Dearnaley (2000) showed that extracellular ATP (ap-
may be involved in catalysis of this ATP. Lew and
ectoapyrases recycle and diminish the hormonal ac-
phosphate or ATP). During animal neurotransmission,
and phosphate transport (using either inorganic phos-
Arabidopsis (1999). Transgenic expression of a pea apyrase in
utes and phosphate transport and mobilization (Thomas et al.,
phosphorylase; Komoszynski and Wojtczak, 1996; Day
mediated extrusion of this antibiotic could make use
ectoapyrase resulted in enhanced resistance to cyclo-
drug resistance transporter) or ectoapyrase resulted in enhanced resistance to cyclo-
the authors suggested that symptom-
mal ATP (Gao et al., 1999).
Important to legume nodulation in response to inocula-
lowers calcium levels were coupled to downstream gene
expression, implying a complete signaling pathway
responsive to ATP.
Membrane depolarization, hormonal activity, cal-
cium oscillations, and signal transduction are all
relevant to legume nodulation in response to inocula-
with rhizobia (for review, see Cohn et al., 1997).
Therefore, ectoapyrases, perhaps through action on
ectoapyrase could be the Nod signal produced
by rhizobia. The Nod signal is essential for the in-
culation of the legume Dolichos biflorus could bind the lipo-chitin Nod signal produced
by rhizobia. The Nod signal is essential for the in-
duction of a nodule structure in response to rhizobial
infection. The ATPase activity of the Dolichos apyrase
was stimulated upon binding the Nod signal, as well
as other related ligands. Etzler et al. (1999) proposed that the D. biflorus apyrase could be the Nod signal
receptor postulated to be essential to nodulation.
However, this now seems unlikely due to the identi-
fication of the LysM domain receptor kinases as the
The soybean apyrase, GS52, was previously characterized as an early nodulin that is expressed in roots and localized to the
plasma membrane. Transgenic Lotus japonicus plants were constructed constitutively expressing the GS52 apyrase. Segregation
and Southern-blot analysis identified four single-copy sense lines, several double-copy sense lines, and one double-copy
antisense line for further analysis. The single- and double-copy sense gs52 L. japonicus lines had enhanced nodulation that
correlated with expression of the transgene. The sense transgenic lines were also found to have increased infection thread
formation and enhanced infection zone length when infected by Mesorhizobium loti, the natural symbiont of L. japonicus. The
data presented show that expression of the GS52 apyrase can enhance nodulation in L. japonicus and points to an important role
for this group of enzymes in nodulation.

Apyrases (nucleotide phosphohydrolases; EC 3.6.1.15) are nonenergy-coupled NTPases that have been observed to play diverse roles, as might be expected of enzymes that can change the ratios of
key energy carriers (e.g. ATP), inorganic phosphorus,
and signaling molecules (e.g. GMP, cAMP). The apy-
rase NTPase catalytic domain can be located both
cytoplasmically (endoapyrases) or extracellularly (ec-
toapyrases; Komoszynski and Wojtczak, 1996; Day
et al., 2000). In animals, the roles of apyrases include
modulation of neurotransmission (Edwards and Gibb,
1993) and blood platelet aggregation (Marcus and
Safier, 1993). In yeast (Saccharomyces cerevisiae), two apyr-
ases facilitate the glycosylation of N- and O-linked oligosaccharides in the Golgi lumen (Abeijon et al.,
1993; Gao et al., 1999).
In plants, apyrases were shown to play a role in
phosphate transport and mobilization (Thomas et al.,
1999). Transgenic expression of a pea apyrase in
Arabidopsis (Arabidopsis thaliana) increased growth
and phosphate transport (using either inorganic phos-
phate or ATP). During animal neurotransmission,
ectoapyrases recycle and diminish the hormonal ac-
tivity of extracellular ATP/ADP (for review, see Clark
et al., 1997). Infection. The ATPase activity of the Dolichos apyrase
approximately 1 mm) would depolarize the membrane
potential of growing Arabidopsis root hairs. Thomas
et al. (2000) showed that transgenic expression of either PGP1 (a multidrug resistance transporter) or ectoapyrase resulted in enhanced resistance to cyclo-

