RESEARCH PAPER

A Medicago truncatula ABC transporter belonging to subfamily G modulates the level of isoflavonoids

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Abstract

Full-sized ATP-binding cassette (ABC) transporters of the G subfamily (ABCG) are considered to be essential components of the plant immune system. These proteins have been proposed to be implicated in the active transmembrane transport of various secondary metabolites. Despite the importance of ABCG-based transport for plant–microbe interactions, these proteins are still poorly recognized in legumes. The experiments described here demonstrated that the level of Medicago truncatula ABCG10 (MtABCG10) mRNA was elevated following application of fungal oligosaccharides to plant roots. Spatial expression pattern analysis with a reporter gene revealed that the MtABCG10 promoter was active in various organs, mostly within their vascular tissues. The corresponding protein was located in the plasma membrane. Silencing of MtABCG10 in hairy roots resulted in lower accumulation of the phenylpropenoid pathway-derived medicarpin and its precursors. PCR-based experiments indicated that infection with Fusarium oxysporum, a root-infecting pathogen, progressed faster in MtABCG10-silenced composite plants (consisting of wild-type shoots on transgenic roots) than in the corresponding controls. Based on the presented data, it is proposed that in Medicago, full-sized ABCG transporters might modulate isoflavonoid levels during the defence response associated with de novo synthesis of phytoalexins.

Key words: ABCG transporters, immune system, isoflavonoids, Medicago truncatula, PDR transporters, phytoalexin.

Introduction

ATP-binding cassette (ABC) transporters form one of the largest and most evolutionarily conserved families of proteins in all kingdoms. They possess a conserved domain-based structure and are classified into eight subfamilies (ABCA–H) (Verrier et al., 2008). Full-sized ABC transporters of the ABCG subfamily [formerly called pleiotropic drug resistance (PDR)] have been identified in plants, fungi, oomycetes, brown algae, and slime molds (Kang et al., 2011). Several full-sized ABCG proteins play a role in the response to biotic stress, especially in a non-specific manner, which confers protection against a wide group of pathogens. For example, RNA interference (RNAi)-mediated silencing of NpPDR1 causes Nicotiana plumbaginifolia to be more sensitive to infection with fungal (Botrytis cinerea and Fusarium oxysporum) and oomycete pathogens (Phytophthora nicotianae) (Bultreys et al., 2009). Expression of Nicotiana tabacum NtPDR1 was shown...
to be induced by treatment with various general elicitors such as flagellin<sup>exo</sup>, yeast extract, and INF1 elicin (Sasabe et al., 2002). In Arabidopsis, the mRNA level of AtPDR8/PEN3/AtABCG36 is elevated during plant infection with virulent and avirulent strains of the bacterial pathogen Pseudomonas syringae. Knockout of AtPDR8 decreases Arabidopsis resistance to inappropriate pathogenic fungi (Kobae et al., 2006; Stein et al., 2006). Expression of Arabidopsis PDR12/AtABCG40, significantly increases after infection with compatible (Sclerotinia sclerotiorum) and incompatible (Alternaria brassicicola) fungal pathogens, as well as after treatment with salicylic acid (SA), ethylene, or methyl jasmonate (MeJA) (Campbell et al., 2003). In wheat (Triticum aestivum), a putative full-size ABCG transporter (LR34) confers a durable resistance to multiple fungal pathogens (Krattinger et al., 2009). Recently, a new opening for the role of ABCG transporters in response to biotic stress has come with the finding of the involvement of the N. tabacum ABCG5 transporter in resistance to Manduca sexta herbivory (Bienert et al., 2012). It was also shown that ABCG32/PEC1 in Arabidopsis and HvABCG31/Eib1 in barley are required for the formation of a functional cuticle, which can act as the first barrier against abiotic and biotic stresses (Bessire et al., 2011; Chen et al., 2011).

