Purification and Biochemical Characterization of a Novel Ecto-Apyrase, MP67, from *Mimosa pudica*

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We have previously reported the presence of an apyrase in *Mimosa pudica*. However, only limited information is available for this enzyme. Thus, in this study, the apyrase was purified to homogeneity. The purified enzyme had a molecular mass of around 67 kD and was able to hydrolyze both nucleotide triphosphate and nucleotide diphosphate as substrates. The ratio of ATP to ADP hydrolysis velocity of the purified protein was 0.01 in the presence of calcium ion, showing extremely high substrate specificity toward ADP. Thus, we designated this novel apyrase as MP67. A cDNA clone of MP67 was obtained using primers designed from the amino acid sequence of trypsin-digested fragments of the protein. In addition, rapid amplification of cDNA ends-polymerase chain reaction was performed to clone a conventional apyrase (MpAPY2). Comparison of the deduced amino acid sequences showed that MP67 is similar to ecto-apyrases; however, it was distinct from conventional apyrase based on phylogenetic classification. MP67 and MpAPY2 were expressed in *Escherichia coli*, and the recombinant proteins were purified. The recombinant MP67 showed high substrate specificity toward ADP rather than ATP. A polyclonal antibody raised against the recombinant MP67 was used to examine the tissue distribution and localization of native MP67 in the plant. The results showed that MP67 was ubiquitously distributed in various tissues, most abundantly in leaves, and was localized to plasma membranes. Thus, MP67 is a novel ecto-apyrase with extremely high substrate specificity for ADP.

Apyrase (nucleotide triphosphate diphosphohydrolase [NTPDase]; EC 3.6.1.5) belongs to the E-type ATPase family and, in the presence of divalent cations, hydrolyzes both the γ- and the β-phosphate on ATP or ADP with broad substrate specificity (Plesner, 1995). Apyrase is widely distributed in both animal and plant tissues and can be classified as endo-apyrase or ecto-apyrase based on localization and biochemical characteristics (Plesner, 1995; Komoszynski and Wojtczak, 1996). Accumulating evidence suggests that animal apyrases participate in several physiological events, such as neurotransmission (Sarkis and Salto, 1991), thromboregulation (Marcus et al., 1997), and protein glycosylation (Abeijon et al., 1993; Gao et al., 1999). In plants, the legume-specific apyrase DbLNP from *Dolichos biflorus* contributes to root nodulation; it localizes to the cell membrane and binds Nod factors produced by rhizobia, playing a role in *Rhizobium*-legume symbiosis (Etzler et al., 1999). GS52, an apyrase of *Glycine soja*, is overexpressed in *Lotus japonicus*, resulting in the enhancement of nodulation of the transgenic plant (McAlvin and Stacey, 2005). In other plants, roles of apyrases in cell growth were reported (Riewe et al., 2008; Clark et al., 2010). In Arabidopsis (*Arabidopsis thaliana*), two ecto-apyrases, AtAPY1 and AtAPY2, play critical roles in controlling the extracellular ATP concentration, thereby contributing to growth (Wu et al., 2007). Like all members of the E-type ATPase family, apyrases share five highly conserved sequences called apyrase conserved regions (ACRs; Handa and Guidotti, 1996; Vasconcelos et al., 1996) that might be important for nucleotide hydrolysis.

We have investigated the seismonastic movement of *Mimosa pudica*. Generally, leaves of this plant droop suddenly in response to mechanical, electrical, or thermal stimulation. This bending is thought to be the result of a sudden change in turgor pressure in specific cells, designated motor cells, of the pulvinus at the base of each leaflet and petiole (Shibaoka, 1962; Allen, 1969). Several reports have confirmed that cytoskeletal proteins are also involved in the movement (Kameyama et al., 2000; Yamashiro et al., 2001); however, the exact mechanism regulating the quick response to stimuli remains to be elucidated. Furthermore, it is uncertain whether the movement is energy dependent. The involvement of ATPase was proposed by the observation that a reduction in ATP content takes place in the motor organ during the movement (Lybimova et al., 1964). These findings suggested that...
ATPase must have a crucial role in the seismonastic movement of *Mimosa* pulvini. Several studies (Biswas and Bose, 1972; Mukherjee and Biswas, 1980) have characterized ATPase from *M. pudica*. However, no definite picture of the properties of *Mimosa* ATPase has been obtained. Previous studies in our laboratory also reported ATP/ADP-hydrolyzing activity in *Mimosa* pulvini, which hydrolyze ADP rapidly and then ATP rather slowly (Ishikawa et al., 1984). Comparison of hydrolyzing activity toward 14 different phosphate compounds revealed that the enzyme is an NDPase distinct from conventional apyrase (Ishikawa et al., 1984). Isolation and cloning of a 36-kD apyrase from *M. pudica* have been reported, and this enzyme plays a role in the light response of *Mimosa* (Ghosh et al., 1998a, 1998b). However, the deduced amino acid sequence of the 36-kD apyrase did not contain an ACR and was quite different from other apyrases (Shibata et al., 2001). Thus, several types of this ATP/ADP hydrolytic enzyme might exist in *Mimosa*.