et al., 2003) also showed that extracellular ATP
(>1 mm) inhibited root gravitropism and polar auxin
transport. Extracellular ATP increased the sensitivity
of roots to exogenous auxin. Demidchik et al. (2003)
showed that ATP could trigger an increase in intracel-
lular calcium levels. More recently, Jeter et al. (2004)
showed that ATP-induced increases in cytoplasmic
calcium levels were coupled to downstream gene
expression, implying a complete signaling pathway
responsive to ATP.
mostly likely receptors for the Nod signal (Limpens and Bisseling, 2003; Madsen et al., 2003; Radutoiu et al., 2003). However, interaction of the Nod signal with an ectoapyrase on the root hair surface could still be relevant to nodulation.

Indeed, a direct role for apyrases in nodulation was suggested by the fact that treatment of roots with antibody directed against either the D. biflorus apyrase (Etzler et al., 1999) or the soybean (Glycine max) GS52 apyrase (Day et al., 2000) inhibited nodulation. These data suggested that the apyrase was localized to the root hair (i.e. the site of rhizobial invasion) and, indeed, Kalsi and Etzler (2000) were able to confirm this by immuno-localization of the apyrase to Dolichos root hairs. Inoculation with compatible, but not with incompatible, rhizobia led to a redistribution of the D. biflorus apyrase to the root hair tips. Day et al. (2000) were also able to localize the soybean GS52 apyrase to root plasma membrane. In addition, both Cohn et al. (2001), working with Medicago truncatula, and Day et al. (2000), working with soybean (L. Merr.), showed that inoculation with the respective, compatible rhizobium resulted in induction of specific apyrase gene expression.

An open question is what unique features are found in legumes, which allow them to be nodulated by rhizobia, compared to other angiosperms. Cannon et al. (2003) compared apyrase gene sequences from various legumes (i.e. M. truncatula, soybean, and Lotus japonicus) to one another and to those from nonlegumes (e.g. Arabidopsis). This phylogenetic analysis identified a potential legume-specific clade, which included the soybean GS52 apyrase and the Nod signal-binding D. biflorus apyrase. Comparisons of rates of change at synonymous and nonsynonymous sites in the GS52 apyrase and sister clades showed rapid evolution in the potential legume-specific clade. The authors suggested that local apyrase gene duplication in an ancestor of the legumes, followed by functional diversification and increased rates of change in the new genes, resulted in the formation of a legume-specific apyrase gene subfamily. This analysis, as well as earlier analyses (Roberts et al., 1999; Cohn et al., 2001), is consistent with the hypothesis that this legume apyrase subfamily plays a unique role in legume biology, and perhaps a critical role in nodulation.

Consistent with this hypothesis, we now report that transgenic expression of the soybean GS52 apyrase in L. japonicus plants significantly increased nodule number upon inoculation with the compatible Mesorhizobium loti. This increase in nodule number was correlated with an increased number of root hair infections and an expansion in the root zone infected.

RESULTS

Construction of gs52 Transgenic L. japonicus

At the time we instigated these studies, the gs52 gene was the only apyrase gene in our possession. Given the difficulties with soybean transformation, we opted to express this gene in L. japonicus, which is more amenable to genetic transformation. The gs52 gene and orthologous LjLNP gene of L. japonicus, isolated by Roberts et al. (1999), share approximately 80% sequence identity, suggesting that the expression of gs52 could result in silencing of the endogenous L. japonicus apyrase genes. Therefore, transgenic L. japonicus plants were constructed in which the Glycine soja gs52 gene (Day et al., 2000) was constitutively expressed from the strong cauliflower mosaic virus 35S promoter in either the sense or antisense orientation. Transformation was via Agrobacterium-mediated hypocotyl transformation as described by Stiller et al. (1997).