Legume ABC transporters additionally attract attention as being possibly implicated in the establishment of symbioses (Sugiyama et al., 2006; Takanashi et al., 2011). Their identification has been accelerated by deciphering of the genome sequences of the model legumes Glycine max and Medicago truncatula (Schmutz et al., 2010; Young et al., 2011). Two M. truncatula so-called half-sized ABCG transporters (STR and STR2) that are present in peri-arbuscular membranes were found to be indispensable for arbuscule development in mycorrhizal symbiosis (Zhang et al., 2010). Expression of several legume ABCG genes was shown to be induced upon treatment with molecules such as SA or with pathogenic fungi (Eichhorn et al., 2006; Jasinski et al., 2009).

The proposed role of ABCGs in the immune system is modulation of the transmembrane transport of signalling/defensive compounds. Thus, efforts have been made to identify the phytochemicals transported by the various ABCGs (Badri et al., 2008; Badri et al., 2009). Recently, it was reported that secretion of strigolactones, signalling molecules found in the initiation of arbuscular mycorrhiza, is the contribu-
tion of the full-sized ABCG transporter (Kretzschmar et al., 2012). Additionally, Arabidopsis ABCG29 has been found as a p-coumaryl alcohol transporter, and it has been suggested that proper function of this protein has a complex impact on phenolic compounds and glucosinolate levels in this plant (Alejandro et al., 2012). Dysfunction of certain ABCG transporters in Arabidopsis results in the accumulation of flavonoid glycosides (kaempferol and quercetin) in the root tissues (Badri et al., 2012). Flavonoids play a particular role in biotic stress responses. This multifaceted group of plant secondary products can function as antimicrobial agents, UV protectants, pollinator attractants, floral pigments, and inducers of the nodulation genes in symbiotic soil bacteria known as rhizobia. A special subclass of flavonoids is composed of iso-
flavonoids, which are limited primarily to the Leguminosae. Isoflavonoids are thought to represent the majority of phytoalexins produced by legume plants (Hassan and Mathesius, 2012). For instance, soybean partial resistance to Fusarium solani seems to be associated with the ability of soybean roots to produce the phytoalexin glyceollin in response to fungal infection (Lozovaya et al., 2004). It has been proposed that ABC-type transporters can be involved in the secretion of (iso)flavonoids from soybean roots (Sugiyama et al., 2007).

In this study, a full-sized ABCG plasma membrane transporter from M. truncatula was characterized. Spatial expression pattern analysis with the β-glucuronidase (GUS) reporter gene revealed the activity of the MtABCG10 promoter in various organs including, roots, leaves, flowers, and fruits. Silencing of MtABCG10 in hairy roots resulted in a lower accumulation of isoflavone precursors of the phytoalexin medicarpin. In addition, faster spreading of F. oxysporum in MtABCG10-silenced Medicago was observed compared with control plants.

### Materials and methods

#### Plant material

*M. truncatula* (Jemalong J5) seedlings were germinated on water-saturated Whatman discs in Petri plates and grown under controlled greenhouse conditions with a mean temperature of 22°C, 50% humidity, and a 16 h photoperiod.

Leaf-originated Medicago suspension cell cultures were maintained in a 16 h photoperiod at 22 °C on an orbital shaker (150 rpm). The cultures were grown in medium (Murashige and Skoog medium plus Gamborg’s vitamins supplemented with 30 g l<sup>–1</sup> of saccharose, 2 mg l<sup>–1</sup> of 2,4-dichlorophenoxyacetic acid, and 0.25 mg l<sup>–1</sup> of kinetin), and were diluted 1:2 every 2 weeks. Plants with silenced MtABCG10 expression were obtained from *M. truncatula* after infection of a radicle with Agrobacterium rhizogenes Arqua1 (http://www.noble.org/medicagohandbook).

Hairy-root cultures were initiated by cutting off the roots and growing them in the dark at 22 °C on solid Fahraeus medium, supplemented with saccharose (10 g l<sup>–1</sup>), myoinositol (100 mg l<sup>–1</sup>), thiamine (10 mg l<sup>–1</sup>), pyridoxine (1 mg l<sup>–1</sup>), biotin (1 mg l<sup>–1</sup>), nicotinic acid (1 mg l<sup>–1</sup>), and glycine (2 mg l<sup>–1</sup>). Fragments of hairy roots were transferred onto fresh medium every 3 weeks.

