

# Protein Kinase Cascade Involved in Rapid ABA-signaling in Guard Cells of *Vicia faba*

Takuya Furuichi<sup>a,\*</sup>, Izumi C. Mori<sup>b</sup>, and (the late) Shoshi Muto<sup>c</sup>

<sup>a</sup> Graduate School of Medicine, Nagoya University, Tsurumai-cho 65, Showa-ku, Nagoya 466-8550, Japan. Fax: +81(0)52-744-2058. E-mail: furuichi@med.nagoya-u.ac.jp

<sup>b</sup> Research Institute for Biosciences, Okayama University, Kurashiki 710-0046, Japan

<sup>c</sup> Graduate School of Agricultural Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

\* Author for correspondence and reprint requests

Z. Naturforsch. **60c**, 769–773 (2005); received January 31/March 9, 2005

Protein kinases are involved in signal transduction for environmental stress responses. In response to drought and salinity, a 48-kDa protein kinase (AAPK; abscisic acid-activated protein kinase (AAPK) in guard cells is activated by abscisic acid (ABA) and phosphorylates several targets such as the carboxy-terminus of inward-rectifying K<sup>+</sup> channel and heterogeneous mRNA binding protein to adapt to the changing environment. The AAPK expressed specifically in guard cells, and recombinant AAPK was phosphorylated only with the extract from ABA-treated guard cells but not from untreated cells. This indicates the presence of an AAPK kinase (AAPKK), which is activated by ABA and phosphorylates AAPK preceding the activation of AAPK. Both AAPK and AAPKK are involved in the protein kinase cascade for the rapid ABA-signaling.

**Key words:** Absciscic Acid (ABA), Protein Kinase, Kinase Cascade

## Introduction

Stomatal aperture is a response to various environmental stimuli such as light and humidity changes, and it controls the transpiration and CO<sub>2</sub> uptake required for photosynthesis under diverse environmental conditions (Zeiger, 1983; Mansfield *et al.*, 1990). Absciscic acid (ABA), one of the members of so-called plant hormones, which accumulate in response to drought and salinity, induces a series of signaling events leading to stomatal closure, to reduce the transpirational water loss (Mansfield *et al.*, 1990). The molecular mechanism of ABA-induced stomatal closure is a major concern for plant physiologists with regards to investigation and improvement of the growth and yield of crops in arid or salt damaged regions. Some of the ABA-insensitive (*abi1-1* and *abi2-1*) and supersensitive mutants (*era1*) supporting that responsiveness to ABA and ABA-induced stomatal closure are necessary for drought tolerance in *Arabidopsis thaliana* (Rodriguez *et al.*, 1998; Pei *et al.*, 1998). Protein kinases are generally involved in various responses to stress, and one of the protein kinases which are activated by ABA was found in *Vicia faba* guard cells, that form and control stomata, and designated AAPK (abscisic acid-activated protein kinase) (Li and Assmann, 1996). Af-

ter the molecular identification of AAPK gene, transformation of guard cells with wild-type AAPK has been tested and no effect on ABA-induced stomatal closure was observed. In comparison, a comparable AAPK mutant lacking adenosine triphosphate (ATP)-binding ability successfully eliminated the ABA-induced stomatal closure due to the reduction or absence of catalytic activity (Li *et al.*, 2000). The transformation of AAPK mutant with no ATP-binding also eliminated the ABA-induced activation of slow anion channel required for controls in turgor pressure and stomatal closure (Li *et al.*, 2000). OST1, the ortholog of AAPK in *Arabidopsis* is identified by a genetic screening for stomatal opening mutant, supporting relevance of AAPK-like kinases in guard cell ABA-signaling among higher plants (Mustilli *et al.*, 2002). Though AAPK is involved in rapid ABA-signaling events in guard cells, the overall mechanism of ABA-signaling is still unclear. As a phosphorylation substrate of ABA-activated AAPK, Li *et al.* (2002) identified one of the AAPK interacting partners termed AKIP1, a heterogeneous nuclear RNA-binding protein (hnRNP). AKIP1 is likely phosphorylated by ABA-activated AAPK and bound to a dehydrin transcript, suggesting the involvement of hnRNPs