Analysis of gs52 Copy Number in Transgenic Plants

Primary (T1) transgenic plants were selfed and segregation of the transgene was analyzed in T2 and T3-generation seeds from individual transformed plants (Table I). Segregation of the transgene was scored based on a comparison of seedling growth on medium with and without G418 antibiotic. Segregation analysis suggested that numerous lines were either single copy (segregating 3:1) or homozygous (copy number unknown) for the transgene. Copy number was subsequently determined by Southern blotting (Fig. 1). Three single-copy sense lines, 45W T2, 45B1K T3, and 45D5 T3, and one double-copy sense line, 45B77 T3, were identified and used for further analysis. In addition, a double-copy antisense line, 44HH T3, was identified and used as a control in further analysis. With the exception of the nodulation assay, these lines were used exclusively for further analyses.

Table 1. Segregation analysis of gs52 L. japonicus plants

<table>
<thead>
<tr>
<th>Line</th>
<th>Resistant:Sensitive Segregation</th>
<th>Southern Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>45 W T2</td>
<td>34:9</td>
<td>3.8:1 1 Copy</td>
</tr>
<tr>
<td>45D5 T3</td>
<td>68:26</td>
<td>2.8:1 15</td>
</tr>
<tr>
<td>45B1K T3</td>
<td>51:15</td>
<td>3.4:1 15</td>
</tr>
<tr>
<td>45B77 T3</td>
<td>62:0</td>
<td>Homozygous a 25</td>
</tr>
<tr>
<td>45A7 T3</td>
<td>75:0</td>
<td>Homozygous 25</td>
</tr>
<tr>
<td>45B38 T3</td>
<td>64:0</td>
<td>Homozygous 25</td>
</tr>
<tr>
<td>45B1.2 T3</td>
<td>92:0</td>
<td>Homozygous 25</td>
</tr>
<tr>
<td>45B1H T3</td>
<td>60:0</td>
<td>Homozygous 25</td>
</tr>
<tr>
<td>45B35 T3</td>
<td>22:0</td>
<td>Homozygous 25</td>
</tr>
<tr>
<td>45B11 T3</td>
<td>100:0</td>
<td>Homozygous 25</td>
</tr>
<tr>
<td>45B16 T3</td>
<td>53:0</td>
<td>Homozygous 25</td>
</tr>
<tr>
<td>45B37 T3</td>
<td>73:0</td>
<td>Homozygous 25</td>
</tr>
<tr>
<td>44HH T3</td>
<td>99:0</td>
<td>Homozygous 2AS</td>
</tr>
</tbody>
</table>

aAll plants were G418 resistant and were homozygous for at least one of the two inserts. bInconclusive.
gs52 Expression Analysis of Transgenic *L. japonicus* Lines

Southern-blot analysis indicated the presence of *gs52* integrated into the genome at independent locations in each of the lines. Northern-blot analysis was carried out to determine whether the *gs52* gene was successfully expressed in these transgenic lines. As shown in Figure 2, 3 single-copy lines had varying levels of *gs52* expression. The double-copy *gs52* line, 45B77 T3, had strong *gs52* expression. In addition, the northern blot indicated that neither the wild-type *L. japonicus* nor the antisense *gs52* line, 44HH T2, had detectable expression of *gs52* mRNA.

Although antisense expression of a transgene can result in gene silencing (e.g. Escobar et al., 2003), significant silencing was not apparent in the case of line 44HH T2 (as evidenced by northern blots; data not shown), consistent with the lack of antisense *gs52* mRNA expression in this line (Fig. 2). It is interesting that very few antisense *gs52* lines were regenerated, perhaps suggesting that significant silencing of apyrase expression is lethal. Studies of Arabidopsis T-DNA mutants showed that a functional apyrase gene was essential for pollen germination (Steinebrunner et al., 2003). Therefore, apyrase function for plant regeneration during the transformation process may be essential. For the purposes of subsequent experiments, the genotype of line 44HH T2 allowed it to serve as a useful control.

gs52 Sense Plants Showed Enhanced Nodulation

Figure 3 shows the nodulation results of 12 lines of *gs52* transgenic *L. japonicus* in comparison to wild-type plants. In every case, the mean nodule number represents the mean results from at least 30 individual plants (n = 30) and data are representative results from 3 independent experiments. Sense *gs52* lines exhibited a 30% to 50% increase in nodulation compared to wild-type controls. In contrast, the control antisense line, 44HH T2, displayed a wild-type nodulation phenotype.