#### Fungal elicitor and MeJA treatment

The *Phoma medicaginis* oligosaccharide elicitor was prepared as described previously (Hahn et al., 1992). The concentration of the elicitor was determined by the phenol/sulphuric acid method (Fry, 1994).

Five-d-old Medicago seedlings were transferred to solid 0.5× Gamborg’s medium supplemented with elicitor (25 µg ml<sup>–1</sup>) or MeJA (10 µM). Water and DMSO were used as controls, respectively. Samples were collected at 1, 2, 3, and 6 h after transfer and immediately frozen.

For metabolomic analysis, 3-week-old root cultures (250 ± 50 mg) were transferred into liquid medium (5 ml) and acclimatized for 24 h. Samples (hairy roots and medium) were collected at 6, 24, and 72 h after treatment with elicitor (25 µg ml<sup>–1</sup>) or water and immediately frozen.

#### Real-time quantitative RT-PCR (qRT-PCR) analysis

RNA was isolated from plant material with an RNeasy Extraction kit (Qiagen). Genomic DNA was removed by on-column DNase treatment. Total RNA (500 ng) was converted to cDNA with Omniscript reverse transcriptase (Qiagen), according to the manufacturer’s protocol. Real-time PCR analysis was...
performed in Rotor-Gene Q Real Time PCR machine (Corbett Research), using the MESA Green qPCR MasterMix Plus SYBR (Eurogentec). Primers sequences were as follows: MtABC10: forward, 5'-AATACTGTATTGCGGCCG-3', and reverse 5'-CCTATTTTCTTTGATATGTC-3', and actin (GenBank no. JQ028731): forward, 5'-TTCTGTAGATTTTCTACACG-3', and reverse 5'-AAGCATACAATACACTCC-3'. The threshold cycle method was used as described by Ruocco et al. (2011).

Quantitative transcript abundance analysis
RNA was isolated from Medicago roots and converted to cDNA as described for qRT-PCR analysis. The genomic DNA was extracted with a DNeasy kit (Qiagen). PCRs on DNA and cDNA as templates (30 cycles) were performed in an MJ Mini Personal Thermal Cycler (Bio-Rad). The sequences of the primers used for the amplification were as follows: forward, 5'-CATATTTGATGATTAGGCG-3', and reverse 5'-CCTATTTTCTTTGATATGTC-3'. PCR/RT-PCR products were cloned into pGEM-T Easy (Promega) and 80 randomly selected clones were sequenced with Sp6/T7 primers.

Preparation of microsomal and plasma membrane fractions
Microsomal fractions were isolated from 150 mg of Medicago hairy-root culture or 4 g of suspension cell culture as described previously (Jasinski et al., 2001). The plasma membranes were purified from microsomal fractions of M. truncatula suspension cell cultures by partitioning in an aqueous two-phase partition system (6 ml of phase mixture), as described by Larsson et al. (1987).

Western blot analysis
Proteins (5 μg) were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore) by electroblotting (semi-dry apparatus; BioRad). The membrane was incubated either with primary polyclonal antibodies against a peptide corresponding to Glu2–Glu27 of MtABCG10 (Eurogentec) or with primary antibodies specific for yyclonal antibodies against a peptide corresponding to Glu2–Glu27, pre-immune serum (1:50), and 2% BSA. Finally, the samples were incubated with the primary antibodies anti-MtABC10 (diluted 1:50), pre-immune serum (1:50), and anti-H'-ATPase (1:400) overnight at 4 °C. The cells were washed as described above and treated with secondary antibody replace by [Alexa Fluor-488-conjugated goat anti-rabbit IgG (Invitrogen), diluted 1:600] for 2 h at room temperature. The cells were observed by laser-scanning confocal microscopy (Nikon A1R-si). Nuclei and vacuoles were stained with 0.1 μM DAPI and 0.1% neutral red, respectively.