In this paper, we purified an apyrase, MP67, to homogeneity by successive column chromatography steps and demonstrated its biochemical characteristics. Furthermore, cDNA clones of *MP67* and a conventional apyrase, *MpAPY2*, were isolated. Biochemical properties of bacterially expressed recombinant proteins were also examined. Then, tissue distribution and cellular localization of native MP67 were examined using a polyclonal antibody raised against the recombinant proteins.

**RESULTS**

**Purification of Native MP67**

We previously reported that an apyrase exhibiting high ADPase activity is present in the main pulvini of *Mimosa* plants (Ishikawa et al., 1984). However, our recent work showed that ADPase activity is ubiquitously present in various tissues of the plant, with the highest activity observed in leaves. An in-gel enzyme assay clearly showed that at least three bands could be detected in all tissues examined, whereas the ratio of the band intensity differed in each tissue (Fig. 1D). Bands on a native PAGE gel were sliced into small pieces and reelectrophoresed by SDS-PAGE to estimate the molecular size of the enzyme. An approximately 67-kD peptide was detected as the major band after silver staining the gels (data not shown).

In this study, we isolated a novel apyrase from shoots of grown plants by monitoring ADP-hydrolyzing activity. Plant tissues were cut off from the root and homogenized vigorously with a Waring blender. After clarification, the crude extract was roughly fractionated by ammonium-sulfate salt fractionation. The sample was loaded onto a butyl column and eluted with a 50% to 0% ammonium sulfate gradient. The fraction containing ADPase activity was further fractionated using an anion-exchange column. A few peptides were detected in the ADPase-containing fractions after DEAE column chromatography. A concanavalin A-agarose column effectively concentrated peptides of around 67 kD (Fig. 1B). However, the band detected after SDS-PAGE was always smeared after purification and thus appeared to consist of several peptides. Therefore, native PAGE was performed to distinguish these peptides. Three major peptide bands were detected by both protein staining and ADPase activity staining (Fig. 1C). Bands on the native PAGE gel were cut into small pieces and were reelectrophoresed by SDS-PAGE. Several approximately 67-kD bands were detected (Fig. 1D). Thus, we considered that these smeared bands were due to the posttranslation modification such as glycosylation or by the presence of other apyrase isoforms. To test this hypothesis, glycosylation of the isolated peptide was detected by a glycoprotein detection kit. As shown in Figure 1B, the isolated peptide appeared to be glyco-
sylated. We designated the novel apyrase as MP67, and the yield of MP67 was 5 to 10 μg per 700 g of fresh tissue.

Biochemical Characterization of the Native MP67

We previously reported that the crude enzyme detected in Mimosa plants expeditiously hydrolyzes ADP rather than ATP in the presence of divalent cations (Ishikawa et al., 1984). However, biochemical characteristics of the purified enzyme were not elucidated. To characterize the enzymatic activity of the purified MP67, enzyme activities were studied using a colorimetric assay or by measuring the hydrolyzed products, namely ATP, ADP, and AMP, by HPLC.

As shown in Figure 2A, substrates and products in the enzyme mixture were separated by HPLC. ATP and ADP were hydrolyzed by the purified MP67 in accordance with Michaelis-Menten kinetics (Fig. 2B). MP67 had a 4-fold lower \( K_m \) value for ADP than for ATP, showing high affinity for ADP. Specific activity of MP67 for ADP was approximately 89 times higher than that for ATP (Table I). The time course of the nucleotide hydrolysis by MP67 was also examined by HPLC (Supplemental Fig. S1). Thus, MP67 preferentially hydrolyzed ADP. Relatively slow hydrolysis of ATP and no hydrolysis of AMP were observed by 4 h. The data show the high substrate specificity of MP67 and the high purity of the purified sample.

The effect of inhibitors on the nucleotide hydrolysis activity of MP67 was examined. As shown in Table II, sodium orthovanadate (Na₃VO₄) had a strong inhibitory effect on the ATPase activity and a weaker effect on the ADPase activity. The presence of erythrosine B did not affect the ATPase activity but inhibited ADPase activity to a minor extent. Sodium fluoride (NaF) inhibited the ADPase activity rather than the ATPase activity. The effect of pH on ADP hydrolysis was also examined using individual buffers (Supplemental Fig. S2). MP67 was active in a physiological pH range.

cDNA Cloning of MP67 and a Conventional Apyrase from Mimosa

To determine the primary sequence of MP67, the purified protein was digested with trypsin and the N-terminal sequences of the four generated peptides were determined by automated Edman degradation (Supplemental Table S1). The sequences showed similarity with other plant apyrases (data not shown).

