Expression of the Apyrase-Like *APY1* Genes in Roots of *Medicago truncatula* Is Induced Rapidly and Transiently by Stress and Not by *Sinorhizobium meliloti* or Nod Factors[1]

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The model legume *Medicago truncatula* contains at least six apyrase-like genes, five of which (*MtAPY1;1*, *MtAPY1;2*, *MtAPY1;3*, *MtAPY1;4*, and *MtAPY1;5*) are members of a legume-specific family, whereas a single gene (*MtAPY2*) has closer homologs in Arabidopsis. Phylogenetic analysis has revealed that the proteins encoded by these two plant gene families are more similar to yeast (*Saccharomyces cerevisiae*) GDA1 and to two proteins encoded by newly described mammalian genes (*ENP5* and *6*) than they are to mammalian CD39- and CD39-like proteins. Northern analyses and analyses of the frequencies of expressed sequence tags (ESTs) in different cDNA libraries suggest that in roots, leaves, and flowers, the more highly expressed genes are *MtAPY1;3/MtAPY2*, *MtAPY1;3/MtAPY1;5* and *MtAPY1;2/MtAPY1;3* respectively. In roots, at least four of the *MtAPY1* genes are induced transiently within 3 to 6 h by a stress response that seems to be ethylene independent because it occurs after treatment with an ethylene synthesis inhibitor and also in the *skl* ethylene-insensitive mutant. This response also occurs in roots of the following symbiotic mutants: *dmi1*, *dmi2*, *dmi3*, *nsp*, *hcl*, *pdl*, *lin*, and *skl*. No evidence was obtained for a rapid, transient, and specific induction of the *MtAPY* genes in roots in response to rhizobia or rhizobial lipochitooligosaccharidic Nod factors. Thus, our data suggest that the apyrase-like genes, which in several legumes have been implicated to play a role in the legume-rhizobia symbiosis (with some members being described as early nodulin genes), are not regulated symbiotically by rhizobia in *M. truncatula*.

The establishment of the legume-rhizobia symbiosis involves a signal exchange between the specific plant and bacterial couples leading to recognition, nodule organogenesis, and controlled infection. On the bacterial side, plant recognition is determined by a set of genes essential for nodulation and host range that specify the production of lipochitooligosaccharidic signals referred to as Nod factors. At pico-micromolar concentrations, these signals can initiate many of the responses characteristic of the rhizobia themselves such as calcium spiking, root hair deformations, and gene expression (Dénarié et al., 1996; Downie and Walker, 1999). How these LCO signals are perceived by the plant is the subject of much research, and genetic, molecular, and biochemical approaches are being pursued (Cullimore et al., 2001; Geurts and Bisseling, 2002).

One candidate for a role in Nod factor perception is a lectin isolated from roots of *Dolichos biflorus* called lectin nucleotide phosphohydrolase (Db-LNP). This protein binds Nod factors preferentially from the *D. biflorus* symbiont and with apparently much higher affinity than chitin fragments (Etzler et al., 1999). Db-LNP is present on the surface of root hairs and relocates to the root hair tips after addition of rhizobia (Kalsi and Etzler, 2000). Antisera raised against the protein inhibit root nodulation by rhizobia. Together these results suggest that Db-LNP plays an important role in nodulation through the binding of Nod factors. A role in nodulation is also supported by work on soybean (*Glycine soja*) showing that a specific antiserum raised to a similar soybean protein inhibits nodulation of this legume (Day et al., 2000).

Db-LNP is related to a large family of eukaryotic proteins that includes the mammalian CP39- and CD39-like proteins and the yeast (*Saccharomyces cerevisiae*) protein GDA1 (Roberts et al., 1999). All these proteins contain a highly conserved domain with four regions characteristic of nucleotide phosphohydrolases (Handa and Guidotti, 1996). Where the catalytic activity has been characterized, it can be described as an apyrase (ATP diphosphohydrolases, EC 3.6.1.5.); however, the activity is usually nonspecific

for various nucleotide tri- and diphosphates. Apyrase-like proteins have been well studied in animals, where they have been shown to be located either extracellularly, associated with the plasma membranes, the Golgi, or the endoplasmic reticulum and to be involved in such diverse functions as neurotransmission, nucleotide recycling, membrane permeability/transport, and glycosylation (Plesner, 1995; Komoszynski and Wojtczak, 1996). In yeast, GDA1 is essential for correct protein glycosylation and cell wall synthesis (Abeijon et al., 1993).

Before the cloning of Db-LNP, the only two plant sequences belonging to the apyrase family were from potato (*Solanum tuberosum*; Handa and Guidotti, 1996), and pea (*Pisum sativum*; Hsieh et al., 1996). The pea protein was originally described as a nuclear ATPase (Matsumoto et al., 1984; Hsieh et al., 1996), but recent studies suggest that it may be associated also with the plasma membrane and involved in mobilizing phosphate from extracellular ATP and in resistance to xenobiotics (Thomas et al., 1999, 2000). It has also been found to be associated with the cytoskeleton (Shibata et al., 1999).

The report that Db-LNP is involved in nodulation in *D. biflorus* has stimulated recent research on this protein in legumes and now clones and sequences are available from soybean, alfalfa (*Medicago sativa*), *Medicago truncatula*, *Lotus japonicus*, and pea (Roberts et al., 1999; Day et al., 2000; Cohn et al., 2001; Shibata et al., 2001). These sequences have been shown to fall into a legume-specific clade containing Db-LNP and another clade that also contains two Arabidopsis apyrase-like proteins (Roberts et al., 1999). Roberts et al. (1999) have shown that the legume-specific alfalfa and pea proteins bind to hog blood group A + H-Sepharose and, hence, can be referred to as LNPs. In soybean, the legume-specific GS52 protein appears to be located in the plasma membrane, whereas the nonspecific GS50 protein seems to be associated with the endomembranes. GS52 is also induced by rhizobial root inoculation and, therefore, has been termed an early nodulin (Day et al., 2000).

We are interested in the perception of Nod factors, particularly in *Medicago* spp., and have characterized two Nod factor-binding sites (NFBS1 and NFBS2) in plant extracts (Bono et al., 1995; Gressent et al., 1999). To establish whether the apyrases could represent one of these sites or an additional one, we initiated a study of the apyrase-like genes in the model legume *M. truncatula*. The small genome, inbreeding genetic system, and ability to transform present a number of advantages for the functional analysis of a gene in this legume (Barker et al., 1990; Cook, 1999). Moreover, expressed sequence tag (EST) databases provide a rich source of clones and expression data (Bell et al., 2001; Journet et al., 2002). A previous study (Cohn et al., 2001) has described four genes in this legume and three of them (*Mtapy1*, *Mtapy3*, and *Mtapy4*) belong to the legume-specific clade and are

tightly linked on linkage group 7, whereas *Mtapy2* is more similar to the Arabidopsis sequences and is located on linkage group 2. *Mtapy1* and *Mtapy4*, but not *Mtapy3*, have been shown to be expressed in roots and to be induced within 3 h by inoculation with *Sinorhizobium meliloti*. *Mtapy1* did not seem to be expressed in roots of two symbiotic mutants, *dmi1* and *pdl*, leading to the suggestion that apyrases play a role early in the nodulation response before the involvement of root cortical cell division leading to the nodule structure (Cohn et al., 2001). Our studies provide additional information on the structure and expression of the apyrase gene families in *M. truncatula* and reveal some fundamental differences to the previously published report.

