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RESEARCH PAPER

FIA functions as an early signal component of abscisic acid signal cascade in *Vicia faba* guard cells

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Abstract

An abscisic acid (ABA)-insensitive *Vicia faba* mutant, *fia* (fava bean impaired in ABA-induced stomatal closure) had previously been isolated. In this study, it was investigated how FIA functions in ABA signalling in guard cells of *Vicia faba*. Unlike ABA, methyl jasmonate (MeJA), H_2O_2, and nitric oxide (NO) induced stomatal closure in the *fia* mutant. ABA did not induce production of either reactive oxygen species or NO in the mutant. Moreover, ABA did not suppress inward-rectifying K^+ (K_{in}) currents or activate ABA-activated protein kinase (AAPK) in mutant guard cells. These results suggest that FIA functions as an early signal component upstream of AAPK activation in ABA signalling but does not function in MeJA signalling in guard cells of *Vicia faba*.

Key words: Abscisic acid, *fia*, guard cell, methyl jasmonate, stomatal closure, *Vicia faba*.

Introduction

Guard cells in pairs surround stomatal pores and respond to various signals including hormones, humidity, light, temperature, and CO_2. In response to drought condition, plants synthesize abscisic acid (ABA), which induces stomatal closure, reducing water loss (Hirayama and Shinozaki, 2007; Shimazaki *et al.*, 2007).

A number of ABA signalling factors, including an ABA receptor complex, have been identified using a model plant, *Arabidopsis thaliana* (Fujii *et al.*, 2009; Ma *et al.*, 2009; Park *et al.*, 2009; Sirichandra *et al.*, 2009; Kim *et al.*, 2010). *Vicia faba* has been widely used for the investigation of ABA signalling in guard cells because it is possible to use experimental techniques which are difficult to apply to model plants (Schwartz *et al.*, 1994; Mori and Muto, 1997).

Iwai *et al.* (2003) reported an ABA-insensitive *Vicia faba* mutant, *fia* (fava bean impaired in ABA-induced stomatal closure). In the *fia* mutant, ABA-induced stomatal closure and seed dormancy are disrupted (Iwai *et al.*, 2003), suggesting that the *fia* mutation affects ABA signalling components and that the *fia* mutant could be a powerful tool for further dissection of the ABA signalling pathway in *Vicia* guard cells.

ABA induces the production of reactive oxygen species (ROS) mediated by NAD(P)H oxidases in guard cells (Pei *et al.*, 2000; Kwak *et al.*, 2003). Bright *et al.* (2006) have reported that nitric oxide (NO) requires ROS production in ABA-induced stomatal closure but Lozano-Juste and León (2010) have proposed an NO-independent regulatory mechanism of ABA-induced stomatal closure, indicating that the roles of NO in ABA signalling are still unsettled. Hydrogen peroxide activates Ca^{2+}-permeable non-selective cation channels, causing the elevation of cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) in guard cells (Pei *et al.*, 2000; Murata *et al.*, 2001). The elevation in $[Ca^{2+}]_{cyt}$ leads to the activation of S-type anion channels, resulting in the depolarization of the plasma membrane, the release of ions, and the reduction of turgor pressure of guard cells (Vahisalu *et al.*, 2008).

The volatile phytohormone, methyl jasmonate (MeJA) regulates various physiological processes including pollen maturation, tendril coiling, and responses to wounding and pathogen attack (Liechti and Farmer, 2002; Turner *et al.*, 2002). MeJA induces production of ROS in guard cells (Suhita *et al.*, 2004; Islam *et al.*, 2010*a*), elevation/oscillation of $[Ca^{2+}]_{cyt}$ (Islam *et al.*, 2010*b*), activation of K^+ efflux, and inactivation of K^+ influx in guard cells (Evans, 2003; Saito *et al.*, 2008). In *Arabidopsis coronatine insensitive1* (*coi1*) mutant, ABA induces stomatal closure but MeJA does not induce stomatal closure (Munemasa *et al.*, 2007) and MeJA-induced stomatal closure requires endogenous ABA in *Arabidopsis* (Hossain *et al.*, 2011).