A degenerate primer was designed from a part of peptide 4 (Supplemental Table S1; LTGFXGT) and used to determine the 3’ region of MP67. The PCR-amplified fragment was cloned and sequenced. The determined sequence of the clone showed approximately 60% sequence similarity with other plant apyrases. Primers for 5’-RACE amplification were designed from the presumed 3’ untranslated region of the clone. Finally, four clones showing sequence similarity with plant apyrase were obtained (Supplemental Fig. S3). The clones had the five ACRs and showed approximately 60% sequence similarity with other plant apyrases. N-terminal sequences of the trypsin-digested MP67 fragments were identified in the sequence and are indicated in Supplemental Figure S3 as a dotted line. Among the four clones, clones 2 and 10 had only three amino acid differences. Clone 7 had many different amino acids compared with the other clones. Clone 3 showed high sequence similarity with clones 2 and 10, but the deduced amino acid sequence was 81 amino acids shorter than the other clones. The length of the coding region of clones 2, 7, and 10 was 1,404 bp, and the deduced amino acid sequence was 468 amino acids, whereas that of clone 3 was 1,161 bp and 387 amino acids. In this study, clone 2 was designated MP67 and was used in the following experiments (Fig. 3). The estimated molecular size of MP67 was 50.9 kD.
MP67 had sequence similarity with other apyrases but was distinct in details. Thus, we considered that conventional apyrase might exist in *Mimosa*. To clone a conventional *Mimosa* apyrase, primers designed from sequences conserved in other plant apyrases and MP67, and from sequences conserved within apyrase but distinct from the MP67 sequence, were used to amplify a conventional *Mimosa* apyrase. Five clones were isolated and sequenced. The protein-coding region of these clones was 1,410 bp and 470 amino acids. From the deduced amino acid sequence, these clones were classified into two groups (Supplemental Fig. S4). Phylogenetic analysis of plant apyrases showed that there are two groups: one, comprising mainly the APY2 apyrases, is distributed in many plants (Cannon et al., 2003). The *Mimosa* apyrase clones obtained have five ACRs and showed high sequence similarity (more than 85%) to APY2-type apyrase. These apyrase clones had only a four-amino acid difference from each other. Thus, in this study, clone 2:2 was designated MpAPY2, and was used in the following experiments (Fig. 3). The estimated molecular mass of MpAPY2 is 51.4 kD.

In this study, the four MP67 clones and five MpAPY2 clones were isolated from the same plant. Like other plant apyrases, MP67 and apyrase are encoded by a multigene family. Southern hybridization analysis clearly showed that MP67 was encoded by many DNA fragments (Supplemental Fig. S5).

MP67 and MpAPY2 have a prospective transmembrane domain (Supplemental Figs. S3 and S4) and show sequence similarity with ecto-apyrases. In addition, phylogenetic classification revealed that MpAPY2 belongs to the conventional apyrases and can be classified as a APY2-type apyrase. However, MP67 is classified to the third group of the apyrase family, which seems to form a novel clade (Fig. 4). Thus, the conventional apyrase and an unconventional apyrase, MP67, exist in *Mimosa*.

Biochemical Characterization of Recombinant MP67

The calculated molecular mass of MP67 is 50.9 kD; however, the molecular mass of the purified apyrase was 67 kD. As described above, glycosylation of MP67 may have caused a mobility shift on SDS-PAGE. Eleven potential N-glycosylation sites were found in the MP67 sequence (indicated by asterisks in Supplemental Fig. S3), whereas two sites were found in the MpAPY2 sequence (indicated by asterisks in Supplemental Fig. S4). Thus, the contradiction between the relative molecular size of the purified MP67 and the calculated size might be due to posttranslational glycosylation. This was supported by the antibody generated against recombinant MP67 recognizing approximately 67-kD peptides, as shown below.

To further characterize MP67 and MpAPY2, recombinant proteins were expressed in *Escherichia coli* (Fig. 5, A and B). Nucleotide-hydrolyzing activities of the recombinant proteins against several triphosphates and diphosphates were studied (Fig. 5C). Specific activity was determined by colorimetric measurement. ADP hydrolysis activity (218.0 ± 2.2 nmol phosphate min⁻¹ mg⁻¹) of recombinant MP67 was five times higher than that for ATP (41.3 ± 1.8 nmol phosphate min⁻¹ mg⁻¹), although ADP hydrolysis activity of recombinant MpAPY2 was slightly lower than for ATP. The ADP-hydrolyzing activity of recombinant MP67 was 5,000 times lower than that of native MP67. However, the recombinant MP67 showed the highest substrate specificity for ADP (Fig. 5C). We also examined the effects of several inhibitors. As in the case of native MP67, vanadate inhibited ATPase activity rather than ADPase activities of recombinant MP67. NaF inhibited ADPase and ATPase activities of recombinant MP67 to a minor extent. In the case of ATP and ADP hydrolysis, recombinant MpAPY2 showed no significant effect in the presence of vanadate (Fig. 6).

Expression and Distribution of MP67 and MpAPY2

As shown in Figure 1A, ADP-hydrolyzing activity was enriched in leaves. Thus, the expression profile of MP67 and MpAPY2 was examined by reverse transcription (RT)-PCR analysis. Total RNA was prepared from tissues of grown plants. Expression of cellular actin was used as an internal control. MP67 was expressed in a tissue-dependent manner, with the highest level in leaves (Fig. 7). Ubiquitous expression of MpAPY2 was observed within all tissues examined (Fig. 7A). However, the expression level was much lower than that of MP67 (Fig. 7B).