for RNA stability in abiotic stressed condition. Mori *et al.* (2000) reported the functional characterization of ABR\* kinase (ABA-responsible protein kinase) which is possibly identical to AAPK due to its molecular size in appearance, guard cell specific expression pattern, and ABA-dependent activation. ABR\* kinase is reportedly activated by pretreatment of guard cell protoplasts (GCPs) with ABA, but not by IAA, 2,4-D or GA<sub>3</sub>, in time- and dose-dependent manners. From the activation time course, ABR\* kinase (AAPK) is activated maximally 10 min after application of 1  $\mu$ M ABA, indicating the existence of some earlier signal mediators activating AAPK acting within 5–10 min following ABA perception. Here, we show that AAPK is phosphorylated by a AAPK kinase in *V. faba* guard cell protoplasts. In addition, subcellular localization and another phosphorylation target of AAPK in drought response are discussed.

## Materials and Methods

### Plant material

Broad bean (*V. faba* L. cv. Otafukusanzu) was grown as described previously (Mori and Muto, 1997).

### Chemicals

Cellulase YC and pectolyase Y23 were purchased from Seishin Pharmaceutical Co. (Tokyo, Japan). [ $\gamma$ -<sup>32</sup>P] ATP was obtained from ICN (Costa Mesa, CA, U.S.A.). ABA [a mixture of (+) and (–) form] and trichloroacetic acid (TCA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). TARON<sup>TM</sup> metal affinity resin was purchased from Clontech (Palo Alto, CA, U.S.A.). (2-Aminoethyl) methanethiosulfonate hydrobromide (MTSEA) was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada).

### Construction and purification of recombinant proteins

The expression plasmid pQE30-AAPK was constructed by inserting desired DNA fragments into pQE30 expression vector (Qiagen, Hilden, Germany) to obtain recombinant AAPK (His)<sub>6</sub>-tagged at the amino-terminus. *E. coli* strain M15 [pREP4] (Qiagen) was transformed with pQE30-AAPK. The expressed recombinant proteins were purified with TALON<sup>TM</sup> affinity resin (Clontech, Palo Alto, CA, U.S.A.) according to the manufac-

turer's protocol with minor modifications. The recombinant peptide of the carboxy-terminus of KAT1 (KAT1C-H) was prepared as described previously (Mori *et al.*, 2000)

### Isolation of guard cell protoplasts, ABA-treatment and sample preparations

GCPs were isolated as previously reported (Mori and Muto, 1997). GCPs were stored on ice at least 1 h before ABA-treatment. ABA was dissolved in ethanol before use. As a solvent control, all samples without ABA-treatment were treated with the same concentration of ethanol. The reaction was immediately stopped by adding the x2 SDS-PAGE sample buffer. When microsomal and soluble fractions were separated, GCPs were immediately frozen by liquid nitrogen instead of adding sample buffer. Cells were dissolved and well disrupted in the buffer containing 10 mM MES [2-(*N*-morpholino)ethanesulfonic acid]-Tris (2-amino-2-hydroxymethyl-1,3-propanediol) (pH 7.0), 10 mM KCl, 1 mM CaCl<sub>2</sub>, 250 mM mannitol, and EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany) with a pestle suitable for 1.5 ml tubes. Cell debris were removed by centrifugation at 10,000  $\times g$  for 5 min at 4 °C and the supernatant was further centrifuged at 400,000  $\times g$  for 20 min at 4 °C. The resultant pellet and supernatant were used as microsomal and soluble fraction. Immunoprecipitation of AAPK, using anti-AAPK anti-serum raised in a rabbit against histidine-tagged recombinant AAPK, was performed by the method of Imamura *et al.* (2002) with minor modification. SDS-PAGE and immunoblot analysis were performed as described previously (Furuichi *et al.*, 2001).