In the *fia* mutant, ABA does not induce stomatal closure but exogenous Ca^{2+} induces stomatal closure, suggesting that the *fia* mutation disrupts ABA signalling between ABA perception and $[Ca^{2+}]_{cyt}$ elevation (Iwai *et al.*, 2003). However, the effects of *fia* mutation on ROS production, NO production, and modulation of potassium channel activities in response to ABA remain to be clarified.

In fava bean, ABA activates 48-kDa ABA-activated protein kinase (AAPK) (Li and Assmann, 1996; Mori and Muto, 1997) and a broad-range protein kinase inhibitor, K252a, inhibits both ABA-induced stomatal closure and ABA activation of AAPK (Mori and Muto, 1997). An in-gel protein kinase assay has demonstrated that AAPK phosphorylates the carboxy-terminus of *Arabidopsis* potassium channel KAT1 (Mori *et al.*, 2000) which is involved in stomatal movement (Kwak *et al.*, 2001). These results suggest that AAPK is a key signal factor in ABA signalling in fava bean.

To understand the details of how the *fia* mutation affects ABA signalling, stomatal closure, the production of second messengers ROS and NO, the suppression of inward-rectifying K^+ (K_{in}) currents, and the activation of AAPK in the *fia* mutant were investigated.

Materials and methods

Plant material and growth

Seeds of *Vicia faba* L., cv. House Ryousai were purchased from Kyowa Seeds Co. (Chiba, Japan) and seeds of *fia* mutant were provided by Kagoshima University. Plants were grown in a growth chamber for 4–8 weeks at 23 °C, 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ under a 18/6 h light/dark cycle. The plants were watered twice a week.

Stomatal aperture measurements

Stomata apertures were measured according to the method described previously (Iwai *et al.*, 2003) with slight modifications. Excised leaves were floated on medium containing 50 mM KCl and 10 mM MES-KOH (pH 6.15) for 2 h in the light. Leaves were transferred to the bathing medium containing 50 mM KCl, 0.01 mM $CaCl_2$, and 10 mM MES-KOH (pH 6.15) and the indicated concentration of phytohormone or reagent, and then incubated for 1 h in the light. The leaves were shredded in a blender and epidermal tissues were collected. Twenty stomatal apertures were measured on each individual experiment and averages were obtained from more than four independent experiments.

Detection of ROS and NO

Production of ROS and NO in guard cells was measured as described previously by Munemasa *et al.* (2007) with slight modifications. For ROS production, epidermal peels were incubated for 3 h in medium containing 5 mM KCl, 50 μM $CaCl_2$, and 10 mM MES-KOH (pH 6.15), and then 50 μM ROS detection fluorescence dye, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA), was added to the medium. The epidermal tissues were incubated for 10 min in the dark and were then washed to remove excess dye. The dye-loaded tissues were treated with 0.1% (v/v) ethanol or 10 μM ABA for 15 min in the light. For NO production, 10 μM NO detection fluorescence dye, 4,5-diaminofluorescein-2 diacetate (DAF-2DA) was added to medium instead of 50 μM H_2DCF-DA. The epidermal tissues were incubated for 1 h in the light and then were washed to remove excess dye. The dye-loaded tissues were treated with 0.1% (v/v) ethanol or 10 μM ABA for 40 min in the light. Fluorescence of guard cells was imaged and analysed using AQUA COSMOS software (Hamamatsu Photonics K. K., Shizuoka, Japan).

Electrophysiology

For whole-cell patch-clamp recording of K_{in} channels, guard cell protoplasts (GCPs) were prepared from epidermal tissues with digestion solution containing 1.0% (w/v) Cellulase R10, 0.5% (w/v) Macerozyme R10, 0.5% (w/v) bovine serum albumin, 0.1% (w/v) kanamycin, 10 mM ascorbic acid, 0.1 mM KCl, 0.1 mM $CaCl_2$, and 500 mM D-mannitol (pH 5.5 with KOH) as described previously (Pei *et al.*, 1997). Whole-cell currents were recorded using a CEZ-2200 patch clamp amplifier (Nihon Kohden, Tokyo, Japan). The resulting values were corrected for liquid junction potential, and leak currents were not subtracted. For data analysis, pCLAMP 8.1 software (Molecular Devices, Sunnyvale, CA, USA) was used. For K_{in} current measurement, the pipette solution containing 150 mM K-Glu, 10 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 5 mM ATP, and 10 mM HEPES-KOH (pH 7.8) and the bathing solution containing 50 mM KCl, 10 mM $CaCl_2$, and 5 mM MES-KOH (pH 5.6) were used. Osmolality was adjusted to 560 mosmol kg^{-1} (pipette solution) and 530 mosmol kg^{-1} (bathing solution) with D-sorbitol.