To further examine the tissue distribution of MP67 and MpAPY2, polyclonal antibodies were generated against recombinant proteins. The antibodies generated were immunoaffinity purified on a nitrocellulose membrane before use in experiments. Mainly, two smeared bands at around 67 kD were detected with the anti-MP67 antibody. As for the isolated native MP67, the immunoreactive bands were not viewed as

**Table I. Nucleotide hydrolysis activities of MP67**

| Parameter           | ATP          | ADP          |
|---------------------|--------------|--------------|
| $K_m$ (mM)          | 1.69 × 10⁻¹  | 4.06 × 10⁻²  |
| Specific activity   | 1.28 × 10³   | 1.14 × 10³   |

**Table II. Effects of inhibitors on nucleotide hydrolysis activity of MP67**

| Inhibitor      | ATPase Activity | ADPase Activity |
|----------------|-----------------|-----------------|
| Control        | 100             | 100             |
| 0.1 mM erythrosine B | 104.4 ± 4.9 | 77.4 ± 3.3 |
| 10 mM NaF      | 92.2 ± 2.9      | 68.0 ± 0.9     |
| 0.5 mM Na₃VO₄  | 8.2 ± 1.5       | 72.4 ± 1.3     |
a sharp band, probably due to the posttranslational modification. MP67 was distributed ubiquitously, and the strongest signal was detected in leaves (Fig. 8). Comparisons of the two major bands detected as MP67 revealed that their ratio seemed to be tissue dependent. However, whether these two bands are isoforms due to posttranslational modification or originate from different genes has not been elucidated. Bands detected with the anti-MpAPY2 antibody were ubiquitously distributed in tissues, and only trace levels of expression were detected in roots (Fig. 8C).

Cellular localization of MP67 and MpAPY2 was examined by indirect immunofluorescence analysis using a confocal microscope. A single-plane image clearly showed that signals detected by both antibodies were mainly localized to the cellular membrane of the mesophyll cells, whereas autofluorescence of the chloroplasts and unknown high background fluorescence of tannin vacuole were detected in control experiments (Fig. 9). Signal for MpAPY2 was more uniformly detected on the plasma membrane than signal for MP67, which appeared in patches on the membrane. Thus, MP67 is an ecto-apyrase and appears to be localized to the plasma membrane, as are conventional apyrases.

DISCUSSION

We describe some biochemical and molecular features of a novel apyrase, MP67. MP67 is a member of the apyrase family; however, it has extremely high nucleotide hydrolysis specificity for ADP, which, to our knowledge, has never been seen before. The primary sequence of MP67 was classified into a unique group by phylogenetic analysis. Thus, its biochemical and molecular characteristics clearly show that MP67 is a novel apyrase.

A previous report from our group showed the presence of a unique apyrase that quickly hydrolyzes ADP in the main pulvinus of Mimosa plants (Ishikawa et al., 1984). At first, we thought that the enzyme might contribute to the nastic movement at the petioles of the plant. However, our studies showed that the ADPase activity was enriched in leaves. Thus, the physiologic importance of the apyrase was controversial. To characterize this unique apyrase, we isolated it and characterized it in detail.

An NTPDase purified from porcine brain has $K_m$ for ATP and ADP of 97 and 95 μM, respectively (Kukulski and Komoszynski, 2003). Biochemical properties of recombinant GS52 were also reported (Tanaka et al., 2011). The $K_m$ values of GS52 for ATP and ADP were 309 and 424 μM, respectively. The $K_m$ of MP67 for ATP, 169 μM, is slightly higher than that of porcine brain NTPDase; however, the $K_m$ value for ADP (40 μM) is lower than that of the NTPDase or GS52, showing the high affinity of MP67 for ADP. In general, apyrase is thought to hydrolyze ATP and ADP almost equally. Indeed, the activities of MpAPY2 against a broad range of nucleotide substrates showed a consistent pattern of almost equal or slightly higher triphosphate-
hydrolyzing activity than diphosphate-hydrolyzing activity. Comparative study on animal and plant apyrases showed that the $K_{sh}$ value, which is the relative ATPase to ADPase activity, is considered an important criterion in distinguishing the members of the apyrase family. In general, apyrase of animals and monocot plants has $K_{sh}$, whereas in dicot plants, it has $K_{sh}$. In that study, the $K_{sh}$ value of apyrases ranged from 0.4 to 1.8, with the lowest value obtained in pea (Pisum sativum). In our work here, the calculated $K_{sh}$ value of native MP67 was 0.01, an extraordinarily small value. MP67 hydrolyzed not only ADP and ATP but also other triphosphates and diphosphate nucleosides; however, it did not hydrolyze monophosphate nucleosides (Fig. 5C).

The biochemical uniqueness of MP67 was further certified by the effect of several inhibitors. Erythrosine B, an inhibitor of proton ATPases, was shown to inhibit animal apyrase activity but was not that strong an inhibitor of plant apyrase activity (Komoszynski, 1993). The native MP67, recombinant MP67, and MpAPY2 showed low sensitivity to erythrosine B, as did other plant apyrases. ADPase and ATPase activities of MpAPY2 were also sensitive to NaF, a well-known inhibitor of the cytosolic phosphoprotein phosphatase and a strong inhibitor of plant apyrase (Knowles and Nagy, 1999), whereas these activities of native MP67 and recombinant MP67 were not as notably sensitive to NaF. Generally, ecto-apyrase is insensitive to inhibitors of P-type, F-type, and V-type ATPases.
ATPase (Plesner, 1995). Activities of recombinant MP67 and MpAPY2 were insensitive to azide, an inhibitor of F-type ATPase, although activities of native MP67 and recombinant MP67 were sensitive to vanadate, an inhibitor of P-type ATPase (Borst-Pauwels and Peters, 1981; Wach and Gra¨ber, 1991). Thus, these experiments clearly show that MP67 exhibits unique biochemical characteristics.

