RESEARCH PAPER

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

Joanna Banasiak1, Wanda Biała2, Anna Staszków1, Barbara Swarczewicz1, Ewa Kępczyńska3, Marek Figlerowicz1 and Michał Jasiński1,2*

1 Institute of Bioorganic Chemistry PAS, Noskowskiego 12/14, 61–704 Poznań, Poland
2 Department of Biochemistry and Biotechnology, Poznań University of Life Sciences, Wołyńska 35, 60–637 Poznań, Poland
3 Department of Plant Biotechnology, Faculty of Natural Science, University of Szczecin, Waska 13, 71–415 Szczecin, Poland

* To whom correspondence should be addressed. E-mail: jasinski@ibch.poznan.pl

Received 8 October 2012; Revised 22 November 2012; Accepted 12 December 2012

**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 phenylpropanoid 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>p</sup> or yeast extract, and INF1 elicitin
(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, signifi-
cantly 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 trans-
porter (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
ABCG6 in mycorrhizal symbiosis (Zhang et al., 2010). For
instance, soybean partial resistance to Fusarium solani seems to be
associated with the ability of soybean roots to produce the
phytoalexin glycineollin 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 trans-
porter from M. truncatula was characterized. Spatial expres-
sion pattern analysis with the β-glucuronidase (GUS) reporter
gene revealed the activity of the MtABCG10 promoter in vari-
sous 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 16h photoperiod.

Leaf-originated *Medicago* suspension cell cultures were
maintained in a 16h photoperiod at 22°C on an orbital shaker (150rpm).
The cultures were grown in medium (Murashige and Skoog medium
plus Gamborg’s vitamins supplemented with 30 g l<sup>–1</sup> of saccharose,
2mg l<sup>–1</sup> of 2,4-dichlorophenoxyacetic acid, and 0.25mg l<sup>–1</sup> of kin-
tin), 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 rhizo-
genus *Arqua* (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, sup-
plemented 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>),
icotinie 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 elic-
tor 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, respect-
ively. Samples were collected at 1, 2, 3, and 6h after transfer and
immediately frozen.

For metabolomic analysis, 3-week-old root cultures (250 ± 50mg)
were transferred into liquid medium (5 ml) and acclimatized for 24h.
Samples (hairy roots and medium) were collected at 6, 24, and 72h
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 RNaseasy
Extraction kit (Qiagen). Genomic DNA was removed by on-
column DNase treatment. Total RNA (500ng) was converted to
cDNA with Omniscript reverse transcriptase (Qiagen), accord-
ing 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: MtABCG10: forward, 5’-AATCTAGTATATGCTGACCC-3’, and reverse 5’-CCTACTTCTTATTGATGATC-3’; and actin (GenBank no. JQ028731): forward, 5’-TTCTTCTAGTACTTTCCACGC-3’, and reverse 5’-AAGCATCAACTCACTCC-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’-CTATTGTTATGAGGATGCGG-3’, and reverse 5’-CATATTGGTATTGGATAGGCG-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 β-tubulin; 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 β-tubulin; BioRad). The sequences of the primers used for the amplification were as follows: forward, 5’-CATATTGGTATTGGATAGGCG-3’, and reverse 5’-CACTATCTTTCATTGATGATC-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).

Genetic constructs and plant transformation
The promoter region of MtABCG10 (710 bp) was amplified with the primers 5’-ATGAGTATCTAAGAGCTCCCAACCGC-3’ and 5’-TAGATCTTCACTTCTGTGTCG-3’. The PCR product was cloned via EcoRI and BamHI restriction sites into the pPR97 vector carrying the nuidA reporter gene (Zabados et al., 1995).

The cDNA fragment (139 bp) used for MtABCG10 RNA silencing was amplified using the primers 5’-GGGGACAAGTTTGTTCATCC-3’ and 5’-GGGGACACCTTGTACAGAAATGC-3’. Cloned into pDONR207/Zeo (Invitrogen), and recombined into a modified Gateway pK7GWIG2(II)-p35S::DsRed binary vector (Limpens et al., 2005).

The binary vectors pPR97 and pK7GWIG2(II)-p35S::DsRed were transferred into the Agrobacterium tumefaciens strain EHA105 and Agrobacterium rhizogenes strain Arqua1, respectively. Finally, the constructs were used to transform M. truncatula according to the procedures described in M. truncatula handbook (http://www.noble.org/medicago/handbook). Transgenic plants carrying the MtABCG10::GUS reporter construct were stained for GUS using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, according to the protocol described by Gallagher (1992). To identify transgenic roots carrying the silencing construct, the plant material was scanned with a Fluor Imager Fla-5100 (Fujifilm) using a 532 nm green laser and an LPG filter.