RESULTS

Characterization of *M. truncatula* cDNA Clones Related to Apyrases

Using primers to conserved regions of plant apyrase genes, a *M. truncatula* apyrase fragment of 386 bp was obtained by reverse transcriptase (RT)-PCR to root mRNA. This fragment was used to screen about 300,000 clones from each of two cDNA libraries made from rhizobial-inoculated roots (Szybiak-Strozycka et al., 1995.) or 4-d-old nodules. The nodule library has been used to provide EST sequences for the *M. truncatula* databases (Journet et al., 2002). Fifty-three clones were identified, some of which were plaque purified and converted into plasmid form. Sequencing of some of these clones and PCR experiments using specific primers on the others resulted in the assignment of the clones to five different genes. A sixth gene was identified from a flower cDNA clone and close analysis of The Institute for Genomic Research (TIGR) *M. truncatula* databases revealed an additional three clones related to this sequence that had been assigned to a cluster of clones related to one of the other genes. One of the longest cDNA clones of each gene was completely sequenced on both strands and the sequences compared with each other (Table I). Five of the sequences are greater than 70% and 77% identical at the protein and DNA levels, respectively, in the coding regions. The other sequence is less than 63% identical at the protein level but shows up to 73% DNA identity to the other clones in the coding region. Comparison with *M. truncatula* sequences in the databases revealed that two of the clones corresponded to the *Mtapy2* and *Mtapy4* genes (Cohn et al., 2001). Two other clones represent genes that had not been described previously (Table II). The remaining two clones were identical in either their 5′ or 3′ moiety to *Mtapy1* (Cohn et al., 2001). Inspection of the cluster containing *Mtapy1* in the TIGR *M. truncatula* Gene Index database (Quackenbush et al., 2001) revealed that all the ESTs are homologous to either the 5′ moiety or the 3′ moiety (i.e. they are homologous to one or the other

Table I. *Percentage sequence identity between the proteins and corresponding cDNA regions of the M. truncatula apyrase-like genes*

	MtAPY1;1	MtAPY1;2	MtAPY1;3	MtAPY1;4	MtAPY1;5	MtAPY2
MtAPY1;1	—	85	84	75	70	59
	—	91[a]	89	83	78	71
MtAPY1;2	85	—	91	73	71	63
	91	—	94	79	79	73
MtAPY1;3	84	91	—	73	72	61
	89	94	—	81	79	72
MtAPY1;4	75	73	73	—	70	56
	83	79	81	—	77	69
MtAPY1;5	70	71	72	70	—	60
	78	79	79	77	—	71
MtAPY2	59	63	61	56	60	—
	71	73	72	69	71	—

[a]The percentages in italics refer to the cDNA.

of our cDNA clones), but none of them span a potential fusion site. Thus, our two clones represent two different genes and the *Mtapy1* cDNA probably represents a chimera produced during the 3'- and 5'-RACE used to obtain this clone. Together, these data suggest that *M. truncatula* contains at least six apyrase-like genes (Table II).

Phylogenetic Analysis of Apyrase-Like Sequences

BLAST analyses were used to identify the closest relatives of the *M. truncatula* apyrase-like proteins from other legumes, Arabidopsis, potato, human (*Homo sapiens*), bovine (*Bos taurus*), mouse (*Mus musculus*), chicken (*Gallus gallus*), and yeast. All of the proteins identified, including all six *M. truncatula* ones, are predicted to contain the GDA1/CD39 (nucleoside phosphatase) domain as described in the Pfam database (Bateman et al., 2002). This prediction was validated by alignment of the sequences where the four "apyrase-conserved regions" (Handa and Guidotti, 1996) are clearly highly conserved (data not shown).

The complete alignment and a truncated alignment (missing the divergent and different length N- and C-terminal regions) were used in various phylogenetic analyses using the PHYLIP package of programs (Felsenstein, 1993). These analyses involved both neighbor-joining and parsimony methods. Only minor variations in tree topology were seen with the different program and the description below is based on conclusions from all the analyses. A representative analysis is shown in Figure 1.

The *M. truncatula* proteins described in this article fall into two clades, one of which seems to be legume-specific (as previously pointed out by Roberts et al., 1999) and includes the legume LNPs. This clade contains five of the six *M. truncatula* sequences. The other clade contains only one *M. truncatula* sequence and is not legume specific because it also contains the two Arabidopsis sequences previously described as Atapy1 and Atapy2. The relative similarity of the proteins in the legume-specific clade is not clear as shown by the low bootstrap values of some of the nodes, and in most analyses the Db-LNP protein is the most divergent. In the nonlegume-specific family, the pea and *M. truncatula* proteins are more similar to the Arabidopsis proteins than they are to the soybean and *D. biflorus* sequences, thus questioning the evolutionary relationship between these proteins. In most analyses the potato protein is the most divergent of the plant apyrase-like sequences.

The phylogenetic analyses also revealed that the closest relatives of the plant apyrase-like proteins are two recently described human and mouse sequences, annotated as ENP5 and ENP6 (ectonucleoside triphosphate diphosphohydrolases), and the yeast protein GDA1. The animal CD39- and CD39-like genes (now renamed as ENP1 and ENP2) are clearly more distantly related and have closer plant relatives

Table II. *Nomenclature for genes of the M. truncatula apyrase-like gene families*

New Gene Name	GenBank cDNA Accession Nos.	Corresponding Gene[a]
MtAPY1;1	AY180377	3' Moiety of *Mtapy1* cDNA
MtAPY1;2	AY180378	New
MtAPY1;3	AY180379	5' Moiety of *Mtapy1* cDNA
		Mtapy3 (only 3' sequence available)
MtAPY1;4	AY180380	*Mtapy4*
MtAPY1;5	AY180381	New
MtAPY2	AY180382	*Mtapy2*

[a]From Cohn et al. (2001)

Figure 1. Phylogenetic tree of various apyrase-related proteins of *M. truncatula* (MtAPY), Arabidopsis (At), potato (St), soybean (Gs), *D. biflorus* (Db), pea (Ps), *L. japonicus* (Lj), chicken, human, bovine, mouse, and yeast. The accession numbers of each sequence are cited in "Materials and Methods." Alignment of the amino acid sequences was performed using ClustalX and the phylogenetic analysis used the PROTDIST (with 100 bootstrap analyses) and KITSCH neighbor-joining program of the PHYLIP package. The number of times each node is supported by the bootstrap analysis is indicated. The tree is rooted arbitrarily. The MtAPY sequences are shown in bold and the legume-specific clade is boxed.

in the form of four proteins predicted from the Arabidopsis genome sequencing project. Interestingly, BLAST analysis to *M. truncatula* EST and other databases did not reveal any close *M. truncatula* relatives to these proteins.