Primary sequence analysis showed that MP67 has sequence similarity with plant apyrases, with the highest similarity (72%) to GS52. Recently, it was reported that the recombinant GS52 has greater activity against diphosphate nucleotides in comparison with triphosphate nucleotides, although the highest activity was shown on pyrimidine nucleotides (Tanaka et al., 2011). MP67 possesses an apyrase conserved region, ACR1 to -5, and has a well-conserved nucleotide-binding motif. Moreover, the presence of a prospective transmembrane domain at the N-terminal region, like MpAPY2 and other plant apyrases, showed that MP67 is a member of the ecto-apyrases. However, phylogenetic analysis based on the deduced amino acid sequence clearly showed that MP67 is distinct from other apyrases and seems to form a novel clade in the phylogenetic tree. One notable feature of the MP67 sequence is a Ser-rich region adjacent to the nucleotide-binding motif. The Ser-rich region might affect the nucleotide selectivity or might be a regulatory site for its activity.

An apyrase with a molecular mass of 36 kD was previously isolated from Mimosa, and its primary sequence was reported (Ghosh et al., 1998a). In this study, we isolated MP67 as a novel apyrase by monitoring ADP-hydrolyzing activity; therefore, we did

Figure 6. Effect of inhibitors of nucleotide hydrolysis activity on the recombinant protein. MP67 (A) and MpAPY2 (B) were purified from inclusion bodies of E. coli. Activity without inhibitor (Control) was defined as 100%. Erythrosine B (100 µM), NaF (10 mM), Na₃VO₄ (0.5 mM), and sodium azide (Azide; 0.5 mM) were used as potential inhibitors. Hydrolysis activities for ATP (white bars) and ADP (black bars) were measured by colorimetric enzyme assay. Results are means ± SE (error bars; n = 9). Different letters above the bars indicate mean values that were significantly different from one another (P < 0.05).

Figure 7. RT-PCR analysis. RT was done with 1 µg of total RNA isolated from leaf, stem, main pulvinus, and root. For the amplification of actin, MP67, and MpAPY2, a 5-fold diluted, 25-fold diluted, and original concentration of cDNA pool were used, respectively. A, Representative images of three independent experiments. B, Relative expression levels of MP67 and MpAPY2 to actin were estimated by calculating the intensity of the gels. Results are means ± SE (error bars; n = 3), and double asterisks indicate P < 0.01.
not know whether such apyrase activity exists in Mimosa plants. We isolated cDNA clones encoding MP67 as well as a conventional apyrase from Mimosa. The predicted molecular mass of the apyrase was 51.4 kD and the native size detected by anti-MpAPY2 antibody was also 52 kD, showing a low level of posttranslational modification. Thus, MpAPY2 is distinct from the 36-kD apyrase. MpAPY2 was classified as an APY2-type apyrase by phylogenetic analysis. In most legumes, two types of apyrase are present; a legume-specific APY1-type apyrase and an APY2-type apyrase. Apyrases of the legume-specific type, such as DbLNP and GS52, are known to function in Rhizobium-legume symbiosis (Day et al., 2000; Kalsi and Etzler, 2000; McAlvin and Stacey, 2005; Govindarajulu et al., 2009). In this study, no cDNA clone belonging to the legume-specific apyrase family was obtained. This is probably because we used cDNAs prepared from leaves. However, a legume-specific type of apyrase might exist in Mimosa, because symbiosis of Rhizobium and M. pudica has been reported (Elliott et al., 2009), and the apyrase might contribute to the symbiosis. We will attempt to isolate a legume-specific apyrase from Mimosa roots in future work.

An anti-MP67 antibody immunoreacted with peptides of approximately 67 and 58 kD. We showed that...
these bands are glycosylated but have still not concluded that these major bands are merely products generated by a posttranslational modification, because we isolated a cDNA clone 81 amino acids shorter than other clones (Supplemental Fig. S3). To evaluate the expression of the shorter MP67 clone, we performed RT-PCR analysis. However, no signal for the shorter clone has been observed (data not shown). The shorter clone might be expressed developmentally.

Recombinant MP67 was detected slightly in the soluble fraction and mainly in inclusion bodies. No significant difference was observed in nucleotide hydrolysis activity of recombinant MP67 purified either from the soluble fraction or from the inclusion bodies. Therefore, the MP67 used in our experiments was prepared from inclusion bodies. The specific activity of the recombinant MP67 was extremely low compared with native MP67. However, the recombinant MP67 preferentially hydrolyzed ADP, as did the native MP67. Furthermore, the effects of inhibitors on MP67 activity were quite similar to those on the native MP67. Thus, the recombinant MP67 was adequately reconstituted for examination of its biochemical properties. MpAPY2 was also purified from the inclusion bodies of bacteria expressing the protein. The recombinant MpAPY2 hydrolyzed ATP and ADP to the same extent. Effects of inhibitors on MpAPY2 activity were quite similar to those previously reported in other plant apyrases (Steinebrunner et al., 2000; Cannon et al., 2003).