In situ immunodetection of protoplasts and cells from M. truncatula suspension cultures
Protoplasts from 6-d-old leaf-organized Medicago suspension cell cultures were isolated as described previously (He et al., 2007), with the exception that the digestion was carried out for 24 h in an enzyme solution consisting of 1.5% cellulase R10 (Serva), 0.6% macerozyme R10 (Serva), and 1% Driiselase* (Sigma-Aldrich). Cells were fixed with 3.7% formaldehyde in MS medium/MTSB buffer (50 mM PIPES, 5 mM MgSO4, 7 mM H2O, 5 mM EGTA) (1:1) for 30 min and then washed with MS/MTSB. To reduce autofluorescence, the cells were treated with 0.1% NaBH4. After washing with 0.5X MTSB, the cell walls were partially digested (except for protoplast immunodetection) with an enzyme cocktail (1.5% cellulase R10, 0.6% macerozyme, and 1% Driiselase* in 0.2 M mannitol, 20 mM KCl, and 20 mM MES, pH 5.8). The plasma membrane was permeabilized with 0.1% Triton X-100 and 0.05% Tween 20 in 0.5X MTSB. The samples were washed with PBS and saturated for 1 h in PBS containing 0.05% Tween 20 and 2% BSA. Finally, the samples were incubated with the primary antibodies anti-MtABCG10 (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 mM 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 80% methanol and subjected to LC/ESI/MS analysis. Profiles of phenolic compounds were acquired using an AgilentR 1200 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 × 106 spores ml⁻¹. A 100 µl sample of spore suspension was deposited onto the roots of 4-week-old composite M. truncatula plants grown in Fahraeus medium. Eighteen empty vector-transformed control composite plants and 18 MtABCG10-silenced composite plants in three biological replications 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’-ACCTTTGTAGACACCATTGCG-3’; reverse: 5’-AGTGCGTAAATGGCTCATTCG-3’) for the β-tubulin gene (Genbank accession no. DQ92478.1) and M. truncatula specific primers for actin.

Results
MtABCG10 (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

---

**Fig. 1.** qRT-PCR time-course expression analysis of *MtABCG10* in *M. truncatula* seedling roots treated (black bars) or not (white bars) with *P. medicaginis* cell-wall oligosaccharides (A) or 10 µM MeJA (B). Transcript levels are shown as values relative to those of actin used as internal control. Data represent means ± standard deviation (SD) of three independent biological experiments, with three plants per condition and four technical replicates of qRT-PCR. Significant differences from the control plants determined by Student's *t*-test are indicated: *P* < 0.05; **P** < 0.005; ***P*** < 0.005.

---

*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
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
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 pK7WGWIWG2(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'-hydroksyformononetin, 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.
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
Surprisingly, in contrast to and several previously
NtPDR1, MtABCG10 from tobacco (Sasabe et al., 2002).
As described here, the expression of MtABCG10 was
rapidly and transiently upregulated following treatment
with a fungal elicitor. This observation is consistent with
the response to various elicitors of NtPDR1, a close
homologue of MtABCG10 from tobacco (Sasabe et al., 2002).
Interestingly, in contrast to NtPDR1 and several previously
described ABCG transporters (e.g. GmPDR12 and SpTUR2;
Smart and Fleming, 1996; Eichhorn et al., 2006), MtABCG10
did not respond to treatment with MeJA and other stress
hormones such as SA or abscisic acid (ABA) (Supplementary
Fig. S8 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 multigene 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
eperiments 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. daid-
zein, formononetin, and vestitone), as well as their precursors
(isoliquiritigenin and liquiritigenin) (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 con-
jugate 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. AtABCG36/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. scolareide) 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 [formerly multidrug resistance-associated protein (MRP)] subfamily of ABC proteins have been implicated in phenolic traffic (Goodman et al., 2004; Zhao and Dixon, 2010). However, data describing the transport of genistein 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 1h 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.

**Acknowledgments**

We thank I. Femiak for excellent technical assistance, M. Stobiecki and D. Muth for their help with LC/MS work, M. Maruniewicz and I. Ziomkiewicz for their help with the confocal microscope, P. Bednarek for critical comments, M. Boutry for W1G and GPDR antibodies, and E. Limpons for the pK7GW1WG2(II)-p35S::DsRED binary vector. National Science Centre Grants supported this work: 2011/03/B/NZ1/02840 and N301 392139.

**References**


