Full-Size ABC Transporters from the ABCG Subfamily in *Medicago truncatula*

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Full-size ATP-binding cassette (ABC) transporters belonging to the ABCG subfamily are unique for plants and fungi. There is growing evidence that certain of these proteins play a role in plant defense or signaling systems. As yet, a complete set of full-size ABCG protein genes has been inventoried and classified in only two plants: *Arabidopsis thaliana* and *Oryza sativa*. Recently, a domain-based clustering analysis has predicted the presence of at least 12 genes encoding such proteins in the *Lotus japonicus* genome. Here, we identify and classify 19 genes coding full-size ABCG proteins in *Medicago truncatula*, a model legume plant. We have found that the majority of these genes are expressed in roots and flowers whereas only a few are expressed in leaves. Expression of several has been induced upon pathogenic infection in both roots and leaves. ABCG messenger RNAs have been detected in root nodules forming during symbiosis of legume plants and nitrogen-fixing bacteria. The data presented provide a scaffold for further studies of the physiological function of *Medicago* ABCG transporters and their possible role in modulating plant–microorganism interactions.

ATP-binding cassette (ABC) proteins occur in all living species. Most of them are involved in transmembrane transport of a great variety of molecules. All members of this protein family have a characteristic modular organization. They are composed of one or two copies of two basic structural elements: a hydrophobic transmembrane domain (TMD) and a cytosolic part containing an ABC transporter-specific nucleotide-binding domain (NBD) (Crouzet et al. 2006; Verrier et al. 2008). Based on two characteristic features, the number and the spatial distribution of structural domains, ABC transporters were divided into several subfamilies. The ABCG subfamily is composed of two major types of proteins: i) half-size transporters, formerly named white-brown complex homologs, comprising only one NBD and TMD in so-called reverse orientation with the NBD domain approaching the amino terminal end of the protein (NBD-TMD); and ii) full-size ABCG transporters, earlier known as plant pleiotropic drug-resistant (PDR)-like subfamily, containing two NBD and two TMD (NBD-TMD₂). The latter probably arose by a single duplication of the ancestral half-size ABCG genes (Crouzet et al. 2006).

A detailed phylogenetic analysis of ABC transporters shows that plant full-size ABCG proteins form clearly separated clusters. Parallel genomic studies demonstrate that full-size ABCG proteins are widespread in plants; 15 members have been identified in *Arabidopsis* and more than 20 in rice (Verrier et al. 2008). Recently collected data revealed the great functional diversity of plant full-size ABCG proteins. In addition, they provide new evidence that members of the ABCG family are involved in plant response to biotic and abiotic stresses. For instance, RNAi-mediated *NpPDR1* silencing caused *Nicotiana glauca* to be more sensitive to infection with pathogenic fungus *Botrytis cinerea* (Stukkens et al. 2005). Moreover, it has been shown that the expression of this gene is upregulated by sclareol (a secondary metabolite with antimicrobial properties), a potential *NpPDR1* substrate (Jasinski et al. 2001). Similarly, knockdown of the PDR8/PEN3 transporter in *Arabidopsis* increased plant susceptibility to bacterial and oomycete pathogens (Kobae et al. 2006; Stein et al. 2006). Other genes belonging to the same subfamily are induced either by pathogens and elicitors derived from them or by plant hormones (e.g. salicylic acid, methyl jasmonate, or ethylene). Abiotic stress such as high salt concentration, cold, presence of lead, or iron deficiency also induces the expression of full-size ABCG proteins (Crouzet et al. 2006). The number of ABC proteins and, in particular, full-size transporters in plants exceeds that reported in other multicellular organisms. This reflects different adaptive strategies of plants and, possibly, a higher number of potential substrates.

Although legumes are the third largest plant family in the world and the second most important crop family; our knowledge concerning legume ABC transporters is limited. One of them, *GmPDR12*, was identified in soybean (*Glycine max*) suspension cell cultures. The expression of this gene has been rapidly induced by salicylic acid but, to date, little is known about *GmPDR12* expression and function in planta (Eichhorn et al. 2006). Recently, several partial sequences of full-size ABCG transporters have been found in *Lotus japonicus*. Based on the conserved domain assembly, the authors identified 12 putative full-size ABCG genes. A few of those are induced in roots upon symbiotic infection (Sugiyama et al. 2006).

Here, we identify and classify 19 full-size ABCG transporters (MtABCG) from barrel medic (*Medicago truncatula* Gaertn.). All of them contain distinctive PDR motives and have been classified together with other known full-size ABCG proteins. The profiles of *MtABCG* expression have been determined. Most of them are transcribed in roots ($n = 14$) and flowers ($n = 11$). Few transcripts have been found in leaves. Moreover, we identified *MtABCG* genes being upregulated upon pathogenic fungal infection in roots and leaves.