A Modified Nomenclature for the *M. truncatula* Apyrase-Like Genes

A phylogenetic analysis using the cDNA sequences related to the plant proteins described above revealed a similar phylogenetic tree topology to the tree obtained with the protein sequences (data not shown). Therefore, the classification of the *M. truncatula* apyrase-like sequences into two clades is sup-

ported by analyses at both the protein and DNA levels and also by the high bootstrap values obtained.

Due to the clear separation of the *M. truncatula* genes and their encoded proteins into two phylogenetic classes, it is proposed to name these two type of genes *APY1* (the legume-specific class) and *APY2*, with the members of the *APY1* class being designated as members of a multigene family (Table II). This nomenclature follows the guidelines of the Commission on Plant Gene Nomenclature (Price and Reardon, 2001) and the guidelines for genetic nomenclature for *M. truncatula* (VandenBosch and Frugoli, 2001). Moreover, it involves a minimum number of changes to the names given by Cohn et al. (2001), but eliminates the problem caused by their potentially chimeric cDNA clone. The prefix Mt for *M. truncatula* is used to distinguish between similarly named but not necessarily orthologous genes of Arabidopsis and other plant species.

Differential Organ Expression of the *M. truncatula* Apyrase-Like Genes by Northern and in Silico Analysis

An "in silico" analysis of the frequencies of specific ESTs in different libraries in the *M. truncatula* databases can be used to elucidate gene expression patterns (Journet et al., 2002). Such an analysis was performed on the 134,477 *M. truncatula* ESTs available in the September 2001 version of the Functional Genomics in *M. truncatula* web site (Journet et al., 2002) from the sequencing of clones from 31 different cDNA libraries. BLAST interrogation of the GenBank *Medicago* ESTs revealed 116 sequences related to the apyrase-like cDNAs. Twenty-five of them were considered redundant because they had homologs in the same bank starting at the same nucleotide and, hence, may have arisen during amplification of the libraries used for sequencing. The frequency of expression of each gene in 12 different conditions (see Web site of Journet et al., 2002) was calculated from the number of specific and total ESTs in banks grouped into these conditions (Table III). Three of the genes, *MtAPY1;1*, *MtAPY1;2*, and *MtAPY1;4*, appear to be poorly expressed with only one to three ESTs each. Interestingly all three *MtAPY1;2* ESTs arose from flower libraries, whereas two of the three *MtAPY1.1* ESTs came from roots infected with arbuscular mycorrhizae. The *MtAPY2* gene, with 14 ESTs, seemed to be expressed in many organs but with a relatively higher number of ESTs from the stem library. The *MtAPY1;3* gene shows the highest level of expression with 38 representatives. This gene and *MtAPY1;5* show a remarkable induction in leaves after insect herbivory stress (18 and 26 ESTs from this library), which is not seen in leaves infected by a fungus. *MtAPY1;3* was the only other gene with representatives (three) in the flower library, whereas *MtAPY1;5* was relatively overrepresented in the stem libraries with four ESTs. Only two ESTs were found

Table III. *In silico expression analysis of the M. truncatula apyrase-like genes by EST analysis*

Origin of Banks[a]	No. of Banks	No. of ESTs	Specific Gene ESTs as Normalized No.[b] (or Actual No.)					
			MtAPY1;1	MtAPY1;2	MtAPY1;3	MtAPY1;4	MtAPY1;5	MtAPY2
Roots (various)	9	23,885	0	0	0.04 (1)	0	0	0.08 (2)
Roots (mycorrhizal)	2	12,246	0.02 (2)	0	0	0	0	0.04 (3)
Roots (rhizobial)	3	9,116	0	0	0.44 (4)	0.11 (1)	0	0.11 (1)
Nodules	5	17,172	0	0	0.06 (1)	0	0	0.06 (1)
Stems	1	10,314	0	0	0.10 (1)	0	0.39 (4)	0.48 (5)
Leaves (various)	3	19,008	0	0	0.32 (6)	0	0.05 (1)	0
Leaves (fungal)	1	6,003	0	0	0.17 (1)	0	0	0
Leaves (insect)	1	9,921	0	0	1.81 (18)	0	2.61 (26)	0.10 (1)
Plantlets	2	9,444	0.11 (1)	0	0.33 (3)	0	0	0
Flowers	1	3,404	0	0.88 (3)	0.88 (3)	0	0	0
Seeds/pods	3	5,038	0	0	0	0	0	0
Cell cultures	1	8,926	0	0	0	0	0.11 (1)	0.11 (1)
Total	32	134,477	0.022(3)	0.022(3)	0.281(38)	0.007(1)	0.236(32)	0.103(14)

[a]The names and origins of the banks are described in "Materials and Methods."   [b]Expressed as no. of specific ESTs per 1,000 ESTs.

in libraries made predominantly from nodule tissue (one each of *MtAPY1;3* and *MtAPY2*), suggesting that the genes are poorly expressed in this organ. No evidence was obtained for induction by phosphate starvation (data not shown).

Northern analysis of root, nodule, leaf, and flower RNA was used to validate part of the "in silico" analysis (Fig. 2). For this experiment, specific probes corresponding to the 3'-untranslated regions of the cDNAs were used, which in Southern experiments with the different apyrase clones were shown to be gene specific (data not shown). Analysis of the abundance of mRNA of each individual *MtAPY* gene confirmed that *MtAPY1;3* and *MtAPY2* are generally the more highly expressed genes. The mRNA of *MtAPY1;1* and *MtAPY1;4* could hardly be detected by northern analysis, and although evidence was obtained that *MtAPY1;2* is expressed in flowers (data

Figure 2. Northern analysis of the expression of the *M. truncatula* apyrase-like genes in various organs. Roots, nodules, leaves, and flowers were harvested from aeroponically grown plants and used for RNA extraction. Ten micrograms of total RNA was run on agarose gels, blotted onto nylon membranes, and hybridized with radiolabeled specific 3' probes from the cDNAs. The blots were exposed for different times depending on the level of hybridization.

not shown), the probe for this gene also cross hybridized with *MtAPY1;3*. In the four organs examined, the northern analysis confirmed that *MtAPY1;3* is relatively highly expressed in flowers compared with the other genes and that *MtAPY1;5* has a very specific and relatively high expression in leaves (Fig. 2). In roots and nodules, *MtAPY1;3* and *MtAPY2* were the more highly expressed genes as predicted even from the low number of apyrase-like ESTs from these organs.