Extraction of phenolic compounds and liquid chromatography/electrospray ionization/mass spectrometry analysis (LC/ESI/MS)
Frozen tissue (250 ± 50 mg fresh weight of roots) was ground at 4 °C with mortar and pestle and extracted with 4 ml of 80% methanol. Culture medium containing root exudates was subjected to solid phase extraction as described previously (Staszkow et al., 2011). Dried extracted samples were dissolved in 5 ml of 80% methanol and subjected to LC/ESI/MS analysis. Profiles of phenolic compounds were acquired using an Agilent1200 liquid chromatograph/microTOF-Q mass spectrometer (Bruker Daltonics). Chromatographic separation was performed using an acetonitrile/water gradient. The spectra were recorded in the targeted mode within the m/z mass range of 50–1000. Metabolite profiles were registered in the positive-ion mode. For details, see Staszkow et al. (2011).

F. oxysporum infection of composite M. truncatula plants
F. oxysporum f. sp. medicaginis strain 179.29 was purchased from the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. F. oxysporum was grown on potato dextrose agar at 24 °C with a photoperiod of 12 h. Suspensions of microconidia were obtained by flooding the Petri dish with sterile water. The spore concentration was determined by counting and was then adjusted to 1.3 × 10^6 spores ml^{-1}. A 100 μl sample of spore suspension was deposited onto the roots of 4-week-old composite M. truncatula plants grown on Fahraeus medium. Eighteen empty vector-transformed control composite plants and 18 MtABC10-silenced composite plants in three biological repetitions were analysed. The infection efficiency was determined as the level of fungal DNA in various Medicago organs. The same amount of genomic DNA (40 ng) for each sample was used for qPCR. Reactions were conducted (as for qRT-PCR) using F. Oxysporum-specific primers (forward: 5'-ACCGTTTAGACACCATTCG-3'; reverse: 5'-AGTCGTTAGTGCCTACCG-3') for the β-tubulin gene (Genbank accession no. DQ924781.1) and M. truncatula specific primers for actin.

Results
MtABC10 (GenBank no. XM_003597771) has been described during studies dedicated to full-sized ABCG
transporters in *M. truncatula* as an expressed gene that was upregulated in roots following infection with pathogenic fungi. *MtABCG10* has two close homologues within the *Medicago* genome, initially described as *MtABCG9* (XM_003597768) and *MtABCG11* (XM_003597770) with 94 and 96% nucleotide identity, respectively (Jasinski et al., 2009). All three genes are adjacent to each other on chromosome 2 and possess conserved organization of gene structure. Thus, this cluster of genes is most likely the product of a single gene multiplication event. Recent insights into *M. truncatula* genomic features demonstrated that high recombination regions are particularly overrepresented among common gene families and especially among resistance-related genes (Paape et al., 2012). To elucidate the expression level of these three genes in roots, quantitative transcript abundance analysis was conducted. For this purpose, two universal primers were designed that permitted the simultaneous PCR- or RT-PCR-based amplification of the fragments of the genes *MtABCG9–MtABCG11* or their corresponding mRNAs. The sequence of the amplified region was distinct enough to allow unambiguous discrimination of homologues. The analysis of transcript abundance based on the sequencing of RT-PCR products showed that less than 2% of the amplicons represented *MtABCG9* or *MtABCG11* mRNAs. The amplicon abundance analysis based on PCR and genomic DNA as a template revealed a 1:1:1 product ratio corresponding to *MtABCG9*, *MtABCG10*, and *MtABCG11*, respectively. This indicated that *MtABCG9* and *MtABCG11* are expressed in roots at very low levels compared with *MtABCG10*. The same results were obtained regardless of whether RNA was extracted from plants grown under standard conditions or from plants treated with pathogenic fungi or symbiotic bacteria.