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RESULTS

Identification of the full-size ABCG transporter genes in *M. truncatula*.

Although the analysis of the expressed sequence tag (EST) indicated the expression of several different members of the ABCG subfamily in *M. truncatula*, to date, those transporters were not systematically classified. To solve this problem, we made a search for the MtABCG genes in all currently available genomic databases. The size of the *M. truncatula* genome was estimated to be 450 to 560 Mbp (Medicago Genome Sequencing Consortium 2006). The first release of the genomic sequence (186.2 Mbp; Mt 1.0 database) contained between 55 and 58% of all genes captured by sequence assembly (*M. truncatula* release 1.0; Medicago Genome Sequencing Consortium 2006). A current release (214.7 Mbp; Mt 2.0 database), raises the percentage of coverage of the protein coding sequences to more than 60%.

In order to identify genes encoding transporters of interest, we performed a BlastP analysis on the translated products. As a query, we used the most conserved fragments of the PDR motifs: i) GLDARAA and TIHQPS, placed in carboxyl terminal part of ABCG proteins; and ii) LLLGPP and GLDSST, located in the amino terminal part (Ducos et al. 2005; Rea 2007; van den Brle and Smart 2002). As a result, we have identified 19 full-size ABCG transporters with at least three PDR motifs in the predicted sequence (Table 1). Because the average full-size ABCG protein is composed of 1,400 amino acids (Rea 2007), we took into account only sequences longer than 700 amino acids. Within shorter fragments, we found a few members of WBC subfamily of half-size ABCG transporters (e.g., IMGA|CT573078_29.5. and IMGA|AC146751_46.4) but these were not classified here. All 19 identified *M. truncatula* ABCG transporters were used for the construction of the phylogenetic tree. The latter also included the previously described complete sequences of full-size ABCG proteins from *Arabidopsis thaliana*, rice (*Oryza sativa*), soybean (*G. max*), spirodela (*Spirodela polyrrhiza*), and *Nicotiana* spp. (Fig. 1). The proteins were named according to the recently proposed unified nomenclature (Supplementary Table S1) (Verrier et al. 2008).

At the current status of genome sequencing, *M. truncatula* displays a high number of ABCG proteins, comparable with that of rice and higher than *Arabidopsis*. Overall sequence identity between *M. truncatula* ABCG proteins is 50 to 70%; however, several of them (e.g., MtABCG1 to MtABCG4 and MtABCG9 to MtABCG11) form tight clades with sequence identity up to 91 and 98%, respectively (Supplementary Table S2). High similarity between these proteins is also reflected in the conserved organization of their gene structures (Supplementary Figure S1). Moreover, we noticed that one group of close homologs, MtABCG9 to MtABCG11, displays 70% amino acid identity to AtABCG40 from *Arabidopsis*. Such a high gene redundancy may indicate that duplication of some full-size MtABCG has occurred in *M. truncatula* as a result of an adaptation to a new function. Interestingly, a close homolog of MtABCG1 to MtABCG4 proteins is the GmPDR12 (GmABCG1) transporter from soybean, sharing 82% amino acid identity with MtABCG1. This identity is higher than any other observed between, for instance, *M. truncatula* and *Arabidopsis* ABCG proteins (Supplementary Table S3), suggesting a "legume" nature of this clade and possible divergence that occurred after *Fabaceae* appearance. Similar species-specific clustering of certain ABCG genes is observed for *Arabidopsis* (e.g. AtABCG30 and 41 to AtABCG43) or rice (OsABCG32 and OsABCG34 to OsABCG37) and has been proposed to be related to acquisition of new functions or substrates (Crouzet et al. 2006).

Full-size ABCG genes expression in *M. truncatula*.

Initially, the accumulation of MtABCG messenger (m)RNAs was analyzed by the reverse-transcriptase polymerase chain reaction (RT-PCR) method. The specific primers were designed and preliminary tests involving genomic DNA allowed us to set an optimal PCR condition (Supplementary Table S4). Obtained products were sequenced, confirming primer specificity (not shown). Only two genes, MtABCG9 and MtABCG11, sharing 95% nucleotide sequence identity (including the 3' untranslated region), were not distinguishable by the PCR method and, therefore, the same pair of primers was applied for their identification. The obtained set of primers was used for the RT-PCR analysis of MtABCG mRNA accumulation in differ-