The Apyrase Genes Are Induced in Roots But Not Specifically by Rhizobia or Rhizobial Nod Factors

Cohn et al. (2001) have shown that certain *M. truncatula* apyrase genes are transiently induced within 6 h postinoculation with *S. meliloti* and have stated that data using rhizobial mutants suggest that only rhizobia that are able to produce Nod signals are able to induce the transcription of these genes. To directly investigate whether addition of Nod factors induces the apyrase-like genes of *M. truncatula*, we grew plants in aeroponic containers, starved them of a fixed nitrogen source for 4 d, and then added 10^{-9} M Nod factors to the root systems. To assess the potential diurnal variation in expression of the apyrase-like genes, some plants were harvested at various times during the day before the Nod factor addition. The expression of the apyrase-like genes were analyzed by northern analysis using a probe that cross hybridizes well with all the *M. truncatula* apyrase genes of the legume-specific (*APY1*) family (Fig. 3). Surprisingly, a strong and transient increase in *APY1* mRNA was observed 6 to 8 h after the start of the harvesting without addition of Nod factors. The following day when Nod factors were added, an increase was also observed but was weaker than before. In contrast, two early nodulin genes, *MtENOD11* (Journet et al., 2001) and *MtRip1* (Cook et al., 1995), showed induced expression only after ad-

Figure 3. Effect of Nod factors on the expression of the *M. truncatula* APY1 genes in roots of aeroponically grown plants. Samples were taken at various hours during Day 4 after nitrogen starvation and then at Day 5 after addition of 10^{-9} M NodSm factors (+NF). T = 0 refers to the first harvest of the day (at 10 AM). Northern analysis was performed on blots, prepared as described in Figure 2, and hybridized with a probe for the *MtAPY1* genes. The expression of the nodulins *ENOD11* and *Rip1* was used as controls for Nod factor induction.

dition of Nod factors and not during the preceding day. It is noteworthy that in accordance with previous studies, *MtRip1* has a much higher basal level of expression than *MtENOD11*, but for both genes the effect of Nod factor addition was clear. In other experiments when separate aeroponic chambers were used, both control and Nod factor-treated plants showed a similar transient induction of *APY1* genes, peaking at about 6 h after the start of harvesting (data not shown), whereas *MtENOD11* was induced only after addition of Nod factors. Thus *APY1* genes show a transient induction response that does not require Nod factors.

Thus, we decided to test whether, in our system, the apyrase-like genes are induced by *S. meliloti* as proposed by Cohn et al. (2001) and to see whether such an induction was dependent on the initiation of the symbiosis. The expression of *MtENOD11* was used as a control for symbiotic induction. Using wild-type plants with a wild-type *S. meliloti* strain, *MtENOD11* induction was observed at 6 h postinoculation, with its mRNA increasing in abundance up to the end of the experiment (96 h postinoculation). When one of the two partners was incapable of forming the symbiosis (the *S. meliloti nodA* mutant or the *M. truncatula dmi1* mutant), no induction of this gene was observed. However, the *APY1* mRNA was transiently induced by 6 h postinoculation in all three plant-bacterial couples and, thus, occurred irrespective of the initiation of the symbiosis (Fig. 4). Therefore, in our aeroponic chambers the level of apyrase mRNA does increase within 6 h of the start of harvesting, but this induction is independent of the addition of either rhizobia or Nod factors.

In the final experiment of this series, plants were grown in growth pouches and inoculated with rhizobia or water mock inoculated, taking care to disturb the plants as little as possible during the experimentation. The roots from several pouches were

harvested either 3 or 6 h later (Fig. 5). In this experiment, no change in the level of *APY1* mRNA was seen by 6 h after treatment, indicating that the apyrase genes are not appreciably induced by rhizobia during this time frame.

To examine whether all the *MtAPY* genes are subject to the induction response we measured, in an induction experiment with wild-type plants, the abundance of mRNA of each individual *MtAPY1* gene by northern analysis using the gene-specific 3'-untranslated region probes (Fig. 6). The results indicated that all four of the tested *MtAPY1* genes show an induction response; however, the mRNA of the *MtAPY1;3* gene was clearly severalfold more abundant in roots than the mRNA of the other *MtAPY1* genes. Cohn et al. (2001) showed induction of *MtAPY1;1* (*Mtapy1*) and *MtAPY1;4* (*Mtapy4*) genes but failed to detect *MtAPY1;3* (*Mtapy3*) mRNA in roots. However, we found that the 3' primer used for *MtAPY1;3* detection in their RT-PCR approach is beyond the polyadenylation point of the mRNAs that we have detected for this gene in root cDNA clones and, thus, would not detect all the mRNA derived from this gene.

Cohn et al. (2001) have also reported that two nodulation deficient mutants of *M. truncatula*, *dmi1*

Figure 4. Effect of rhizobial inoculation on the expression of the *M. truncatula* APY1 genes in roots of aeroponically grown plants. Wild-type (Wt) or mutant *dmi1* plants were starved of nitrogen for 4 d and then inoculated with either wild-type or *nodA S. meliloti* at T = 0. Samples were harvested at various hours after inoculation. Blots were prepared as described in Figure 2 and hybridized with a probe for the *MtAPY1* genes or for *ENOD11*.

Figure 5. Effect of rhizobial inoculation on the expression of the *M. truncatula APY1* genes in roots of plants grown in growth pouches. Plants were grown in growth pouches without a nitrogen source for 8 d before inoculation with *S. meliloti* or mock inoculation with water and roots were harvested 3 or 6 h later. Blots were prepared as described in the legend to Figure 2 and hybridized with a probe for the *MtAPY1* genes.

and *pdl*, do not express apyrases to any detectable level, whereas another mutant, *lin*, showed wild-type basal levels and rhizobial induction of apyrase mRNA. We used these three mutants and several other symbiotic mutants (Table IV) to look at both basal levels of apyrase mRNA in roots and the levels of apyrase mRNA at 6 h during an induction experiment. It was clear from this experiment that *MtAPY1;3* is the most highly expressed gene in the roots of all the mutants and that all the mutants showed the induction response (Fig. 6). Although the level of abundance of the mRNA corresponding to the *MtAPY1;1*, *MtAPY1;4*, and *MtAPY1;5* genes were toward the limit of detection, these genes also appeared to be expressed in all the mutants and to show the induction response. The level of mRNA of *MtAPY1;2* could not be detected specifically. The *MtAPY2* gene was clearly the second most highly expressed apyrase gene in roots of all the mutants and did not show a consistent induction response, in accordance with the results of Cohn et al. (2001). Thus, our experiments did not show any clear differences in the expression of the apyrase genes in any of the symbiotic mutants; all the mutants, even those that are deficient in almost all symbiotic responses, showed the induction phenomenon that appears to occur for *MtAPY1;1*, *MtAPY1;3*, *MtAPY1;4*, *MtAPY1;5*, and not *MtAPY2*.