Figure 1. *gs52* genomic Southern blot. Eight-microgram genomic DNA was digested with *Hind*III and separated by agarose gel electrophoresis. A 1.4-kb PCR product complementary to the *gs52* cDNA was used as a probe. 1, 45W T2 (single-copy line); 2, 45D5 T3 (single-copy line); 3, 45B1.2 T3; 4, 45B1H T3; 5, 45B1K T3; 6, wild type; 7, 44HH T2 (double-copy antisense line); and 8, 45B77 T3 (double-copy sense line). The enzyme *Hind*III cuts once within the T-DNA of pGA941 (but not within the *gs52* sequence); therefore, each band represents an independent insertion into the plant genome.

Figure 2. Northern-blot analysis of wild-type and *gs52* transgenic *L. japonicus*. A 1.4-kb PCR product complementary to *gs52* cDNA was used to detect *gs52* mRNA expression levels (top). Lanes 1 to 6 represent the results of 20 μg total RNA from each sample. 1, Wild type; 2, 44HH T2 (double-copy antisense line); 3, 45D5 T3 (single-copy sense line); 4, 45W T2 (single-copy sense line); 5, 45B1K T3 (single-copy sense line); and 6, 45B77 T3 (double-copy sense line). A soybean actin probe was used as a RNA loading control (bottom).
Nodulation Results of GS52 Transgenic Plants

Figure 3. Nodulation results of gs52 transgenic L. japonicus plants. Compared to wild-type and line 44HH T2 plants, sense gs52 plants had enhanced nodulation. Seeds from each line were sterilized, germinated, and grown as described in "Materials and Methods." Three-week-old plants were equally inoculated with M. loti NZP2235 and then grown for an additional 4 weeks. The plants were scored for their nodulation phenotype 4 weeks postinoculation. Bars indicated ± †SE. Groups marked (a) and (b) are statistically different from one another (α = 0.05) by Student's t test. The data shown are from 1 experiment, but are representative of the 3 independent trials that were conducted (n ≥ 10 for each treatment in each trial). Line 45M T2 (not listed in Table I) shown in this figure was a sense gs52 line that exhibited greater variation with regard to nodulation and, therefore, did not differ from the wild type. It is included here to indicate that not all sense lines showed greater nodulation, although most did. The differences are probably due to positional effects of the various T-DNA insertions.

Table II. Infection thread analysis of GS52 L. japonicus plants

<table>
<thead>
<tr>
<th>Data</th>
<th>WT</th>
<th>44HH T2</th>
<th>4DS5 T3</th>
<th>45W T2</th>
<th>45B1K T3</th>
<th>45B77 T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copy no.</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Avg total root length (mm)</td>
<td>210.2 (a)</td>
<td>144.4 (b)</td>
<td>231.8 (a)</td>
<td>195.2 (a)</td>
<td>185.4 (c)</td>
<td>182.3 (c)</td>
</tr>
<tr>
<td>Avg nodule no/plant</td>
<td>8 (a)</td>
<td>7 (a)</td>
<td>12 (c)</td>
<td>13 (c)</td>
<td>11 (c)</td>
<td>9 (c)</td>
</tr>
<tr>
<td>Avg tap root infection threads/plant</td>
<td>83 (a)</td>
<td>50 (b)</td>
<td>144 (c)</td>
<td>229 (d)</td>
<td>316 (e)</td>
<td>207 (d)</td>
</tr>
<tr>
<td>Avg lateral root infection threads/plant</td>
<td>55 (a)</td>
<td>42 (b)</td>
<td>137 (c)</td>
<td>164 (d)</td>
<td>270 (e)</td>
<td>123 (c)</td>
</tr>
<tr>
<td>Avg infection threads/plant</td>
<td>138 (a)</td>
<td>92 (b)</td>
<td>281 (c)</td>
<td>393 (d)</td>
<td>566 (e)</td>
<td>330 (c)</td>
</tr>
<tr>
<td>Avg root infection zone length (mm)</td>
<td>28.3 (a)</td>
<td>30 (a)</td>
<td>39 (c)</td>
<td>59 (d)</td>
<td>85 (e)</td>
<td>57 (c)</td>
</tr>
<tr>
<td>Avg root infection zone length (mm)</td>
<td>11 (a)</td>
<td>7 (b)</td>
<td>17 (c)</td>
<td>20 (d)</td>
<td>20 (d)</td>
<td>13 (c)</td>
</tr>
<tr>
<td>Avg root infection threads/mm in the avg total root length (mm)</td>
<td>0.65 (a)</td>
<td>0.64 (a)</td>
<td>1.21 (c)</td>
<td>2.01 (d)</td>
<td>3.16 (e)</td>
<td>1.81 (d)</td>
</tr>
</tbody>
</table>
plants. Such a result could be explained by either an increase in the number of infections or by an increase in the percentage of infections that can lead to nodule formation. It is well known that not all infections actually result in nodule formation (Calvert et al., 1985). We tested these two possibilities by directly counting the number of infections, as visualized using a *M. loti* strain constitutively expressing β-galactosidase. The results showed a significant enhancement in infection thread formation when the GS52 sense plants were inoculated with *M. loti*.