### Protein kinase activities

AAPK activity was detected by the in-gel kinase assay as reported previously (Mori and Muto, 1997) with minor modification. KATC-H (0.5 mg/ml) was immobilized in 10% polyacrylamide gels. AAPK kinase (AAPKK) activity was detected by an *in vitro* kinase assay. The kinase samples prepared from ABA-treated or -untreated GCPs were dissolved and mixed into 100  $\mu$ l of reaction buffer [20 mM Tris-HCl, pH 7.5, 5 mM MgCl<sub>2</sub>, 1 mM 2-mercaptoethanol, 1 mM EGTA, 0.5 mg recombinant AAPK, 10 mM cold ATP (not radioactive) and 20 mCi (740 kBq) of [ $\gamma$ -<sup>32</sup>P]ATP] to initiate the reaction. After incubation for 30 min at 30 °C,

most of proteins were precipitated by adding 10% TCA. After neutralization by washing with 80% acetone, precipitated proteins were dissolved into the sample buffer and electrophoresed in a SDS-PAGE gel. The radioactive bands were visualized by a Bioimaging Analyzer BAS 2000 (Fuji Photo Film Co., Tokyo, Japan).

## Results and Discussion

### Purification of the recombinant AAPK

A recombinant AAPK (with a histidine-tag) was produced in *E. coli* cells, and purified with the respective affinity columns. As estimated from the amino acid sequence of AAPK, the size of recombinant AAPK after the SDS-PAGE was 40 kDa, while the AAPK gene was identified by a peptide sequence corresponding to a protein, which possesses 48 kDa of apparent molecular mass (Li *et al.*, 2000). This indicates that some posttranscriptional modification of AAPK occurs *in planta*. The identity between AAPK and ABA-activated 48-kDa-protein kinase and tissue-specific expression of this protein was confirmed by immunochemical methods with rabbit IgG against a recombinant AAPK. As shown by Fig. 1A, a 48-kDa-protein was clearly detected only in GCPs,

not in other tissues. Because the population of guard cells in intact leaves is very low, detection of the corresponding band in extracts of both young and mature leaves was hardly successful, although a 55-kDa unknown protein was detected only in the extract of young leaves. This 55-kDa-band may not be a modified AAPK, since we did not observe a corresponding kinase activity at 55-kDa in young leaf extract (data not shown). Posttranslational modifications of proteins with lipid chains often affect not only the electrophoresed mobility but also the subcellular localization of the proteins. For example, if the amino-terminus of a host protein was modified with palmitate, it should be localized near the plasma- or endomembrane as an anchoring protein (Little *et al.*, 1998). To identify the subcellular localization of AAPK, extracts from GCPs were separated into microsomal and soluble fractions and analyzed by immunoblotting (Fig. 1B). AAPK was collected mainly in the microsomal membrane fraction, implying that AAPK is posttranscriptionally modified with a lipid chain, although no consensus sequence for palmitoylation nor myristoylation is conserved in AAPK. It is also possible that AAPK indirectly associates with membranes through interaction with another membrane protein or protein complex. In addition, no catalytic activity of recombinant AAPK was detectable even after treatment with ABA or ABA-pretreated guard cell extract (data not shown). Likely in addition to the AAPK-activating signal molecules, some posttranslational modifications are necessarily required for catalytic activity of AAPK.

### Immunoprecipitation of AAPK activated by ABA

For further confirmation, the 48-kDa-protein was immunoprecipitated from GCPs pretreated with 50  $\mu$ M ABA and the catalytic activity was tested by an in-gel kinase assay in the gels containing recombinant peptides as the substrate, which corresponded to the carboxy-terminus of KAT1, an inward-rectifying K<sup>+</sup> channel of *Arabidopsis thaliana* expressed primarily in guard cells (Anderson *et al.*, 1992; Mori *et al.*, 2000). A band corresponding to the protein kinase activity was detected in the immunoprecipitant from ABA-treated GCP extracts, but not in those from untreated cell extracts (Fig. 1C). These results support the view that AAPK is identical to an ABR\* kinase involved in K<sup>+</sup> channel phosphorylation.