Table 1. Details of the *Medicago truncatula* ABCG genes

Name	TC number, TIGR	Name, IMGAG	Name, MIPS	Size (bp)	Spliced size (bp)	Size (amino acids)	PDR ^a	Contig
MtABCG1	TC131744	IMGA AC171532_22.4	AC171532_22	8,343	4,866	1,469	4	meth2-5a15 (chr07_pseudomolecule_IMGAG_V2)
MtABCG2	...	IMGA AC171532_32.4	AC171532_32	8,028	4,388	1,462	4	meth2-5a15 (chr07_pseudomolecule_IMGAG_V2)
MtABCG3	TC123185	IMGA AC171532_16.4	AC171532_16	8,014	4,510	1,462	4	meth2-5a15 (chr07_pseudomolecule_IMGAG_V2)
MtABCG4 ^b	...	IMGA AC147517_9	AC147517_9	8,285	4,329	1,443	4	chr07_pseudomolecule_IMGAG
MtABCG5	...	IMGA AC171532_21.4	AC171532_21	7,120	4,413	1,471	4	meth2-5a15 (chr07_pseudomolecule_IMGAG_V2)
MtABCG6	...	IMGA AC171532_26.4	AC171532_26	7,089	4,575	1,433	4	meth2-5a15 (chr07_pseudomolecule_IMGAG_V2)
MtABCG7	TC136665	IMGA AC144617_13.5	AC144617_13	8,440	4,494	1,425	4	meth2-7m9 (chr01_pseudomolecule_IMGAG_V2)
MtABCG8	TC114314	IMGA AC144617_23.5	AC144617_23	10,538	5,649	1,442	4	meth2-7m9 (chr01_pseudomolecule_IMGAG_V2)
MtABCG9	TC107192	IMGA AC144340_29.4	AC144340_29	7,261	4,479	1,493	4	meth2-7k2 (chr02_pseudomolecule_IMGAG_V2)
MtABCG10 ^b	TC107191	IMGA AC144340_22	AC144340_22	7,078	4,527	1,429	4	chr02_pseudomolecule_IMGAG
MtABCG11	...	IMGA AC144340_9.4	AC144340_9	7,820	5,631	1,428	4	meth2-7k2 (chr02_pseudomolecule_IMGAG_V2)
MtABCG12	...	IMGA AC170988_18.5	AC170988_18	7,406	4,284	1,428	4	meth2-53m12 (chr07_pseudomolecule_IMGAG_V2)
MtABCG13	...	IMGA AC170988_6.5	AC170988_6	7,320	4,368	1,456	4	meth2-53m12 (chr07_pseudomolecule_IMGAG_V2)
MtABCG14	...	IMGA AC170988_17.5	AC170988_17	9,304	4,677	1,473	4	meth2-53m12 (chr07_pseudomolecule_IMGAG_V2)
MtABCG15	TC102393	IMGA AC174352_33.5	AC174352_33	8,260	4,522	1,429	4	meth2-7o1 (chr08_pseudomolecule_IMGAG_V2)
MtABCG16	TC107302	IMGA AC123976_31.5	AC123976_31	10,684	5,221	1,462	4	meth2-30j23 (chr08_pseudomolecule_IMGAG_V2)
MtABCG17	...	IMGA CR954192_34.5	CR954192_34	8,686	3,738	1,246	3	meth2-15h3 (chr05_pseudomolecule_IMGAG_V2)
MtABCG18	TC97256, TC104271, TC109879	IMGA AC170856_10.4	AC170856_10	8,763	4,088	1,251	3	meth2-13d14 (chr02_pseudomolecule_IMGAG_V2)
MtABCG19	...	IMGA AC202577_4.4	AC202577_4	6,878	3,558	1,115	3	meth2-176a4 (chr08_pseudomolecule_IMGAG_V2)

^a Pleiotropic drug-resistant (PDR) signatures.

^b Reference from Mt 1.0 database (Medicago Genome Sequencing Consortium 2006).

ent *M. truncatula* organs. A vast majority of ABCG genes were expressed in the 6-week-old plants (Fig. 2A). Only four transcripts (*MtABCG2*, *MtABCG5*, *MtABCG6*, and *MtABCG12*) were not detected. For them, a new set of primers was designed and tested but, again, no product was detected (not shown), indicating that these genes were most likely not transcribed in the tested condition. The latter hypothesis is supported by the fact that no EST of the corresponding transcripts were found in the available databases. We have observed a high number of genes expressed in roots (14 of 19); namely, *MtABCG1*, *MtABCG3* to *MtABCG4*, *MtABCG7* to *MtABCG11*, and *MtABCG14* to *MtABCG19* (Fig. 2A). In contrast to roots, only eight genes were expressed in leaves (*MtABCG8* to

MtABCG14 which encode transporters sharing a high percentage of amino acid identity (91%) specifically accumulated in two different organs, flowers and roots, respectively (Fig. 2A).

Full-size ABCG gene expression in *M. truncatula* roots.