The number of nodules formed on legume roots is under autoregulatory control (for review, see Caetano-Anolles and Gresshoff, 1991) The net result is that only a small segment of the developing root is susceptible to nodulation. This is termed the infection zone. We measured this area by counting the number of infections as a function of root length. The GS52-expressing *L. japonicus* plants had significantly larger infection zones. Taken together, the infection thread and infection zone data suggest that the GS52 apyrase plays a role in early nodulation at the stage of infection thread formation. This would be consistent with earlier reports of Nod signal enhancement of apyrase enzyme activity (Etzler et al., 1999).

Recently, LysM receptor-like kinases were identified as the likely receptors for the Nod signal. These proteins were identified by positional cloning of their corresponding genes starting with plant mutants blocked in the earliest steps in nodulation (Limpens and Bisseling, 2003; Madsen et al., 2003; Radutoiu et al., 2003). The LysM domain was first found in bacterial proteins involved in binding murein (peptidoglycan) found in bacterial cell walls (Bateman and Bycroft, 2000). Peptidoglycan, a polymer of N-acetylmuramic acid and GlcNAc, is structurally similar to chitin and, therefore, the presence of the LysM domain in the nodulation-related receptor-like kinases suggests a role in direct binding of the lipo-chitin Nod signal. Although direct biochemical evidence for Nod signal binding to these proteins is still lacking, the mutant phenotype supports their role as Nod signal receptors. Given these findings, the earlier suggestion (Etzler et al., 1999) that apyrases, due to Nod signal-binding activity, could be the postulated, essential Nod signal receptor seems unlikely. However, available evidence, including the information reported here, supports a critical role for apyrases in the early infection mechanism.

If GS52 does not play a role as a bona fide Nod signal receptor, then what other possible roles exist for this protein in nodulation? Our results do not allow a definitive answer to this question. However, the work of Demidchik et al. (2003) and Jeter et al. (2004) suggest one possible function. Demidchik et al. (2003) showed that extracellular ATP could increase intracellular calcium levels. It is known that an increase in cytoplasmic calcium is an essential component in the initial phases of rhizobial infection of the root hair (for review, see Cohn et al., 1997). Jeter et al. (2004) recently reported that extracellular ATP triggers an increase in cytoplasmic calcium levels, resulting in enhanced expression of stress-related transcripts, including those involved in ethylene biosynthesis. Ethylene is known inhibitor of nodulation and, therefore, the ability to control extracellular ATP levels via apyrase activity could allow fine control of cellular responses both beneficial (e.g. calcium oscillations) and detrimental (e.g. ethylene production) to nodulation.
Nucleic Acid Isolation and Manipulation

Plasmid DNA Isolation

Plasmid DNA was isolated from E. coli and A. tumefaciens using the alkaline lysis method described in Sambrook et al. (1989) or by using the Wizard Plus Miniprep DNA purification system (Promega) for automated DNA sequencing. DNA concentrations were determined using a DU-800 spectrophotometer (Hoefer Pharmacia Biotech, San Francisco).