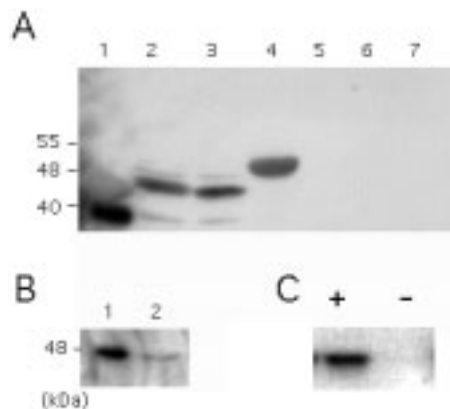


Fig. 1. Western blot analysis of AAPK (ABA-activated protein kinase). (A) Guard cell specific expression of AAPK. 1, Recombinant peptide purified from *E. coli* extract; 2, GCPs (guard cell protoplasts) treated with ABA; 3, GCPs without ABA treatment; 4, young leaves; 5, mature leaves; 6, roots; 7, stems. (B) Subcellular localization of AAPK. 1, Microsomal membrane fraction (10  $\mu$ g protein); 2, soluble fraction (10  $\mu$ g protein). (C) Detection of AAPK activity in immunoprecipitant with anti-AAPK antibody. Isolated guard cell protoplasts of *V. faba* were pretreated with (+) or without (–) 50  $\mu$ M ABA.

Phosphorylation of the distal carboxy-terminus has been reported to modulate the voltage-dependent activation of the *Shab*-related slow voltage-dependent  $K^+$  channel Kv2.1 (Murakoshi *et al.*, 1997). It implies that the ABA-induced phosphorylation of the carboxy-terminus could alter the activity of KAT1-like channel in *V. faba* guard cells. Then,  $K^+$  and the following water influx may cause stomatal closure.

#### *Immunodetection of a potent phosphorylation target of AAPK*

Because AAPK is a membrane-anchored protein and AAPK, by pretreatment of guard cell protoplasts (GCPs) with ABA, phosphorylates the carboxy-terminus of KAT1 (Fig. 1C), activation of a KAT1-related  $K^+$  channel in *V. faba* might be a putative phosphorylation target of AAPK. To obtain the evidence, GCPs pretreated with or without 50  $\mu M$  ABA for 5 min were treated with 1 mM (2-aminoethyl) methanethiosulfonate hydrobromide (MTSEA), a membrane-permeable disulfide cross-linking reagent to conjugate AAPK and its substrates during AAPK possesses a high catalytic activity and interacts with its substrates. The microsomal fractions of MTSEA-treated GCP extracts were exposed to SDS-PAGE without reducing reagent in the sample buffer. Then immunoblot analysis was performed. In addition to the band of AAPK (48 kDa), a weak band of approx. 300 kDa was found only in GCP extracts pretreated with ABA (Fig. 2). The apparent molecular weight of AAPK is 48 kDa, and the predicted molecular weight of KAT1-tetramer is 312 kDa (Anderson *et al.*, 1992). The apparent molecular weight of the detected band is smaller than the predicted molecular weight of the AAPK/KAT1-tetramer complex. These samples were electrophoresed without reducing reagent to abolish the

secondary and 3-dimensional structure, and ABA-activated AAPK clearly phosphorylates the carboxy-terminus of KAT1 (Fig. 1C) as reported by Mori *et al.* (2000). The possibility may be assumed that this band of a 300-kDa-protein complex is identical with a complex of AAPK and  $K^+$  channel-tetramer. The exact membrane protein being phosphorylated should be identified in future studies.