Especially high abundance of the MtABCG gene products in roots prompted us to more detailed studies of their expression in this organ. At first, we used roots of plants grown in sterile conditions. The roots were dissected into three arbitrarily chosen zones—zone 1 (0 to 0.5 cm from a root tip) followed by zone 2 (0.5 to 2 cm) and zone 3 (2 to 4 cm)—and the accumulation of MtABCG mRNAs was analyzed by RT-PCR. We observed that transcripts of at least three genes (*MtABCG3*, *MtABCG8*, and *MtABCG14*) were especially abundant in the region close to a root tip (zone 1). The rest of the MtABCG mRNAs were detected along the entire root, with the exception of *MtABCG4*, found exclusively in zone 2, and *MtABCG19*, found in zone 3 (Fig. 3A).

Symbiosis of the legume plants and prokaryotes known as rhizobia (*Rhizobium* and *Sinorhizobium* spp.) is a particular

example of a plant–microbe interaction, which results in the formation of a new organ known as a root nodule. The symbiotic nodules are a place of intense trade in which ABC transporters can be involved. Proteomic studies of nodule bacteria led to the identification of several ABC transporters, certain of them being necessary for the exchange of metabolites (Dupont et al. 2004). Considering the above observation, we attempted to determine MtABCG gene expression in nodules forming on seedling roots upon a *Sinorhizobium meliloti* infection (Fig. 3B). Undertaken RT-PCR analysis showed a particularly high level of *MtABCG16* mRNA accumulation. It was maintained up to 21 days after inoculation and then decreased (Fig. 3B). Interestingly, other MtABCG transcripts accumulated in nodules to visibly lower levels than in noninoculated roots (Fig. 3). Observation concerning *MtABCG16* is consistent with the DNA microarray data obtained for a corresponding EST (TC107302) during symbiotic nodules analysis (Benedito et al. 2008).

To determine whether MtABCG are up- or downregulated during symbiotic interactions, the pattern of their expression was also examined in seedling roots after inoculation with *S. meliloti*. For the majority of the genes, there were no significant differences in the level of MtABCG mRNA accumulation compared with noninfected control plants at the arbitrarily chosen time points (Fig. 4A). Several genes were expressed constitutively at a low level (*MtABCG4*, *MtABCG13*, *MtABCG17*, and *MtABCG19*) and certain others were not expressed at all (*MtABCG2*, *MtABCG5*, *MtABCG6*, and *MtABCG12*). As a result, only two genes belonging to different clades (Fig. 1)—*MtABCG10* (appearing as upregulated) and *MtABCG16* (showing high level of expression in nodules)—were chosen for the further analysis by real-time quantitative (q)RT-PCR, which revealed only slight changes in the level of expression of both genes. However, they were statistically significant in the case of *MtABCG10* at the early stage of infection (Fig. 4B).

Changes in the full-size MtABCG expression upon fungal pathogen infection.

The expression of several ABCG transporters was shown as induced by biotic stress; for example, NpPDR1 (NpABCG1) (Stukkens et al. 2005) and AtPDR12 (AtABCG40) (Campbell et al. 2003); and dysfunction of certain others was associated with an increased plant susceptibility to pathogenic fungal infections (Cruzet et al. 2006). Taking into account the above facts, we examined how the MtABCG gene expression is affected by infection with two common pathogenic fungi, *Fusarium culmorum* and *Phoma medicaginis*, both causing severe diseases of forage and grain legumes.

F. culmorum is a necrotrophic, soilborne pathogenic fungus, infecting root system and causing spring black stem and root rot (Tivoli et al. 2006). The infection of *M. truncatula* roots with *F. culmorum* resulted in an increased transcript accumulation, especially that of *MtABCG10* and *MtABCG16* (Fig. 5). It is worth noting that MtABCG10 shares 70% amino acid identity with AtPDR12, expression of which has been induced in *Arabidopsis* upon fungal pathogen infection, including *Fusarium* spp. (Campbell et al. 2003). MtABCG16 is a close homolog (72% amino acid identity) of AtPDR8/PEN3 (AtABCG36) (Kim et al. 2007; Stein et al. 2006) which, in *Arabidopsis*, contributes to defense against pathogenic fungi such as *Erysiphe pisi* and *Phytophthora infestans* (Stein et al. 2006).

A similar tendency was observed for both genes (*MtABCG10* and *MtABCG16*) when we analyzed the material derived from the *M. truncatula* leaves infected with the host-specific fungus *Phoma medicaginis* (Fig. 6). The genus *Phoma*

Fig. 2. Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of the full-size MtABCG gene expression in different organs. **A**, cDNAs were synthesized from total RNA isolated from roots (R), leaves (L), stems (S), and flowers (F) and subjected to RT-PCR (30 cycles). As a reference, the expression of actin was determined. **B**, Western blot analysis of the MtABCG proteins in microsomal fractions (30 µg) extracted from roots (R), leaves (L), stems (S), and flowers (F).

is a necrotrophic pathogen responsible for frequently occurring diseases of legumes, such as leaf spot (Kamphuis et al. 2008). Although, in general, a low number of different *MtABCG* mRNAs were present in leaves, the detected ones showed a clear response to *P. medicaginis* (Fig. 6). *MtABCG10* upregulation is in agreement with DNA microarray data obtained for corresponding EST (TC107191 and MtD00930_GC) (Journet et al. 2002).