The Induction of Apyrases in Roots Is Stress-Related But Is Not Mediated by Ethylene

The previous experiments have shown a rapid induction of *APY1* mRNA in the aeroponic chambers that occurs independently of rhizobial addition. Several experiments were carried out to investigate this response further.

In the first experiment, we investigated whether this response was mediated by ethylene. Two mechanisms were used to inhibit ethylene responses: Either the ethylene synthesis inhibitor, L-alpha-(2-aminoethoxyvinyl)-Gly (AVG), was included in the medium of wild-type plants, or the mutant *skl* was used that is ethylene insensitive (Penmetsa and Cook, 1997). The results in Figure 7 show that the level of *APY1* mRNA increased by 6 h whether or not ethylene signaling had been inhibited; thus, the induction response is independent of ethylene.

Another possible reason for the increased expression of the *APY1* genes during our experiments could be an endogenous rhythm. In all our experiments, plants were grown in a 16-h-light/8-h-dark regime and the zero time point was taken at 10 AM Greenwich Mean Time (10 h), 6 h into the light period. In the following experiment, two aeroponic chambers were used (Fig. 8A). The first samples were harvested at 4 PM (corresponding to a normal 6-h induced time), and then the next day more samples were harvested at 0, 3, 6, and 24 h, starting at either 10 AM or at 4 PM. (In this experiment, care was taken not to disturb unduly the root systems to be harvested at later time points.) The results show that the level of *APY1* mRNA is not at the high level at 4 PM unless preceded by other harvests. Moreover, although an increase in *APY1* mRNA was observed at the 3-h time point when harvesting was started at 4 PM, this was not maintained at 6 h. One reason for this result could be that by this time the plants were already in the dark period; thus, the induction response could be influenced by light.

To investigate this possibility further, plants were grown in growth pouches in a 16-h-light/8-h-dark regime with their root systems either exposed to the

Figure 6. Expression of the apyrase-like genes in roots of aeroponically grown symbiotic mutants of *M. truncatula*. Wild-type (Wt) or various mutant plants were starved of nitrogen for 4 d and then treated with 10^{-9} M NodSm factors and samples were harvested at T = 0, 3, 6, and 24 h. For the mutants, only the T = 0 and 6 h samples are shown. Blots were prepared as described in Figure 2 and hybridized with specific 3' probes from the various *APY1* or the *APY2* cDNAs.

Table IV. *Plant lines and rhizobial strains used in this study*

Designation	Line/Strain	Relevant Characteristics[a]	Reference/Source
M. truncatula			
Wild type	Jemalong A17	Nod$^+$Fix$^+$ with *S. meliloti*	Penmetsa and Cook (1997)
dmi1	Y6	Has$^+$, Inf$^-$, Ccd$^-$, and Nod$^-$	Penmetsa and Cook (1997); Catoira et al. (2000)
dmi2	TR25	Has$^+$, Inf$^-$, Ccd$^-$, and Nod$^-$	Catoira et al. (2000)
dmi3	TRV25	Has$^+$, Inf$^-$, Ccd$^-$, and Nod$^-$	Catoira et al. (2000)
hcl	B56	Hab$^+$, Inf$^-$, Ccd$^+$, and Nod$^-$	Catoira et al. (2001)
nsp	B85	Hab$^+$, Inf$^-$, Ccd$^-$, and Nod$^-$	Catoira et al. (2000)
pdl (Poodle)	BC$_1$F$_4$	Hab$^+$, Inf$^-$, Ccd$^-$, and Nod$^-$	R.V. Penmetsa and D.R. Cook (unpublished data)
lin (Lumpy infections)	M4	Hab$^+$, Inf$^{+/-}$, Ccd$^+$, and Nod$^-$	R.V. Penmetsa and D.R. Cook (unpublished data)
skl	BC$_1$F$_4$	Nod^{+++}, ethylene insensitive	Penmetsa and Cook (1997)
S. meliloti			
Wild type	GMI6526	2011(pXLGD4), Nod$^+$Fix$^+$ on *M. truncatula*	Ardourel et al. (1994)
NodA	GMI6702	2011nodA::Tn5#2208(pXLGD4). Hab$^-$, Inf$^-$, Ccd$^-$, and Nod$^-$ on *M. truncatula*	Debellé et al. (1986)

[a]Responses to rhizobia: root hair swelling (Has) or branching (Hab), infection threads (Inf), cortical cell divisions (Ccd), and nodulation (Nod).

light or not (Fig. 8B). Root systems were then harvested at 10 AM ($T = 0$) and 3 and 6 h later and subjected to northern analysis. It is clear that after prolonged growth with the root systems in the light the level of root *APY1* mRNA is higher than if the roots are kept in the dark. Moreover, the level of *APY1* mRNA did not increase during the day of harvesting; in fact, the dark-grown root systems seemed to decrease at the 6-h time point. If the roots of plants with dark-grown root systems were exposed to the light at 10 AM, no increase in *APY1* mRNA was observed either 3 or 6 h later, thus suggesting that the induction response is not due to short-term exposure of the root systems to light. If similarly grown plants were covered in black plastic to also place the shoots in the dark, the level of *APY1* mRNA decreased even further at the 6-h time point. Finally, if the roots were switched to the light and the shoots to the dark, the level of the mRNA again decreased by 6 h. These experiments show that exposure of the shoots or the roots to light does have some influence on the level of root *APY1* mRNA, but that it does not lead to short-term increases and, therefore, probably is not the cause of the induction of the *APY1* mRNA in the aeroponic chambers.

Figure 7. Role of ethylene in the induction of the *M. truncatula APY1* genes in roots of aeroponically grown plants. Wild-type (Wt) plants grown either in the presence or absence of an ethylene inhibitor (AVG) or plants of the mutant defective in ethylene perception (*skl*) were starved of nitrogen for 4 d and harvesting was started at 10 h ($T = 0$). Northern analysis was performed on blots prepared as described in Figure 2 and hybridized with a probe for the *MtAPY1* genes.

DISCUSSION

The report that a *D. biflorus* apyrase-like protein (Db-LNP) binds Nod factors and that antibodies raised against it inhibit root nodulation (Etzler et al., 1999) suggests that this protein plays an important role in the establishment of the symbiosis between this legume and rhizobia. If these proteins play an essential rather than facultative role in nodulation, it would be expected that Db-LNP orthologs play similar roles in other legume species. Day et al. (2000) have shown that apyrase-specific antisera inhibit nodulation in soybean and that a soybean apyrase-like gene is induced early during nodulation. However, *D. biflorus* and *G. soja* both belong to the Phaseoleae and produce determinate nodules in which cell division begins in the outer cortex. Many other species of importance to temperate agriculture (for example, those belonging to the genera *Medicago*, *Pisum*, *Vicia*, and *Trifolium*) are taxonomically more distant and produce indeterminate nodules in which cell division begins in the inner cortex and pericycle (Gualtieri and Bisseling, 2000). Thus, it is important to establish whether apyrase-like proteins play an important symbiotic role in these species. Among these species is the model legume *M. truncatula*, in which genetic and genomic tools have been developed (Cook, 1999).