Expression of *MtABCG10* is modulated by elicitors

It is known that, in addition to pathogen infection, various elicitors or signal molecules affect the expression of several full-sized ABCG transporters. Among these are fungal oligosaccharides also described as pathogen-associated molecular patterns (PAMPs) and MeJA (Sasabe et al., 2002; Grec et al., 2003). In legumes, fungal PAMPs cause the de novo synthesis of isoflavonoid phytoalexins. MeJA regulates the mobilization of preformed phytoalexin intermediates stored in vacuoles, as well as terpenoid biosynthesis (Suzuki et al., 2005; Naoumkina et al., 2007; Farag et al., 2008). To assess PAMP effects on *MtABCG10* expression, *Medicago* plants were treated with oligosaccharides isolated from the cell walls of *P. medicaginis*, a pathogenic fungus causing severe diseases of forage and grain legumes. Consecutive qRT-PCR analysis revealed a rapid and transient increase in *MtABCG10* mRNA in plant roots. The highest level of *MtABCG10* mRNA accumulation was observed 1 and 2 h after elicitation (Fig. 1A). In addition, we observed that the expression of two defence-related genes, encoding key enzymes in isoflavonoid biosynthesis namely phenylalanine-ammonia-lyase (PAL; XM_003625614) and isoflavone synthase (IFS; AY939826) was also upregulated. In contrast, the expression of other defence-related genes, such as the gene encoding β-amyrin synthase (β-AS; XM_003604073), which is associated with terpenoid biosynthesis, remained unchanged (Supplementary Fig. S1 at *JXB* online).

MeJA induces the expression of several homologues of *MtABCG10* identified in other plants. This is the case for *NtPDR1* (74% sequence identity with *MtABCG10*), expression of which is specifically induced even at low concentrations of MeJA (between 1 and 20 µM; Sasabe et al., 2002). The same effect was observed for tobacco *NpPDR1* and soybean (*G. max*) GmPDR12 (Grec et al., 2003; Eichhorn et al., 2006). Although these genes are close homologues of *MtABCG10* (Supplementary Fig. S2 at *JXB* online), the latter did not respond to MeJA at a concentration of 10 µM (Fig. 1B).

*MtABCG10* expression pattern

To determine the expression pattern of *MtABCG10* in *Medicago*, transgenic plants were generated that carried the *MtABCG10* promoter fused with the β-glucuronidase (gusA) reporter gene (*MtABCG10::GUS*). Six independent lines were obtained, and the plants from the T1 generation were then analysed histochemically for GUS activity. In the roots of 8-week-old plants grown in soil, GUS expression was localized to the stele, which contains the conductive tissues...
(Fig. 2A–C, H, and I). In the leaves, a staining pattern was observed that was restricted to the junctions of three leaflets of the petiole and, to a lesser extent, the veinlets (Fig. 2D, E). In the flowers, GUS activity was observed in the anthers and in the pollen grains (Fig. 2F). In the fruit, GUS expression was seen in the conductive tissues (Fig. 2G).

Subcellular localization of MtABCG10

In situ immunolocalization of MtABCG10 in cultured Medicago suspension cells showed a clear signal coming from the plasma membrane (Fig. 3A). Under the same parameters of image acquisition, the control with pre-immune serum did not give any signal (Fig. 3B). These images were analogous to those observed for antibodies against H+-ATPase, a plasma membrane marker (Fig. 3C) and control with secondary antibodies (Fig. 3D), respectively. In situ immunolocalization with culture cells was performed because these do not require embedding, which was found to destroy antigenic epitopes. When protoplasts were used for MtABCG10 immunolocalization again, signals from membranes surrounding the cells were observed (Fig. 3E), compared with the control of pre-immune serum (Fig. 3F). The nucleus was visualized by DAPI staining (Fig. 3G). The absence of MtABCG10 from the vacuolar membrane (tonoplast) might be assumed as the vacuole in suspension culture used for immunolocalization can readily be stained by neutral red and appeared fragmented into several large vesicles that could not be