In general, the analysis of *MtABCG* mRNAs accumulation presented above suggests that symbiotic and pathogenic interactions especially affect two genes: *MtABCG10* and *MtABCG16* (Figs. 4A, 5A, and 6A). Their expression is particularly elevated in leaves inoculated with *P. medicaginis* (approximately fivefold increase of mRNA accumulation) (Fig. 6B). A less substantial although statistically significant increase of *MtABCG10* and *MtABCG16* mRNA accumulation is observed in *S. meliloti*- or *F. culmorum*-inoculated roots (Figs. 4B and 5B). Based on a Western blot analysis involving ABCG subfamily-specific antibodies, one can conclude that the upregulation of ABCG genes is also observed at the protein level (Supplementary Fig. S2).

DISCUSSION

Sequencing of several eukaryotic genomes revealed a significantly higher number of putative ABC transporter genes in plants (>120) than in other multicellular organisms (approximately 50) (Jasinski et al. 2003; Sanchez Fernandez et al. 2001; Sugiyama et al. 2006). Although this fact suggests that ABC proteins are of special importance for plants, our knowledge about their functioning is still very poor. To better recognize this problem, we attempted to characterize full-size ABCG transporter genes from *M. truncatula*, a model legume plant. We focused on the ABCG subfamily because some of its members were shown to be implicated in plant-microbe inter-

actions (Campbell et al. 2003; Kobae et al. 2006; Stein et al. 2006; Stukkens et al. 2005). Out of all *M. truncatula* genes we analyzed, 19 were classified as members of this subfamily. The number of the identified ABCG genes was higher than in *Arabidopsis* and close to that of rice (Cruzet et al. 2006). In addition, we found, in various *M. truncatula* cDNA resources, only one EST (TC 102516) without a corresponding full-length genomic sequence present in the Mt 2.0 database. TC 102516 seems to be a homolog of *MtABCG10*, sharing 82% nucleotide identity. Unfortunately, we did not know the whole coding sequence of this gene; thus, the product of its expression was not included in the phylogenetic tree (Fig. 1). Altogether, these suggest that at least 20 full-size *MtABCG* genes are present in the *M. truncatula* genome and that the majority of them have been included in our studies.

Sugiyama and coworkers have made a similar attempt to inventory ABC transporter genes in *L. japonicus* (Sugiyama et al. 2006). Among 91 identified genes, only 12 have been classified as coding for full-size ABCG proteins. The applied methodology (domain-based clustering analysis) is most likely the reason why a relatively low number of full-size ABCG genes (*LjABCG*) has been found in *Lotus* spp. (for 70% of the genome coverage). The genome-wide screening, based exclusively on two domains (TMD and NBD), could make the identification of close homologs difficult. *MtABCG9* to *MtABCG11* well exemplify the situation. The overall identity of their nucleotide sequence is more than 90% and only full sequence alignment enabled us to distinguish these three genes.

Closer analysis of 12 *LjABCG* revealed that at least 5 of them are homologs of *Arabidopsis* AtPDR12 (AtABCG40). Interestingly, we have also identified three transporters (*MtABCG9* to *MtABCG11*) having 70% amino acid identity with AtABCG40 and four more (*MtABCG1* to *MtABCG4*) for which identity to the latter protein is greater than 65%. The

Fig. 3. Reverse-transcriptase polymerase chain reaction analysis (30 cycles) of the *MtABCG* gene expression in roots. **A**, *MtABCG* mRNA accumulation in the arbitrary selected root segments of the 7-day-old *Medicago truncatula* seedlings: zone 1 (0 to 0.5 cm) from a root tip followed by zone 2 (0.5 to 2 cm) and zone 3 (2 to 4 cm). **B**, *MtABCG* mRNA accumulation in the root nodules. The cDNAs were synthesized using total RNA isolated from nodules collected 14, 21, and 30 days postinoculation (dpi). The actin gene transcript was analyzed as an internal control.

above-mentioned TC 102516 also displays a high similarity to *MtABCG10*. Accordingly, one can state that at least eight homologs of *AtABCG40* are encoded in the *M. truncatula* genome. It seems that such a redundancy of *AtABCG40*-like genes may be a unique feature of legume genomes because it is observed in both *M. truncatula* and *Lotus* spp. but not in rice.

An RT-PCR analysis showed that, in the 6-week-old *M. truncatula* plants, only seven *MtABCG* genes were expressed in all examined organs. The mRNAs of 14 *MtABCG* genes were detected in root tissues. Among them there were several with an especially high level of accumulation in roots (*MtABCG3*, *MtABCG4*, *MtABC 7*, *MtABCG14*, and *MtABCG18*), root nodules (*MtABCG16*), or in particular root zones (*MtABCG3*, *MtABCG4*, and *MtABCG19*). The number of *MtABCG* expressed in other organs was markedly smaller. Similar observation was made by Sugiyama and coworkers.

