizal colonization, specifically negatively regulating arbuscule formation, rather than influencing fungal invasion and accommodation. This is consistent with the fact that the na-1 and la cry-s mutants specifically regulated marker genes expressed during the later stages of mycorrhizal colonization, including arbuscule formation, and that these marker genes are negatively regulated by GA3 application (Fig. 6). Enhanced GA signalling due to the DELLA mutations in la cry-s was correlated with significant reductions in the expression of all three genes, while GA-deficient na-1 plants have somewhat (although not significantly) elevated expression of two genes, which could be significantly reduced by exogenous GA3 application.

Although bioactive GAs play an important role in root growth and development (Yaxley et al., 2001), which may have indirect effects on mycorrhizal development, GA3 application studies indicate that GA levels and/or signalling affect arbuscule formation more directly. As mycorrhizal colonization can occur in mature and developing root tissue, it is important to note that arbuscule formation was inhibited in all parts of the GA3-treated na-1 root system, including the short thick ‘na roots’ that matured before GA3 treatment (Fig. 2). This clearly indicates that it is the GA content of the root, rather than the root structure, that determines the arbuscule formation.

In addition to their important role in root development, GAs are essential for shoot elongation. This is reflected in the fact that the GA mutants employed in this study have strong effects on shoot elongation; na-1 is a severe dwarf and la cry-s has an overelongated ‘slender’ shoot phenotype (Reid et al., 1983; Weston et al., 2008). However, studies with brassinosteroid- and auxin-deficient dwarf mutants of pea indicate that dwarfism per se does not affect mycorrhizal development. Unlike the elevated arbuscule formation observed in na-1 plants (Figs. 1 and 2), the auxin-deficient bsh mutant has reduced arbuscule formation (Foo, 2013), and the brassinosteriod-deficient lkb dwarf mutant appeared to have no effect on mycorrhizal colonization (Fig. 7). It should be noted, however, that the BSH gene has not been characterized, and aspects other than auxin physiology may be altered in the bsh mutant. Furthermore, given the leaky nature of the lkb mutation, which results in some brassinosteroid production, further studies are needed to clarify if more severe reductions in brassinosteroids affect mycorrhizal development.

 Severely GA-deficient na-1 plants have previously been shown to produce more ethylene than wild-type plants (Ferguson et al., 2011), and their altered root structure is similar to roots treated with ethylene (Ferguson et al., 2005b). However, ethylene is thought to inhibit AM formation, although as discussed in the Introduction the evidence is at best inconsistent on this point. The elevated level of arbuscule formation observed in na-1 roots is the reverse of what would be expected and raises two questions. First, it highlights the need for more work using well-defined and specific ethylene mutants to clarify the issue. Secondly, if the effect of ethylene is to inhibit arbuscule development, this argues that the clear effect seen in the GA mutants must be independent of any interaction or effect on ethylene levels.

A picture of plant hormone regulation of mycorrhizal development is emerging, with potential roles for almost all of the plant hormones, including evidence presented here for a novel role for GAs. However, significant gaps in our understanding remain, including the somewhat contradictory reports on the roles of ethylene and JA. In addition, despite the significant role defined for cytokinin in the related symbiosis with rhizobial bacteria (Gonzalez-Rizzo et al., 2006; Tirichine et al., 2007), recent studies using the M. truncatula cre1 mutant (Plet et al., 2011) suggest that cytokinins may not play a major role in regulation of mycorrhizal development (F. Frugier and P. Bonfante, pers. comm.). Exciting areas of future research include more detailed analysis of the role of GAs, and investigating the potential dual role of strigolactones as both potential internal and external signals in mycorrhizal symbioses (Foo et al., 2013b).

ACKNOWLEDGEMENTS
We wish to thank Dr Laura Quittenden, Caine Barlow, Sara Blake, Casey Balmer, Tracey Winterbottom and Michelle Lang for technical support. This work was supported by the Australian Research Council (DP1095478 and DP110102085) and the University of Tasmania.

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