Tissue distribution and cellular localization of MP67 and MpAPY2 were examined. RT-PCR and western-blots analyses as well as an in-gel enzyme assay clearly showed that MP67 is preferentially expressed in leaves. To our knowledge, no other apyrase has been reported to be preferentially expressed in leaf. Differential expression of apyrase isoforms has been previously reported. In particular, legume-specific apyrases are highly expressed in roots (Etzler et al., 1999; Day et al., 2000). MP67 expression in seedlings was at the trace level; however, isoforms of APY2 apyrase cannot be distinguished by anti-APY2 antibody. Similarly, isoforms of APY2 apyrase cannot be distinguished by other antibodies. The legume-specific apyrases such as DbLNP (Etzler et al., 1999) from D. biflorus and GS52 (Day et al., 2000; Kalsi and Etzler, 2000) from G. soja are localized to the cell membrane and are known to function in the nodulation of legumes. Potato (Solanum tuberosum)-specific apyrase has a signal sequence and is apoplastically localized in tissues (Riewe et al., 2008). Most non-legume-specific apyrases localize in plasma membranes and seem to contribute to phosphate catabolism (Thomas et al., 1999; Wu et al., 2007, 2008). For non-legume-specific apyrase, double knockdown of AtAPY1 and AtAPY2 led to a complete inhibition of pollen germination in Arabidopsis, indicating a role of apyrase in plant reproduction (Steinebrunner et al., 2003). The expression of MP67 seems to gradually increase in a development-dependent manner (data not shown). Thus, we consider that MP67 may contribute to phosphate catabolism in leaves. In particular, extracellular adenine nucleotides are a major target for MP67 and may play a role in ATP-mediated signal transduction via kinase activity that is localized on the surface of the plasma membrane.

MATERIALS AND METHODS

**Plant Material and Chemicals**

*Mimosa pudica* plants were grown under standard conditions in a greenhouse. Grown plants approximately 2 months old were harvested and separated from their roots. The freshly excised plants were immediately used for protein purification. We purchased chemicals and enzymes from Sigma-Aldrich.

**Purification of MP67**

Grown excised *Mimosa* leaves (700 g) were homogenized in 1 L of extraction solution (100 mM Tris-HCl, pH 8.0, 0.5 mM CaCl2, 0.4 M sorbitol, 32 mg mL−1 polyvinylpolypyrrolidone, 10% glycerol, 1 μM L−1 leupeptin, 1 μM L−1 pepstatin, 1 μM L−1 phenylmethlysulfonyl fluoride [PMSF], 7 mM 2-mercaptoethanol [2-ME], and 1% Triton X-100) using a Waring blender at full speed for 3 min and then centrifuged at 2,500g for 15 min to remove debris. The crude extract was obtained by further centrifugation of the supernatant at 10,000g for 30 min. Ammonium sulfate was added to the crude extract to a final concentration of 70%, and the mixture was incubated for 2 h with gentle stirring. After centrifugation at 10,000g for 30 min, the supernatant was mixed using a batch procedure with butyl-Toyopearl 650M column beads (TOSOH) equilibrated with 70% ammonium sulfate in buffer A (20 mM imidazole-HCl, pH 7.0, 1 mM EDTA, 10 mM PMSF, and 7 mM 2-ME). The resin, after washing with the same equilibration solution, was cast into an open column and washed with 50% ammonium sulfate in buffer A. Bound protein was eluted with a linear gradient of 50% to 0% ammonium sulfate in buffer A. Fractions containing ADPase activity were pooled and dialyzed against 20 mM Tris-HCl (pH 7.5) buffered solution. The dialysate was centrifuged at 10,000g for 15 min, and the supernatant was loaded onto a DEAE-Toyopearl 650M
column (TOSOH) equilibrated with 20 mM Tris-HCl (pH 7.5). Elution was by a linear gradient of 50 to 200 mM NaCl in 20 mM Tris-HCl (pH 7.5). ADPase-containing fractions were pooled, NaCl was added to approximately 0.5 M, and the fractions were subsequently subjected to affinity chromatography on a concanavalin A-agarose column (Seikagaku) equilibrated with buffer B (0.5 M NaCl and 20 mM Tris-HCl, pH 7.4). Bound protein, which has affinity for concanavalin A, was eluted with 0.5 M di-o-t-r-Z-Man in buffer B. Eluates were used as the purified protein. The purity of the ADPase-containing fraction was assessed by SDS-PAGE. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

**Gel Electrophoresis**

Protein purification was analyzed by SDS-PAGE using 10% polyacrylamide gels (Laemmli, 1970). Protein bands were detected with Coomassie Brilliant Blue staining. Native PAGE was performed according to an established protocol (Davis, 1964) with slight modifications. Briefly, a 7.5% acrylamide separating gel buffered with 0.375 M Tris-HCl (pH 8.8) and a 5% acrylamide stacking gel buffered with 0.125 M Tris-HCl (pH 8.8) were cast using a thin-slab gel system. Electrophoresis was carried out at 4°C in a 25 mm Tris, 0.192% gly (pH 8.8) buffered solution. Following native PAGE, an in-gel enzyme assay was performed as reported by Nimmo and Nimmo (1982). In brief, gels were soaked in the buffer (100 mM Tris-HCl, pH 8.0, 18 mM CaCl$_2$, 50 mM KCl, and 1.5 mM ADP) used for enzyme assay at 37°C for 30 min. They were then transferred to a staining mixture containing 180 mM Ca$_2^+$ ions. The white bands of the precipitated calcium salt were viewed and photographed by reflected light against a dark background. The bands were excised from the gels and reelectrophoresed by SDS-PAGE to estimate the molecular mass of the enzyme.