Six of eight *LjABCG* they examined also have a higher transcript level in roots than in other organs (Sugiyama et al. 2006). In addition, they observed the increased accumulation of six *LjABCG* transcripts (out of eight examined) in roots 3 weeks after inoculation with *Mesorhizobium loti*. We have also noted some changes in *MtABCG* expression in *Medicago truncatula* roots during symbiotic interactions with *S. meliloti*. However, it is difficult to compare Sugiyama's data concerning the whole roots with ours obtained for seedling roots (representing the early stage of infection) and fully developed nodules. Nevertheless, altogether, they indicate that *LjABCG* and *MtABCG* can be involved in symbiosis between legumes and rhizobia.

As yet, little is known about the function of the plant ABC transporters in roots. The rhizosphere is a place of intense interactions with bacteria, fungi, insects, and other plants.

Fig. 4. Expression of the full-size *MtABCG* genes in roots upon *Sinorhizobium meliloti* infection. **A**, Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis (27 cycles) of the *MtABCG* mRNA accumulation in noninfected (control) and infected seedlings roots at the indicated time points. The actin gene transcript was used as an internal control. **B**, Quantitative (q)RT-PCR analysis of the *MtABCG10* and *MtABCG16* gene expression. Transcript levels are shown as values relative to those of actin used as the internal control. White bars = noninfected and black bars = infected material. Data represent the means $\pm$ standard deviation of two independent biological experiments. Each experiment involved five plants per condition and four technical replicates of qRT-PCR. Significant differences from the control plants determined by Student's *t* test are indicated (*, **, and *** = $P < 0.05$, 0.02 , and 0.01 , respectively).

Thus, an efficient transport through cellular membranes is necessary to ensure effective flow of different molecules (e.g., root exudates with defense or signaling properties). In the rhizome of *Coptis japonica*, one member of the ABCB subfamily was recognized as an importer of alkaloids (Shitan et al. 2003). Other ABCG from *Arabidopsis*, namely PGP1 (AtABCB1) and PGP4 (AtABCB4), were shown to be particularly expressed in the roots and root tip, where they contribute to distribution of auxin (Geisler et al. 2005; Santelia et al. 2005; Terasaka et al. 2005). To date, ABCG proteins have not been associated with auxin transport. However, *Arabidopsis* PDR9 (AtABCG37) has been shown to be capable of transporting synthetic auxin 2,4-D.

It seems that the AtPDR9 can specifically efflux 2,4-D out of plant cells without affecting endogenous auxin transport (Ito and Grey 2006). Because of the high expression in the lateral root cap and epidermis, it was suggested that AtPDR9 can also participate in communication between plant roots and other organisms (Ito and Grey 2006). In addition, the involvement of two other *Arabidopsis* ABCG proteins, AtPDR6 (AtABCG34) and AtPDR7 (AtABCG35), in the metabolite secretion was postulated, based on the observation that the deletion of their genes affected the composition of root exudates (Badri et al. 2008).

Infection of *M. truncatula* roots with *F. culmorum* resulted in the slightly higher expression of *MtABCG10* and

Fig. 5. Expression of the full-size MtABCG genes in roots upon *Fusarium culmorum* infection. **A**, Total RNA was isolated at the indicated time points from noninfected (control) and infected seedlings roots. cDNAs obtained after a reverse transcription were subjected to polymerase chain reaction (PCR) analysis (27cycles). The actin gene transcript was used as an internal control. **B**, Quantitative reverse-transcriptase (qRT)-PCR analysis of *MtABCG10* and *MtABCG16* gene expression in untreated (white bars) and infected (black bars) material. Transcript levels are shown as values relative to those of actin used as the internal control. Data represent the means $\pm$ standard deviation of two independent biological experiments. Each experiment involved five plants per condition and four technical replicates of qRT-PCR. Significant differences from the control plants determined by Student's *t* test are indicated (*, **, and *** = $P < 0.05$, 0.02 , and 0.01 , respectively).

MtABCG16 than during symbiotic interactions with bacteria. However, both genes were much more strongly induced by *P. medicaginis* in leaves, thus suggesting a relation between their activation and fungal infections. Accordingly, one can hypothesize that ABCG proteins are involved not only in plant–symbiont but also in plant–fungus interactions in *M. truncatula*.

In addition, the collected data indicate that both transcripts (*MtABCG10* and *MtABCG16*) are under the influence of the diurnal cycle, showing a relatively low level of accumulation at the early hours of the light period (Fig. 6B, T0 = 10:00 a.m.).