Fig. 2. Expression of the MtABCG10P::GUS reporter construct in M. truncatula. Whole mounts (A–F) of MtABCG10P::GUS transgenic plants were stained for GUS activity. (A–G) Root stele (A–C), leaf (D), young leaf (7-d-old) (E), flower (F), and fruit (G). (H, I) Longitudinal (H) and transverse (I) sections through a root of an MtABCG10P::GUS transgenic plant. Bars, 1.25 cm (A–C); 2.5 cm (D–G); 100 µm (H, I).
misidentified as the plasma membrane. (Fig. 3H). Finally, Western blot analyses confirmed that MtABCG10 accumulates in phase partition-purified plasma membrane fractions similarly to H+-ATPase (Fig. 3I). The specificity of the MtABCG10 antibodies was assayed by Western blotting with protein extracts from MtABCG10-silenced and non-silenced plant material additionally treated or not with fungal oligosaccharides (Supplementary Fig. S3 at JXB online).

RNAi-mediated knockdown of MtABCG10 in Medicago hairy roots

A. rhizogenes-mediated RNAi is a fast and effective method to study gene function in legumes. Transformed hairy roots are a good alternative to stable transgenic lines and can be propagated clonally (Limpens et al., 2004). To suppress MtABCG10 expression, a 139 bp fragment from the coding region (nt 3156–3295 of the cDNA) was obtained and introduced into the pK7CGWVG2(II)-p35S::DsRED binary vector (Limpens et al., 2005). The ability of the RNAi construct (MtABCG10 RNAi) to silence the expression of the MtABCG10 gene in the roots was tested. A significant reduction in the MtABCG10/MtABCG10 level was observed when assayed by real-time PCR and Western blotting (Supplementary Fig. S3).

Upon elicitation, in the roots of Medicago, induction of MtABCG10 expression proceeded along with that of PAL and IFS (Supplementary Fig. S1). Because one of the proposed functions of the full-sized ABCG transporters in plants is the translocation of secondary metabolites (Jasinski et al., 2001; Badri et al., 2008; Sugiyama et al., 2008), a search for phenotypic differences at the metabolome level was initiated. Hairy roots represent a fully differentiated tissue that tends to produce tissue-specific secondary metabolites and thus are a suitable material for such an approach (Pistelli et al., 2010).

Six independent control lines carrying empty vector (EV) and six MtABCG10-silenced lines of hairy roots were analysed. A strong reduction in MtABCG10 levels was not accompanied by any visible morphological changes in the transgenic roots (Supplementary Fig. S3). To mimic fungal infection, the transgenic roots were exposed to an elicitor (oligosaccharides isolated from P. medicaginis cell walls) that induces the expression of MtABCG10 and the phenylpropanoid biosynthesis pathway.

Both control and MtABCG10-silenced root tissue (treated or not with elicitor), as well as root exudates, were analysed by LC/ESI/MS. The profiling and identification of flavonoids and their glycoconjugates in the samples were based on standards, LC retention times, and high-resolution mass spectra.

LC/MS analysis revealed that free aglycones like the chalcone isoliquiritigenin and its derivatives liquiritigenin, 5-deoxyisoflavones (e.g. daidzein, formononetin, 2'-hydroxyformononetin, and vestitone) and medicarpin differentially accumulated in elicited and non-elicited samples (Fig. 4, and Supplementary Figs. 4 and 5 at JXB online). This was also true for naringenin and 5-hydroxyisoflavone (e.g. biochanin A), although to a lesser extent (data not shown). This effect was visible in control and MtABCG10-silenced root tissue.
Medicago ABCG and (iso)flavonoids

and root exudates. The amount of the above-mentioned free aglycones was markedly reduced in MtABCG10 RNAi material compared with controls. The most significant differences were observed in the root exudates, especially after elicitor treatment. The content of the corresponding glycosidic conjugates of the analysed aglycones (e.g. formononetin-Glc, formononetin-MalGlc, and medicarpin-MalGlc) and the flavones (e.g. apigenin) was unaffected by elicitor treatment or MtABCG10 silencing at any time point (6, 24, and 72 h). However, the levels of aglycones in the exudates declined over time; for example, medicarpin was detected only until 6 h after elicitation (Fig. 4 and Supplementary Figs. 4 and 5).