As a first step to a functional analysis of the role of apyrase-like proteins in indeterminate nodulation, we have characterized the gene families encoding these proteins in *M. truncatula*. Through isolation of cDNA clones from root and nodule libraries, we have identified five genes related to plant apyrase-like proteins and an additional gene has been identified from the *M. truncatula* EST databases. Phylogenetic analysis has revealed that the proteins encoded by five of these genes fall into the legume-specific class of sequences identified by Roberts et al. (1999), whereas the sixth gene is more similar to two Arabi-

Figure 8. Effect of time of harvesting and the light regime on the expression of the *M. truncatula APY1* genes in roots. A, Aeroponically grown plants were starved of nitrogen for 4 d and the roots harvested at different times during the daylight periods of 16-/8-h diurnal cycles (light period from 4–20 h). The first harvest on Day 5 is T = 0. B, Plants were grown in diurnal cycles (16/8 h) in growth pouches with the root systems either in the light (L) or the dark (D). At the start of harvesting, the exposure to light of some of the plants with root systems in the dark was altered: transfer of root systems to light (D-L), transfer of shoots to dark (D-D), and transfer of roots systems to light and shoots to dark (D ⇌ L). Roots were harvested at 3 and 6 h starting at 10 h (T = 0). Northern analysis was performed on blots prepared as described in Figure 2 and hybridized with a probe for the *MtAPY1* genes.

dopsis proteins. We have confirmed and refined the position of one of the legume-specific genes on linkage group 7 (A. Kereszt, A. Niebel, J. Cullimore, and T. Huguet, unpublished data), which has been shown previously to be part of a cluster of four tightly linked genes, whereas the other gene is located on linkage group 2 (Cohn et al., 2001). Based on the phylogenetic analysis and in accordance with the guidelines for naming plant genes (Price and Reardon, 2001), we propose to denote the five genes as members of the *MtAPY1* gene family (*MtAPY1;1, 1;2, 1;3, 1;4,* and *1;5*), whereas the other gene is referred to as *MtAPY2*. *APY* is preferred as the gene mnemonic to *LNP* because *APY* is the precedent to *LNP* in the literature and the four regions of the apyrase domain are clearly very highly conserved (data not shown), whereas the position of the putative lectin domain has yet to be defined.

Do these six genes represent the total number of apyrase-like genes in *M. truncatula*? This question may only be definitively answered by the complete sequencing of the whole genome. Cohn et al. (2001) identified four of these genes by a combination of library screening, RACE experiments, and analysis of the *M. truncatula* EST banks. The possibility raised here that the gene they named *Mtapy1* may be based on a chimeric clone of *MtAPY1;1* and *MtAPY1;3* illustrates the dangers of using RACE to elongate

clones related to multigene families. By renaming the genes as members of the *MtAPY1* multigene family, we have kept as close as possible to their preceding nomenclature while eliminating the confusion caused by their potentially chimeric clone. Close analysis of the current EST banks (TIGR version 5.0) has revealed that there may be an additional *APY1* and an *APY2* gene. Moreover, a 6-bp deletion near the encoded C terminus in several clones that are otherwise identical may suggest that there is an additional gene closely related to *MtAPY1;1*. However, at present we cannot exclude that these variants have arisen either by posttranscriptional modification or are alleles due to polymorphisms in the seed stocks. In pea, which is closely related to *Medicago* spp., two different types of cDNA and genomic clones differing only in six point mutations near the N terminus have been identified, but again, whether they are alleles or different genes is not clear (Shibata et al., 2001). These clones are interestingly putative orthologs to *MtAPY1;1*, suggesting that this gene may be particularly susceptible to variation.

In addition to the *MtAPY1;1* and pea gene orthologs, it is also clear that the alfalfa gene named *MsLNP* (Roberts et al., 1999) is the ortholog of *MtAPY1;4* (Fig. 1). In relation to the aim of this work, this analysis failed to discover a clear single ortholog of *Db-LNP* in *M. truncatula*, which would be the likeliest candidate to play a similar symbiotic role. At present, studies of the symbiotic role of *Db-LNP*-related genes in *M. truncatula* would need to include all five members of the *MtAPY1* family.

The phylogenetic analyses (Fig. 1) also revealed that the closest mammalian homologs of the plant apyrases are not CD39- and CD39-like genes (now named ENP1 and ENP2) as previously described (Roberts et al., 1999) but are a recently reported group of apyrase-like proteins from rat, human, and mouse named ENP5 and ENP6 (Chadwick and Frischauf, 1998). Comparative studies of these related proteins may lead to new ideas of their function in different organisms.

Another way in which a potential symbiotic function of a gene is inferred is by its expression pattern. In this respect, it is interesting to note that the soybean GS52 gene has been described as an early nodulin gene (Day et al., 2000), and that two of the *M. truncatula* genes, *Mtapy1* (*MtAPY1;1* or *1;3*) and *Mtapy4* (*MtAPY1;4*) have been reported to be induced by rhizobia (Cohn et al., 2001). In this latter paper, transient increases in the abundance of mRNA of the two genes were observed at 3 and 6 h postinoculation and data not shown suggested that this increase was dependent on the ability of the rhizobia to produce Nod signals. Such an expression pattern could be consistent with the encoded protein acting in Nod signal perception because there are examples in the literature of receptor genes being induced by their ligands (Tata, 2000; Beuschlein et al., 2001).

In this paper, we have confirmed that the abundance of mRNA of the *MtAPY1* genes increases transiently in roots after inoculation with rhizobia in aeroponic chambers; an increase was seen at 3 and 6 h and then decreased by 24 h (Fig. 4). These results are similar to those obtained by Cohn et al. (2001), who also used aeroponic chambers for some of their experiments. However, we found that no such response to rhizobia was seen if the plants were grown in growth pouches (Fig. 5). Further analysis of this surprising result showed that the response in aeroponic chambers was not dependent on Nod factors (Fig. 3), the ability of the rhizobia to produce Nod factors (Fig. 4), or the addition of the rhizobia itself: A similar response occurred without any additions. Thus, we have not found any evidence to support the notion that the expression of the apyrase-like genes in *M. truncatula* are induced transiently by rhizobia or by Nod factors in the early stages of the legume-rhizobia symbiosis. Therefore, our studies do not support a description of *MtAPY1;1* and *MtAPY1;4* as ENOD (early nodulin) genes.