#### *Phosphorylation of AAPK by AAPK kinase in guard cell protoplast extracts*

Potential membrane-associated ABA receptor(s) of *V. faba* guard cells were found using biotinylated ABA, although molecular identification has still to be done (Yamazaki *et al.*, 2003). Many ABA-signaling components, such as ion channels, protein phosphatase and protein kinase are known but no one can be directly bound to ABA, therefore ABA-signaling must be initiated by the membrane-associated ABA receptor(s) as mentioned above. In case of ABA-dependent activation of AAPK, this enzyme is maximally activated 5–10 min after the addition of 1  $\mu M$  ABA (Mori *et al.*, 2000). ABA-dependent activation of AAPK is inhibited by protein kinase inhibitors, like K252a and staurosporine, suggesting the involvement of upstream protein kinase in AAPK activation (Mori and Muto, 1997). In addition, ABA activation of AAPK does not occur *in vitro*, suggesting that a signaling cascade for the activation of AAPK is required. As an activation mechanism of protein kinase, phosphorylation of MAP kinase by MAPK kinase (MAPKK) is a well-known phenomenon (Haystead *et al.*, 1992; Gomez *et al.*, 1992). By analogy, we performed an *in vitro* phosphorylation assay with ABA-treated GCP extracts using recombinant AAPK as a substrate (Fig. 3). As a result, a band indicating phosphorylation of AAPK was detected after 1 min of 50  $\mu M$  ABA treatment. We called this protein AAPK kinase (AAPKK). Al-

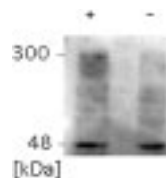


Fig. 2. Immunodetection of the conjugated protein complex of ABA-activated AAPK and phosphorylation targets. Microsomal fractions of MTSEA treated GCPs (guard cell protoplasts) pretreated with (+) or without (-) 50  $\mu M$  ABA.

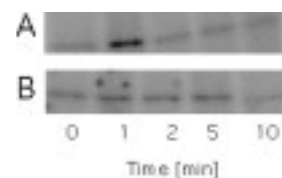


Fig. 3. The time course of AAPK kinase activation by ABA. GCPs were incubated with 50  $\mu M$  ABA for indicated times. (A) GCPs treated with ABA. (B) GCPs without ABA treatment.



though AAPK was activated 5–10 min after ABA-treatment (Mori *et al.*, 2000), AAPKK was shown to be inactivated within 2 min. This rapid activation and inactivation of AAPKK and AAPK implies that the ABA-signaling cascade in guard cells is strictly regulated by other components for ABA-signaling or for housekeeping.

Phosphorylation of recombinant AAPK was analyzed in gel to identify the molecular weight and the activation profiles of AAPKK, but no catalytic activity was detected. Also MTSEA-treatment could not trap the AAPK-AAPKK complex possibly because this reaction is too rapid and the amount of AAPKK in guard cells might be very low. In future, the exact amino acid residue responsible for phosphorylation and modification with lipid chain should be identified. This may help the molecular identification of AAPKK. In ABA-signaling, many signal components interact in collaboration. In the present study, we revealed the existence of AAPKK which phosphorylates AAPK in *V. faba* guard cells. AAPKK is activated and inacti-

vated within 2 min after the ABA-perception. This ABA-activated protein kinase cascade may play an essential role in drought response, because AAPK likely phosphorylates K<sup>+</sup> channel(s) required for stomatal closure, and a hmRNP that modifies stability of heterogeneous nuclear RNA-binding protein (hmRNP). Further elucidation of the members of the ABA-signaling pathway may reveal the control of stomatal gating and following plant development.

#### Acknowledgement

The authors are grateful to Dr. Tomonori Kawano for helpful comments and critical reading of the manuscript. This work is supported by Grant-in-Aid for Scientific Research (B) (No. 11 440235) to S.M. from the Ministry of Education, Sciences, Sports, and Culture of Japan and JSPS Research Fellowship for Young Scientists (15000639) to T.F. from the Japan Society for the Promotion of Science.