**Glycoprotein Detection**

The sugar moiety bound to the purified protein was detected by a glycoprotein detection kit (G.P. Sensor; Seikagaku Biobusiness) according to the manufacturer's protocol.

**Tissue Distribution of MP67**

Crude extracts were obtained from leaf, stem, root, and main pulvinus by homogenization with 100 mM Tris-HCl, pH 8.0, 1 μg mL$^{-1}$ leupeptin, 1 μg mL$^{-1}$ pepstatin, 1 mM PMSE, 7 mM 2-ME, and 1% Triton X-100 using a glass homogenizer. Aliquots (30 μg) of each sample were resolved by native PAGE, and ADPase activity was detected as above.

**Measurement of ATP/ADPase Activity by Colorimetric Assay**

The protein sample was mixed with assay mixtures consisting of 60 mM KCl, 2 mM CaCl$_2$, and 40 mM Tris-HCl (pH 7.5). The reaction was started by adding substrate to a final concentration of 1 mM, and the mixture was incubated at 25°C. The reaction was terminated by adding acid malachite green solution, and inorganic phosphates were subsequently determined by a colorimetric procedure (Chen et al., 1986) with slight modification as described previously (Kanzawa et al., 1993). To assess the effect of inhibitors, 100 μM erythrosine B, 10 μM NaF, 0.5 mM Na$_2$VO$_4$, or 0.5 mM sodium azide was added to the assay mixture. The significance of differences was analyzed with Student’s t test.

**HPLC Separation of the Reaction Products**

The enzymatic reaction was started as above. Aliquots of the reaction mixture were taken at different time points, and the reaction was terminated by adding SDS solution to a final concentration of 1%. An aliquot of the mixture (30 μL) was separated on a TSK-gel ODS-120T column (TOSOH) in 30 mM diethylaminoethanol, pH 3.0, containing 20 mM phosphoric acid and 2% methanol. Concentration of nucleotides in the sample was determined spectrophotometrically at 260 nm by comparison of the retardation time with that of appropriate standards. To determine the $K_m$ and $V_{max}$, 43 ng of the purified MP67 in the reaction mixture was used for ATP hydrolysis, whereas 1 ng of MP67 was used for ADP hydrolysis.

**Amino Acid Sequence Analysis**

The purified MP67 was separated by SDS-PAGE (10% acrylamide), and the protein in the band was digested in-gel with trypsin. The extracted fragment from the gel was placed upon a TSK-gel ODS-80Ts column (TOSOH) equilibrated with 0.1% trifluoroacetic acid and eluted with a linear gradient of 0% to 90% acetonitrile. The N-terminal sequences of the eluted peptides were subsequently determined by a HP G1055A protein-sequencing system (Hewlett-Packard).

**cDNA Cloning of MP67 and a Conventional Apyrase**

Primers used in this study are summarized in Supplemental Table S2. Total RNA was prepared from grown leaves of the plant. A cDNA pool was synthesized using dT-Ad primer, oligo-dT (40) followed by an adaptor sequence. Degenerate forward primer (MP67_F) designed from a part of peptide 4 (Supplemental Table S1; LTGFXGT) and the adaptor primer were used to amplify a cDNA fragment. An approximately 800-bp DNA fragment was amplified, and the fragment was subcloned into a TA-cloning vector using the pGEM-T Easy cloning vector system (Promega). The determined sequence showed approximately 60% similarity with GS52, Glycine soja apyrase. Reverse primers (MP67_R1) for 5′-RACE were designed from the prospective 3′-untranslated region of the clone. 5′-RACE was performed using the SMART RACE cDNA amplification kit (Takara) with UPM primer from the kit for the forward primer and MP67_R1 as the reverse primer. An approximately 1,700-bp amplified band was cloned in pGEM-T Easy vector and sequenced on an ABI model 3101 DNA Sequencer (Applied Biosystems). Four clones encoding MP67 were isolated (Supplemental Fig. S3). Clone 2 was used as MP67 in the following experiments.

**Partial clones of Mimosa conventional apyrase were obtained by 5′-RACE using primer designed from the sequence conserved within the plant apyrase family and MP67 in the first PCR (UPM from the kit as forward primer and Apy_R1 as reverse primer). For the nested PCR, primer was designed from the sequence conserved within plant apyrases but distinct from MP67 (NUP from the kit as forward primer and Apy_R2 as reverse primer). Finally, the amplified 1,100-bp fragment was cloned into pGEM-T Easy vector and sequenced. The determined sequence showed a high sequence similarity with other plant apyrases, especially Apy2-type apyrase (data not shown). To perform 3′-RACE, primers Apy_F1 and Apy_F2 designed from the sequence of the 5′-RACE clone of Mimosa conventional apyrase were used. Apy_F1 and adaptor primers were used for the first PCR, and Apy_F2 and adaptor primers were used for the nested PCR. The 1,700-bp amplified fragment obtained was cloned into pGEM-T Easy vector and sequenced. Full-length apyrase clones were further amplified using primers designed from the prospective 5′-untranslated region sequence of the 5′-RACE clone (Apy_F3) and from the prospective 3′-untranslated region sequence of the 3′-RACE clone (Apy_R3) with a cDNA pool prepared from leaves of grown plants. Five clones were isolated and classified into two groups (Supplemental Fig. S4).