It is worth noting that the exogenous application of isoliquiritigenin and liquiritigenin onto Medicago roots induced the expression of MtABCG10 (Supplementary Fig. S6 at JXB online).

MtABCG10 silencing increases M. truncatula susceptibility to F. oxysporum

One question is whether the silencing of MtABCG10 expression will enhance Medicago susceptibility to pathogen infection. To address this issue, composite plants with transgenic roots were challenged with F. oxysporum f. sp. medicaginis in a plate assay. The in vitro-induced composite plant consisted of a wild-type shoot with transgenic roots and represents a significant advance because it decreases the amount of time required to generate transgenic plant tissue. Such composite plants have been used previously for functional studies of Medicago genes implicated in resistance to soilborne pathogenic fungi (Anderson et al., 2010), as well as for the assessment of the role of isoflavonoids in plant defences against Fusarium sp. (Lozovaya et al., 2004). F. oxysporum is a vascular wilt fungus that infects plants through the roots. It invades
described ABCG transporters (e.g. GmPDR12 and SpTUR2; Surprisingly, in contrast to NtPDR1 and several previously from tobacco (Sasabe et al., 2002). Nevertheless, MtABCG10 homologues were not detected in wild-type M. truncatula plants (Supplementary Fig. S7 at JXB online). Analysis of the MtABCG10 promoter sequence (http://www.dna.affrc.go.jp/PLACE/) revealed a large number (n=57) of W-box cis-acting elements with a core TGAC sequence, which is recognized by WRKY transcription factors, located in the 1500 bp proximal to the promoter region. It is known that perception of the PAMP signal triggers a downstream mitogen-activated protein kinase (MAPK) cascade that regulates the expression of numerous defence-related genes through WRKY (Eulgem and Somssich, 2007). Therefore, the expression of MtABCG10 may be influenced by PAMP/MAPK/WRKY pathways and may be independent of the pathways mediated by MeJA and SA.

As, following elicitation, MtABCG10 is co-expressed with early flavonoid pathway genes, it was of interest to determine whether this transporter might influence flavonoid fluxes in Medicago or not. To address this issue, an RNAi silencing approach was employed. Within this multigenic family, expression of the MtABCG10 homologues was not affected by the silencing construct (Supplementary Fig. S9 at JXB online). This was not the case for MtABCG9 and MtABCG11. The fact that MtABCG9 and MtABCG11 transcripts were affected by the silencing construct and could not compensate for the absence of MtABCG10 might be in favour for the experiments undertaken. However, both homologues were expressed at scant levels compared with MtABCG10 under our experimental conditions. Therefore, the silencing effects appeared to be representative of MtAGCG10 suppression. Metabolite analyses conducted with MtABCG10-silenced hairy-root cultures revealed that the silencing of MtABCG10 resulted in a much lower content of the isoflavone aglycones, especially from the 5-deoxyisoflavonoids branch (e.g. daidzein, formononetin, and vestitone), as well as their precursors (isoliquiritinigenin and liquiritinigenin) (Supplementary Fig. 10 at JXB online). In certain legumes (e.g. Medicago sp. and Glycine sp.), this branch leads to phytoalexin biosynthesis and seems to be crucial for plant disease resistance (Graham et al., 2007; Kamphuis et al., 2012).

The production of phytoalexins depends on the nature of the elicitation. Previously, it has been reported that, in M. truncatula suspension cell culture and in Lupinus seedlings, fungal oligosaccharides (e.g. yeast extract) induce changes in the profiles of isoflavonoid aglycones but not their glycosidic conjugates and flavones (Farag et al., 2008). It is thought that PAMPs cause the de novo synthesis of medicarpin, and that MeJA stimulates the release of its conjugate precursors from the vacuole (Naoumkina et al., 2007; Farag et al., 2008). MtABCG10 is a plasma membrane transporter, and expression of the corresponding gene is highly induced by PAMPs but not by MeJA. Silencing of its expression results in decreased amounts of free medicarpin precursors (aglycones) and does not influence the levels of their conjugates. Thus, it may be proposed that MtABCG10 is a modulator of isoflavonoid levels during the defence response associated with the de novo synthesis of Medicago phytoalexin.
Owing to the pleiotropic substrate profiles that are often associated with ABCG proteins (e.g. MtABCG36/AtPDR8; Stein et al., 2006; Kim et al., 2007; Strader and Bartel, 2009), it cannot be excluded that MtABCG10 might transport several different molecules. The latter might be represented by isoliquiritigenin and/or liquiritigenin. These compounds strongly induced the expression of MtABCG10, and it has been shown that certain substrates for ABCG transporters (e.g. scclareolide) induce the expression of their transporter (e.g. NpABC1) (Jasinski et al., 2001).