We also examined the levels of apyrase-like mRNA and the induction response in roots of a number of symbiotic mutants (Fig. 6). Some of these are mutated in components of a Nod factor signal transduction pathway (*dmi1*, *dmi2*, and *dmi3*) and others have later defects in nodulation (*hcl*, *nsp1*, *pdl*, *lin*, and *skl*). The results showed that there was no great difference in the levels of *MtAPY1* mRNA in roots of any of the mutants compared with wild type and that the induction response occurred in all the mutants. Although the mRNA of some of the individual *MtAPY1* genes was near the limit of detection, it seems that at least four of the *MtAPY1* genes show an induction response and that this occurred in all the mutants. Thus, most of the *MtAPY1* genes are affected by the induction response and studies with plant mutants suggests that this induction occurs independently of the establishment of the symbiosis. Our results are in contrast to those of Cohn et al. (2001), who failed to detect *MtAPY1* mRNA in the *dmi1* and *pdl* mutants and suggested that *MtAPY1* expression is correlated with the formation of nodule primordia. We have no explanation for the discrepancy between the two studies.

If the induction response is not due to rhizobia or rhizobial Nod factors, what could it be due to? The following possibilities were examined: (a) an endogenous rhythm/diurnal variation, (b) exposure of the roots to light, or (c) stress related to the harvesting of multiple samples from the aeroponic chambers. Because the higher level of mRNA is not observed at different times of the day unless it is preceded by at least one harvest, the response does not appear to be due either to an endogenous rhythm or diurnal variation. Moreover, in plants grown in pouches short term (3–6 h), exposure of the roots to light did not lead to an increase in the *APY1* mRNA levels, sug-

gesting that light is not the key factor triggering the induction although it may modulate the degree or maintenance of the response (Fig. 8). Together, these results suggest that the induction response seems to be related to the harvesting of multiple samples from the aeroponic chambers.

In most experiments, the plants were densely planted in the aeroponic chambers, and it is possible that harvesting of some samples caused physical damage to the remaining roots; hence, the *APY1* gene induction could be a direct wounding response. Consistent with this possibility is the fact that *APY1;3* and *APY1;5* ESTs are highly overrepresented in the leaf herbivory library compared with leaf libraries prepared from either pooled developmental stages, phosphate-starved plants, or fungus-infected plants (Table III), suggesting that the *APY1* genes may be induced by tissue damage. However, in Figure 8A care was taken not to physically damage the remaining plants in the aeroponic systems and yet induction still occurred, albeit at a lower level. Alternative explanations are that the induction is due to mechano-stimulation (see Johnson et al., 1998) due to movement of the root systems in the aeroponic containers during harvesting, or induction by volatiles (see Baldwin et al., 2002) released from the harvested plants. Whatever the stimulus, it is clear that the induction response is independent of ethylene because induction occurred both in wild-type plants treated with an inhibitor of ethylene synthesis and in *skl* mutant plants that are insensitive to ethylene (Fig. 7). Ethylene independence is also a characteristic of certain responses to mechanostimulation (Johnson et al., 1998) and elicitation of some defense genes (Yamamoto et al., 1999).

Expression via both EST and northern analysis have shown that, in the conditions studied, *MtAPY1;3*, *MtAPY1;5*, and *MtAPY2* are clearly more highly expressed than *MtAPY1;1*, *MtAPY1;2*, and *MtAPY1;4*, whose mRNAs are rare (less than 0.003% of total ESTs). *MtAPY2* mRNAs have been detected in most organs studied; hence, this gene shows a rather constitutive expression, whereas the *MtAPY1* genes show a more differential organ expression (*MtAPY1;3* and *MtAPY1;2* are higher in flowers and *MtAPY1;5* is higher in leaves). *MtAPY1;3*, with *MtAPY2*, is the most highly expressed *MtAPY* gene in roots, in contrast to the results of Cohn et al. (2001) who failed to detect *MtAPY1;3* mRNA in this organ. The observation that all five *MtAPY1* genes can be transiently induced in roots suggests that this ethylene-independent stress-like response is imposed on other controls regulating the qualitative and quantitative expression of these genes. What is the purpose of this response? Our data have shown clearly that it is not specific to the rhizobial symbiotic interaction and, thus, may have a role in other processes. Thomas et al. (2000) have shown that Arabidopsis plants overexpressing apyrases are more re-

sistant to xenobiotics; hence, one possibility is that a stress induction may be in anticipation of toxin exposure.

In conclusion, *M. truncatula* contains at least five (*MtAPY1*) genes, which from phylogenetic analysis are members of a legume-specific clade including the *D. biflorus* Db-LNP. In addition, there is a *MtAPY2* gene that is more similar to Arabidopsis apyrase-like genes. Expression studies have shown that the *MtAPY1* genes are induced in roots by stress and that this response is not mediated by ethylene. No evidence has been found for a transient induction of these genes by rhizobia or Nod factors. Whether these genes could, however, play a role in the establishment of the symbiosis, independently of a symbiotic-specific regulation of expression, is under investigation.

MATERIALS AND METHODS

Growth Conditions and Plant Material

All plant lines and bacterial strains are listed in Table IV. *Medicago truncatula* Gaernt. cv Jemalong lines A17 or J5 (these lines are probably identical; T. Huguet, personal communication) were grown in aeroponic chambers (unless otherwise indicated) in a 16-h photoperiod on a medium containing 5 mM NH_4NO_3 for 2 weeks essentially as described by Journet et al. (2001). The media were then replaced by similar media lacking a nitrogen source, and the plants were then starved of nitrogen for 4 d before either inoculation with *Sinorhizobium meliloti* (Table IV), treatment with 10^{-9} M NodSm factors, or other treatments. Plant mutant lines were obtained from Prof. D. Cook (University of California, Davis), Dr. G. Duc (INRA, Dijon, France), or Dr. J. Dénarié (INRA, Toulouse, France) as indicated in Table IV and grown in the same way. Treatment with the ethylene inhibitor AVG involved the addition of 0.88 μM AVG 17 h before the first harvest. Note that the plants for different time points of an experiment were grown in the same aeroponic chamber; thus, all the root systems were exposed to light and potential stress during the few minutes of harvesting. In most experiments, about 150 plants were grown in an area of 0.16 m^2 and roots corresponding to at least five plants were harvested for each sample and frozen immediately in liquid nitrogen and stored at -80°C. In the experiment in Figure 8A, the plants were grouped in five lots of 25 plants and one lot was harvested per time point. In some experiments (Figs. 5 and 8B), plants were grown in growth pouches for 8 d using media lacking a nitrogen source essentially as described by Vernoud et al. (1999). After the various treatments, roots of plants from several whole pouches were harvested for each time point.

Isolation and Characterization of cDNA Clones

RT-PCR was used to obtain a fragment corresponding to nucleotides 173 through 559 of *MtAPY1;3*, and this fragment was radiolabeled with ^{32}P and used to screen about 300,000 plaque-forming units of each of two *M. truncatula* cDNA libraries prepared, in the Lambda ZAP vector (Stratagene, La Jolla, CA), from either 4-d old nodules or from roots inoculated with *S. meliloti* and harvested 6, 24, and 48 h later. The phage were eluted from agar plugs containing the hybridizing phage, and these were used either for purification of the hybridizing phage or for PCR analysis. Purified positive phage were converted by in vivo excision to plasmid form into the vector pBluescript SK$^-$ (Stratagene) and characterized either by PCR using specific gene primers or sequencing. At least one cDNA clone encoding full-length proteins of each of the identified six apyrase-like genes was sequenced on both strands.