Numerous MtABCG mRNAs were found in *M. truncatula* flowers (Fig. 2). One of them (*MtABCG13*) was detected exclusively in this organ. Its close homologs are NpPDR2

(NpABCG2) from *N. plumbaginifolia* and AtPDR11 (AtABCG39) from *Arabidopsis*, both displaying 70% amino acid identity. Expression of the *NpPDR2* gene was shown to be dependent on pollination and its transcript was found only in pollinated style. No specific phenotype was observed in plants silenced for NpPDR2 (Trombik et al. 2008). The *AtPDR11* was also shown as flower-specific (carpel and ovary) (Zimmermann et al. 2004). The full-size ABCG proteins were proposed to transport floral fragrances in flowers (Stukkens et al. 2005), signaling molecules or hormones induced by processes such as pollination (Trombik et al. 2008). Surprisingly, we found that *MtABCG14*, a close homolog of *MtABCG13*, is exclusively expressed in roots. This suggests that either both

Fig. 6. Expression of the full-size MtABCG genes in leaves upon *Phoma medicaginis* infection. **A**, Total RNA was isolated at the indicated time points from noninfected (control) and infected leaves. cDNAs obtained after reverse transcription were subjected to polymerase chain reaction (PCR) analysis (25 cycles). The actin gene transcript was used as an internal control. **B**, Quantitative reverse-transcriptase (qRT)-PCR analysis of *MtABCG10* and *MtABCG16* gene expression in untreated (white bars) and infected (black bars) leaves. Transcript levels are shown as values relative to those of actin used as the internal control. Data represent the means $\pm$ standard deviation of two independent biological experiments, five plants per condition, and four technical replicates of qRT-PCR. Significant differences from the control plants determined by Student's *t* test are indicated (*, **, and *** = *P* < 0.05, 0.02, and 0.01, respectively).

fulfill similar roles but in different organs or they have different substrates.

Identification of the substrates for the ABCG proteins still remains an essential and unsolved issue. Members of the ABCG subfamily are capable of transporting a wide range of unrelated molecules. In addition, as shown, for instance, for the *Arabidopsis* mutants *Atpdr6* and *Atpdr7*, one compound may be a substrate for different transporters, indicating their functional redundancy (Badri et al. 2008). On the other hand, one ABCG protein can transport different molecules; for example, *Arabidopsis* PDR8 (AtABCG36) (close homolog of MtABCG16) both contributes to defense against pathogenic fungi (probably by transporting chemicals that mediate pathogen resistance) and functions as an efflux pump for cadmium or cadmium conjugates (Kim et al. 2007; Kobae et al. 2006; Stein et al. 2006). Therefore, a high level of *MtABCG16* mRNA in leaves and nodules can have different biological meanings. The expression of several MtABCG transporters in different organs such as stems, leaves, and flowers (e.g., *MtABCG10* and *MtABCG15* to *MtABCG17*) also suggests their possible functional diversity.

The ABC transporters have been shown to be active in transport of various natural products, including plant secondary metabolites. Although, currently, nothing is known about the MtABCG substrates, it should be noted that, during *P. medicaginis* infection, a higher accumulation of medicarpin (isoflavonoid phytoalexin) and its precursors (e.g., formononetin glucoside) was observed in *M. truncatula* leaves (Deavours and Dixon 2005). The medicarpin is a possible substrate for the ABC transporters, as shown in biochemical assays with vacuolar membrane vesicles of mung bean (*Vigna radiata*) (Li et al. 1997). Vacuolar uptake experiments indicated that, depending on the plant species and type of conjugated moiety (glucoside or glucuronide), different transporters (either directly energized ABC-type transporters or secondarily energized antiporters) are involved in the trafficking of phenolic compounds (Yazaki 2005). Molecular identity of such transporters starts to be recognized (Goodman et al. 2004; Marinova et al. 2007). To date, most of the characterized ABCG transporters have been localized in plasma membrane (Ito and Gray 2006; Jasinski et al. 2001; Kobae et al. 2006). However, one cannot exclude the possibility that they are also anchored in the other types of cellular membranes or membrane vesicles; participating, for instance, in compartmentation or turnover of secondary metabolites or their precursors. In soybean, the translocation of flavonoid compounds through the plasma membrane was shown to be fulfilled by ABC transporters most likely being members of the ABCG subfamily (Sugiyama et al. 2007). However, also in this case, identification of the concrete transporter or transporters is still necessary.