The relatively widespread expression of MtABCG10 in many organs might support the term ‘pleiotropic’, not only for the substrate profile but also for the function fulfilled by MtABCG10 in a particular organ. Apart from its role in roots and an effect on isoflavonoids as revealed by gene silencing, its precise role in other plant parts (e.g. flowers) remains to be elucidated.

The translocation of phenolic compounds, especially flavonoids and isoflavonoids, is still a matter of debate. Several mechanisms have been proposed, including vesicle-mediated transport and membrane transporter-mediated transport (Hassan and Mathesius, 2012). To date, members of the ABC family of ABC proteins have been implicated in phenolic traffic (Goodman et al., 2004; Zhao and Dixon, 2010). However, data describing the transport of genistin in soybean (Sugiyama et al., 2008) and the fact that silencing of MtABCG10 is associated with changes in isoflavonoid composition bring new insights into the possible role of ABCG proteins in modulating the amount of phenolic compounds in legumes.

It is also worth considering that the MtABCG10 substrate may be an unknown signalling molecule that regulates defence mechanisms that rely on isoflavonoid biosynthesis/transport. This regulatory mechanism probably does not affect, at least at the transcriptional level, key enzymes of isoflavonoid biosynthesis, such as PAL or IFS, because their expression profile does not vary in control and silenced lines following elicitation. The tissue expression pattern of MtABCG10 visualized with the GUS reporter system and its plasma membrane localization suggested that the MtABCG10 protein might translocate such molecules through the stele. Membrane transporters are important players in the regulation of metabolite biosynthesis and fluxes (Zhao and Dixon, 2010). In view of the presented data, a new potential role for plant ABCGs as modulators of isoflavonoid levels in legumes during biotic stress can be postulated.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1 RT-PCR time-course expression analysis of MtABCG10, PAL, IFS, and β-AS in M. truncatula seedling roots treated (+) or not (–) with P. medicaginis cell-wall oligosaccharides.

Fig. S2 Phylogenetic tree of the MtABCG10 homologues.

Fig. S3 MtABCG10 silencing in hairy root cultures and specificity of the anti-MtABCG10 antibodies.

Fig. S4 Relative levels of selected (iso)flavonoids and isoflavonoid conjugates in M. truncatula control (EV) and MtABCG10-silenced (RNAi10) hairy root cultures.

Fig. S5 Relative levels of selected (iso)flavonoids and isoflavonoid conjugates in M. truncatula control (EV) and MtABCG10-silenced (RNAi10) root exudates.

Fig. S6 Time-course expression analysis of MtABCG10 in M. truncatula seedlings roots treated (+) or not (–) with isoliquiritigenin (100 μM) (A) or liquiritigenin (100 μM) (B).

Fig. S7 Pictures of control and MtABCG10-silenced plants infected with F. oxysporum.

Fig. S8 Time-course expression analysis of MtABCG10 transcript (A) and protein (B) levels in M. truncatula suspension cell cultures treated (+) or not (–) with ABA, SA, or MeJA.

Fig. S9 Comparison of MtABCG genes expression in control (EV) and MtABCG10-silenced (RNAi10) hairy roots 1 h after treatment (+) or (–) with P. medicaginis cell-wall oligosaccharides.

Fig. S10 Outline of the biosynthetic pathways leading to the major classes of flavonoids in M. truncatula.

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**References**