Sequence and Phylogenetic Analyses

Sequence data were analyzed using the GCG software (Genetics Computer Group, Madison, WI), and derived protein sequences were analyzed

using the SwissProt Expasy server. Sequences showing homology were identified using the National Center for Biotechnology Information BLAST server (Altschul et al., 1997) and recovered from either the GenBank or SwissProt databases. The following sequences (with their accession nos.) were used: PsNTPA (P52914); MsLNP (AAF00611); LjLNP (AAF00609); Gs52 (AF207688); Atapy1 (AF093604); Atapy2 (AAF66599); PsAPY2 (AB071370); Gs50 (AF207687); DbLNP (AAD31285); DbAPY2 (AAF00610); StAPY (P80595); YEAST_GDA1 (P32621); HUMAN_ENP6 (O75354); HUMAN_ENP5 (O75356); MOUSE_ENP5 (Q9W429); At-NP_565293, At-NP_172876, At-NP_172877, At_NP_563942, and BOVIN_CD39 (O18956); MOUSE_CD39 (P55772); HUMAN_CD39 (P49961); CHICK_CD39 (O93295); CHICK_ENP2 (P79784); HUMAN_ENP2 (O55026); and MOUSE_ENP2 (O55026). ClustalX (Thompson et al., 1997) was used to align the sequences, and minor adjustments were made either to improve the alignments or to delete extruding ends using GeneDoc version 1997 (Nicholas and Nicholas, 1997). Phylogenetic analyses were performed either on a 32-sequence amino acid alignment of 632 residues or on a derived truncated alignment of 521 residues using the PHYLIP package of programs (Felsenstein, 1993) on the Pasteur Institute Server (http://bioweb.pasteur.fr/seqanal/phylogeny/). PROTDIST with 100 bootstrap analyses was used to calculate the distance between the sequences before analysis with the neighbor-joining programs NEIGHBOR, KITSCH, and FITCH, and calculation of the consensus tree using CONSENSE. The parsimony method used the PROTPARS program, again with 100 bootstrap analyses. The sequences of the 17 plant cDNAs were similarly aligned and used in phylogenetic analyses either using the above neighbor-joining programs or the DNAPARS parsimony program. All trees were viewed and edited using TREEVIEW (Page, 1996).

Comparisons with *M. truncatula* ESTs and Electronic Northern Analyses

The full-length cDNAs reported here were used in BLAST analyses with the GenBank *Medicago* ESTs and the *M. truncatula* Gene Index Release 4.0 database at TIGR (http://www.tigr.org/tdb/tgi/mtgi/) or the *M. truncatula* EST database (version September 2001) at the Institut National de la Recherche Agronomique (http://medicago.toulouse.inra.fr/Mt/EST/DOC/MtB.html), and 116 ESTs were identified. Duplicate ESTs of the same gene from the same cDNA library that started at the same nucleotide (potentially arising from the same cloning event) were eliminated from further analyses. Alignments were perused manually and each apyrase-like EST could be classified into one of six clusters centered on the cDNAs. It is noteworthy that the clustering of certain ESTs did not correspond with the database clusters, particularly for the TIGR database. To derive data on the differential expression of the six genes (electronic northern analyses), the 32 cDNA banks were classified into 12 classes of similar origin and the frequency of expression of each gene in each class was calculated by the number of ESTs divided by the total ESTs and expressed per 1,000 ESTs. The 12 classes were slightly modified from that described at http://medicago.toulouse.inra.fr/Mt/EST/DOC/MtB.html and contained the following banks (all are prefixed by Mt): roots, various (NF-RT, RHE, RP, MHRP, KV0, BA, DSIR, MGHG, and HOGA); roots, rhizobial (KV1, KV2, and KV3); roots, mycorrhizal (MHAM and BC); nodules (BB, R108, NF-NR, GVN, and GSVN); stem (NF-ST); leaves (DSLC, NF-LF, and NF-PL); leaves, fungal (DSIL); leaves, insect challenged (NF-IN); plantlets (NF-DT and NF-IR); flowers (NF-FL); seeds/pods (GESD, NF-GS, and GPOD); and cell cultures (NF-EC).

RNA Isolation and Northern Hybridization

The plant material corresponding to a sample was crushed in a mortar and pestle with liquid nitrogen, and aliquots corresponding to about 180 mg fresh weight were used for total RNA isolation using the EXTRACT-ALL kit (Eurobio, Les Ulis, France). The total RNA was dissolved in 30 μL of sterile water, and the quality and quantity of the RNA was assessed by migration on agarose gels with ethidium bromide staining and by spectrophotometry. RNA gels were run in phosphate buffer (Lummerzhein et al., 1994) using 10 μg of total RNA per track, and the RNA was then blotted on to Nytran+ membranes (Schleicher & Schuell, Dassel, Germany). Probes were prepared by labeling with ^{32}P using the Ready-To-Go kit (Amersham Biosciences, Buckinghamshire, UK) using the DNA fragments that were isolated either using restriction enzymes or by PCR. The following fragments were used to produce *MtAPY* probes: *MtAPY1* general probe (whole cDNA of *MtAPY1*;

3), *MtAPY2* probe (whole cDNA), *MtAPY1;1*-specific probe (nucleotides 1,354–1,469), *MtAPY1;3*-specific probe (nucleotides 1,345–1,444), *MtAPY1;4*-specific probe (nucleotides 1,323–1,466), and *MtAPY1;5*-specific probe (nucleotides 1,326–1,588). Probes were also prepared using cDNA fragments of the *MtENOD11* (Journet et al., 2001), *MtRip1* (Cook et al., 1995), and rRNA genes.

Prehybridization was carried out for at least 2 h using the phosphate-based solution (Lummerzheim et al., 1994). Hybridization was carried out overnight using the same solution and about 2×10^6 cpm mL^{-1} of labeled probe. The blots were then washed twice in SSC (0.15 M NaCl and 0.015 M sodium citrate) containing 0.1% (w/v) SDS at 65°C and the patterns of hybridization to the blots were analyzed after exposure to a Phosphor screen using a PhosphorImager. Different blots and probes were exposed for different times to emphasize the relative rather than quantitative differences.

Distribution of Materials

Upon request, all novel materials described in this publication (the six *MtAPY* cDNA clones) will be made available in a timely manner for non-commercial research purposes, subject to the requisite permission from any third party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor. The cDNAs have the following GenBank accession numbers: *APY1;1*, AY180377; *APY1;2*, AY180378; *APY1;3*, AY180379; *APY1;4*, AY180380; *APY1;5*, AY180381; and *APY2*, AY180382.

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