Isolation of DNA Fragments

DNA restriction endonuclease fragments used in cloning were separated by agarose gel electrophoresis, as described by Sambrook et al. (1989). Gel-purified fragments were isolated using the DNA gel extraction kit (Qiagen, Valencia, CA).

Genomic DNA Isolation

Genomic DNA was isolated from L. japonicus using the protocol described by Dellaporta et al. (1983). DNA concentrations were determined as described previously in Sambrook et al. (1989).

Isolation of Total RNA

Total RNA was isolated from L. japonicus plants using the protocol described by Verwoerd et al. (1989). Briefly, plant material (approximately 100 mg) was ground in liquid nitrogen and 500 μL of hot (80°C) extraction buffer were added (phenol 0.1 M LiCl, 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS [1:1]) and homogenized by vortexing for 30 s. Next, 250 μL of chloroform:isoamyl alcohol (24:1) were added and the samples were vortexed again. The samples were centrifuged in a microfuge at 14,000g for 10 min at 4°C. The water phase was removed and mixed with 1 volume of 4 M LiCl. RNA was precipitated overnight and collected by centrifugation as described above. The resultant pellet was dissolved in 250 μL of nuclease-free water and 0.1 volume of 3 M NaOAc, pH 5.2, and 2 volumes of ethanol were added. The sample was incubated for 30 min at –80°C and the RNA was collected by centrifugation as described above. Finally, the pellet was washed with 70% ethanol and allowed to air dry for 10 min before dissolving in 50 μL of sterile nuclease-free water. RNA quantity and integrity were determined by monitoring the optical density as 260 nm and by agarose gel electrophoresis, respectively.

DNA Sequencing

Automated DNA sequencing was performed by Dr. Neil Quigley (University of Tennessee). Plasmid DNA sequencing was performed with the ABI Prism dye terminator cycle sequencing reaction kit on an ABI 373 DNA sequencer (Perkin-Elmer, Foster City, CA). Both strands of two independent clones were sequenced to ensure the fidelity of sequences.

LITERATURE CITED


Nodulation Assays

Lotus seeds were sterilized and germinated as described above. One-week-old seedlings were planted in 4-inch plastic pots containing sterile vermiculite and given Broughton and Dilworth nutrient solution (Broughton and Dilworth, 1971). After planting, the seedlings were allowed to grow for 2 additional weeks (as described above) and then inoculated with a M. loti culture as described above. The plants were placed back into the growth chamber and allowed to grow for an additional 4 weeks (as described above). At 4 weeks postinoculation, the plants were harvested and scored for nodule number.

Infection Thread Assays

Lotus seeds were sterilized and germinated as described previously. After 1 week, the germinated seedlings were transferred to sterile Leonard jars containing sterile vermiculite for further growth and nodulation assays. To ensure sterility, all containers, vermiculite, and water and/or nutrient solutions used in these experiments were autoclaved. The plants were watered with Broughton and Dilworth nutrient solution (Broughton and Dilworth, 1971) and grown for an additional 2 weeks as described above.

The 5-week-old plants were inoculated with 1 mL of a 3-day-old M. loti (hemA-lacZ) culture washed with sterile water and diluted to an optical density 600 of 0.1. The culture inoculant was manually applied to each plant with a pipette. The plants were allowed to grow for an additional 2 weeks postinoculation before the roots were harvested, fixed, and stained for visualization of infection threads. At the time of harvest, the plants were removed from the Leonard jars by flooding gently with water. The roots were further washed gently in water and subsequently detached from the plant using a razor blade. The detached roots were immediately fixed in glutaraldehyde and stained for LacZ (β-galactosidase) expression as described by Boivin et al. (1990). Roots were stored in the dark in sterile, distilled water at 4°C until use.

The roots were measured and photographed using a stereoscope (Olympus SZX12) equipped with a Nikon DXM1200 digital camera. The infection zone was defined as an area on the root showing the most abundant infections (compare with Calvert et al., 1985). The edges of the infection zone were arbitrarily determined as the point where no additional infections were apparent within 3 mm.