**Sequence Analysis**

Multiple alignments of MP67, MaAPY2, and other plant apyrase family members were generated using a Clustal program (Thompson et al., 1997). Phylogenetic analysis was performed by SEQBOOT, NEIGHBOR, and CONSENSE programs from the PHYLIP package (Felsenstein, 1989). Prediction of N-glycosylation sites of the sequences was performed on the NetNGlyc server (http://www.cbs.dtu.dk/services/NetNGlyc/; Blom et al., 2004). Transmembrane domain prediction was performed on the SOSUI server (http://bip.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html; Hirokawa et al., 1998).

**Tissue Expression Analysis by RT-PCR**

Total RNA was prepared from leaf, stem, main pulvinus, and root of mature plants. RT was done with 1 μg of total RNA isolated from each tissue using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Primers MP67_RT_F and MP67_RT_R were used for the amplification of a DNA fragment of MP67. Primers Apy_F3 and Apy_RT_R were used for the amplification of a DNA fragment of MaAPY2. Actin was used as an internal control by amplification using primers Actin_F and Actin_R. For actin, an aliquot (1 μL) of the 5-fold-diluted cDNA pool was used. For MP67 and MaAPY2, aliquots (1 μL) of 25-fold diluted and original concentrations of the cDNA pool were used, respectively. Amplified fragments were electrophro-
Cedroppings without the transmembrane domain of MP67 and MpAPY2 were amplified by PCR with primer pairs Xho_MP67_F/\nPst_MP67_R and Eco_APY_F/Sal_APY_R, respectively. The amplified fragments were ligated into the pCold I expression vector (TaKaRa) at the XhoI/\nPstI site for MP67 or the EcoRI/Sall site for MpAPY2. The sequence-verified plasmid vectors were used to transform \nEscherichia coli BL21 (DE3), and positive transformants were cultured overnight at 37°C in Luria-Bertani medium. An aliquot (100 \nµL) of the culture was added to 400 mL of Luria-Bertani medium and cultured at 37°C for 2 h, followed by 15°C for 30 min.\nExpression of the recombinant His-tag at the N-terminal of \nT. denticolens was induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM, and incubation was continued overnight at 15°C. The cells were collected by centrifugation at 5,000 \n×g for 10 min and suspended in 20 mM Tris-HCl (pH 7.5), 5 mM imidazole, and 1 mM 2-ME (solution I) containing 6 M guanidine-HCl at 4°C. The clarified sample was applied to a HIS-Select nickel affinity column (Sigma). Purification was verified by SDS-PAGE, and the eluates were dialyzed against solution I and then used in experiments. Recombinant proteins were also\nprepared from the inclusion bodies of the cell lysate. The inclusion bodies were suspended overnight in 20 mM Tris-HCl (pH 7.3), 5 mM imidazole, and 1 mM 2-ME (solution II) containing 6 M guanidine-HCl at 4°C. The clarified sample was applied to a HIS-Select nickel affinity column, which was previously equilibrated with solution II containing 6 M urea. Bound recombinant proteins were washed and renatured by a linear gradient of 6 to 0 M urea in solution II. Elution was performed with solution II with the imidazole concentration changed to 150 mM. Eluates were dialyzed against solution I and used in experiments.

Indirect Immunofluorescence Analysis
Tissue sections were prepared as described previously (Kanzawa et al., 2006). Briefly, paraformaldehyde-fixed samples were dehydrated and mounted in a low-melt wax, and sections (15 µm thickness) were rebathed and used for immunofluorescence analysis. Nonspecific binding of antibody was blocked by preincubation with 1% (w/v) bovine serum albumin and 3% (v/v) normal goat serum in PBS. After a rinse in PBS, the specimens were incubated with either anti-MP67 antibody at 1:100 dilution or anti-MpAPY2 antibody at 1:100 dilution. Secondary antibody was Alexa Fluor 488-conjugated anti-rabbit IgG antibody (Molecular Probes). Specimens were examined with a confocal scanning microscope (LSM700; Carl Zeiss).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure S1. Time course of nucleotide hydrolysis.
Supplemental Figure S2. pH dependence of ADP hydrolysis.
Supplemental Figure S3. MP67 clone sequences.
Supplemental Figure S4. MpAPY2 clone sequences.
Supplemental Figure S5. Southern blot analysis of MP67.
Supplemental Table S1. N-terminal sequences of MP67.
Supplemental Table S2. Primer list.

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AB600993; MP67; AB600994; MP67-10, AB600995; MpAPY2-1, AB600996; and MpAPY2-2, AB600997.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: MP67-2, AB600992; MP67-3,