Apart from phenolic compounds, terpenoids also appeared as possible substrates for the ABCG transporters (e.g., NpPDR1 and diterpene sclareol) (Jasinski et al. 2001). In legumes, a group of sesquiterpene lactones have been identified as a root-released signal inducing the hyphal branching of mycorrhizal fungi (Akiyama et al. 2005). Secretion of such terpenoid signaling molecules can be a function of certain ABCG transporters (Cruzet et al. 2006). Analysis of EST data from the electronic northern recourses revealed that certain MtABCG transporters (e.g., *MtABCG16* and MtC00386_GC) are clearly upregulated after *Glomus* spp. infection. However, it must be emphasized that caution is needed in the prediction of homologous gene functions in different species. A recent comparative transcriptome analysis of *M. truncatula* versus *Arabidopsis* revealed a significant divergence in the developmental expression profiles of highly similar genes, which indi-

cates that a phylogenetic analysis alone is insufficient for the elucidation of their possible function (Benedito et al. 2008). Identification of transporters and their putative substrates is essential for better understanding interactions of legume plants with other organisms. The data presented here represent a scaffold for further detailed studies of physiological functions of particular MtABCG proteins and their role in the transport of various molecules throughout plant tissue.

MATERIALS AND METHODS

Plant material.

Plants (*M. truncatula* Gaertn. ecotype Jemalong J5, provided by A. Kondorosi, CNRS, Gif-sur-Yvette, France) were grown in pots containing a mixture of sand and perlite (1:1) under controlled greenhouse conditions, with an average temperature of 25°C, 50% humidity, and a 16-h photoperiod. Plants were watered every 2 days and supplemented once per week with N-P-K fertilizer (6:3:6).

P. medicaginis infection of *M. truncatula* leaves.

Six-week-old plants were inoculated with *P. medicaginis*. To this end, conidia of *P. medicaginis* Malbr. et Roum. (strain PH 33, from the collection of the Institute of Plant Genetics, Polish Academy of Sciences) were plated on potato dextrose agar and incubated at 28°C for 3 weeks until the appearance of pycnidia. Pycnidiospores were collected by flooding the plate with sterile 0.1% (vol/vol) Tween 20. Next, the suspension was filtered to remove parts of mycelia, and spore concentration was determined by counting. Routinely, an inoculation mixture containing 2×10^6 spores/ml was sprayed on the plants. Leaves in control and infected plants were collected 3, 6, 9, 24, 48, and 72 h after inoculation and immediately frozen in liquid nitrogen.

S. meliloti or *F. culmorum* infection of *M. truncatula* seedlings.

M. truncatula seedlings were germinated on a water-saturated Whatman disks in petri plates and kept in a greenhouse as described above. Seven-day-old seedlings were inoculated with *S. meliloti* Rm1021 strain (approximately 2×10^6 cells per milliliter in 10 mM MgSO₄, 0.2 ml per root) (Meade et al. 1982) or *F. culmorum* strain Cul-3 from the collection of the Institute of Plant Genetics, Polish Academy of Sciences (5×10^6 spores per milliliter in sterile water, 0.5 ml per root). Seedling roots in control and infected plants were collected 3, 6, 9, 24, 48, and 72 h after the inoculation and immediately frozen in liquid nitrogen.

Semiquantitative RT-PCR and real-time qRT-PCR analysis.

RNA was isolated with the RNeasy plant RNA extraction kit (Qiagen). Genomic DNA was removed by on-column DNase (Qiagen) treatment. RNA samples used for qRT-PCR were additionally treated with DNA-free DNase (Ambion). Total RNA (500 ng) was converted to cDNA with Omniscript reverse transcriptase (Qiagen) according to the manufacture's protocol. PCR reactions were performed in an MJ Mini personal thermocycler (Bio-Rad) using 30, 27, or 25 cycles. qRT-PCR analysis was performed in Rotor-Gene RG-3000 Real-Time PCR machine (Corbett Research) using the SYBR Green PCR Master Mix (Applied Biosystems). To determine specificity of the primers used for qRT-PCR (single-product formation), melting curves were analyzed. Primers were accepted as specific if a single amplification product was detected. The threshold cycle method (Rasmussen 2001) was used to calculate mRNA concentration. The actin mRNA (TC107326) was used as a reference.

Microsomal fraction preparation and Western blot analysis.

The microsomal fractions were isolated from 300 mg of plant material as described by Jasinski and associates (2001). Proteins (30 µg or, when mentioned, 40 µg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (7% polyacrylamide) and transferred to a polyvinylidene difluoride membrane (Millipore) by electroblotting (semidry transfer; Bio-Rad). The membrane was incubated with rabbit antibodies specific for the ABCG subfamily (GPDR) obtained against the conserved PDR motive (Ducos et al. 2005) and antibodies specific for H⁺-ATP-ase (W1G) (Morsomme et al. 1998).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- J. Craig Venter Institute EST database: www.tigr.org
- Medicago EST Navigation System website: medicago.toulouse.inra.fr/Mt/EST
- The *Medicago* Genome Sequence Consortium Mt2.0 release: www.medicago.org/genome/downloads/Mt2
- The German Research Center for Environmental Health *Medicago* genome database: mips.gsf.de/proj/plant/jsf/medi/index.jsp