- Anderson J. A., Huprikar S. S., Kochian L. V., Lucas W. J., and Gaber R. F. (1992), Functional expression of a probable *Arabidopsis thaliana* potassium channel in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **89**, 3736–3740.
- Furuichi T., Mori I. C., Takahashi K., and Muto S. (2001), Sugar-induced increase in cytosolic Ca<sup>+</sup> in *Arabidopsis thaliana* whole plants. *Plant Cell Physiol.* **42**, 1149–1155.
- Gomez N., Traverse S., and Cohen P. (1992), Identification of a MAP kinase kinase in phaeochromocytoma (PC12) cells. *FEBS Lett.* **314**, 461–465.
- Haystead T. A., Dent P., Wu J., Haystead C. M., and Sturgill T. W. (1992), Ordered phosphorylation of p42mapk by MAP kinase kinase. *FEBS Lett.* **306**, 17–22.
- Imamura F., Maeda S., Doi T., and Fujiyoshi Y. (2002), Ligand binding of the second PDZ domain regulates clustering of PSD-95 with Kv1.4 potassium channel. *J. Biol. Chem.* **277**, 3640–3646.
- Li J. and Assmann S. M. (1996), An abscisic acid-activated and calcium-independent protein kinase from guard cells of fava bean. *Plant Cell* **8**, 2359–2368.
- Li J., Wang X. Q., Watson M. B., and Assmann S. M. (2000), Regulation of abscisic acid-induced stomatal closure and anion channels by guard cell AAPK kinase. *Science* **287**, 300–303.
- Li J., Kinoshita T., Pandey S., Ng C. K.-Y., Gygi S. P., Shimazaki K., and Assmann S. M. (2002), Modulation of an RNA-binding protein by abscisic-acid-activated protein kinase. *Nature* **418**, 793–797.
- Little E. B., Edelman G. M., and Cunningham B. A. (1998), Palmitoylation of the cytoplasmic domain of the neural cell adhesion molecule N-CAM serves as an anchor to cellular membranes. *Cell Adhes. Commun.* **6**, 415–430.
- Mansfield T. A., Hetherington A. M., and Atkinson C. J. (1990), Some aspects of stomatal physiology. *Annu. Rev. Plant Physiol. Mol. Biol.* **41**, 55–75.
- Mori I. C. and Muto S. (1997), Abscisic acid activates a 48-kilodalton protein kinase in guard cell protoplasts. *Plant Physiol.* **113**, 833–839.
- Mori I. C., Uozumi N., and Muto S. (2000), Phosphorylation of the inward-rectifying potassium channel KAT1 by ABR kinase in *Vicia* guard cells. *Plant Cell Physiol.* **41**, 850–856.
- Murakoshi H., Shi G., Scannevin R. H., and Trimmer J. S. (1997), Phosphorylation of the Kv2.1 K<sup>+</sup> channel alters voltage-dependent activation. *Mol. Pharmacol.* **52**, 821–828.
- Mustilli A. C., Merlot S., Vavasseur A., Fenzi F., and Giraudat J. (2002), *Arabidopsis* OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *Plant Cell* **14**, 3089–3099.
- Pei Z. M., Ghassemian M., Kwak C. M., McCourt P., and Schroeder J. I. (1998), Role of farnesyltransferase in ABA regulation of guard cell anion channels and plant water loss. *Science* **282**, 287–290.
- Rodriguez P. L., Benning G., and Grill E. (1998), ABI2, a second protein phosphatase 2C involved in abscisic acid signal transduction in *Arabidopsis*. *FEBS Lett.* **421**, 185–190.
- Yamazaki D., Yoshida S., Asami T., and Kuchitsu K. (2003), Visualization of abscisic acid-perception sites on the plasma membrane of stomatal guard cells. *Plant J.* **35**, 129–139.
- Zeiger E. (1983), The biology of stomatal guard cells. *Annu. Rev. Plant. Physiol.* **34**, 441–475.