In-gel protein kinase assay

The in-gel kinase assay was performed as described previously by Mori and Muto (1997) with slight modifications. Proteins were isolated from 4×10^4 GCPs and dissolved in Laemmli's sample buffer containing phosphatase inhibitor cocktail 2 (Sigma-Aldrich, St Louis, MO, USA). The artificial substrate protein (myelin basic protein, MBP) for protein kinase was mixed with SDS-PAGE gel (10% polyacrylamide) before polymerization. After fractionation of proteins by SDS-PAGE, SDS in gels was removed by washing with 20% (v/v) isopropanol in 20 mM TRIS-HCl (pH 8.0). Fractionated proteins in gels were denatured completely by the treatment with 6 M guanidine-HCl and then renatured by washing the gels with 20 mM TRIS-HCl (pH 8.0) containing 1 mM 2-mercaptoethanol and 0.03% (w/v) Tween 20. The gels were equilibrated with 25 ml of the reaction mixture containing 20 mM TRIS-HCl, pH 7.5, 5 mM $MgCl_2$, 4 mM 2-mercaptoethanol, and 1 mM EGTA and incubated in 10 ml of the reaction mixture supplemented with 25 μM [γ-^{32}P]ATP (740 kBq) and 37.5 μM ATP. Phosphorylation was carried out for 1 h at 30 °C and terminated by removing the reaction mixture and adding 30 ml of 5% (w/v) TCA-PPi. Unreacted ^{32}P was removed by successive washing with TCA-PPi. The washed gels ware dried on filter paper and the radioactivity of the gels was detected with X-ray film.

Immunoblot analysis

Protein samples were fractionated by 10% SDS-PAGE and transfer to PVDF membranes (Millipore, Billerica, MA, USA) with blotting buffer containing 25 mM TRIS, 192 mM glycine, and 20% (v/v) methanol. After blocking with 5% (w/v) skim milk in TBS-T buffer

(10 mM TRIS-HCl, pH7.4, 0.1 M NaCl, and 0.1% (v/v) Tween 20), membranes were incubated with the anti-AAPK antibody at a dilution of 1:100 (v/v) for 1 h at room temperature. The membrane was washed with TBS-T four times, and then the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:2000 (v/v) for 1 h at room temperature. After washing as described above, AAPK proteins were visualized using Chemi-Lumi One (Nacalai tesque, Kyoto, Japan) and AAPK activities were evaluated using LAS3000 (FUJIFILM, Tokyo, Japan).

Statistical analysis

Significance of differences between data sets was assessed by Student's *t* test analysis in all parts of this article. Differences at the level of $P < 0.05$ were regarded as significant.

Results

ABA- and MeJA-induced stomatal closure in fia *mutants*

Abscisic acid and MeJA induce stomatal closure with their signalling cross-talk (Suhita *et al.*, 2004). ABA- and MeJA-induced stomatal closure was investigated in the *fia* mutant. Application of 1 and 10 μM ABA induced stomatal closure in the wild type but did not in the *fia* mutant (Fig. 1A), which is consistent with our previous result (Iwai *et al.*, 2003). Application of MeJA induced stomatal closure in the wild type and the *fia* mutant (Fig. 1B). These results indicate that FIA functions in early ABA signalling in guard cells of fava bean but not in MeJA signalling. Therefore, responses to ABA were analysed in the *fia* mutant in the following experiments.

ROS production and ROS-induced stomatal closure in fia *mutant*

ABA induces ROS production and H_2O_2 elicits $[Ca^{2+}]_{cyt}$ elevation/oscillation, resulting in stomatal closure (Pei *et al.*, 2000; Murata *et al.*, 2001; Zhang *et al.*, 2001). ROS production was measured in guard cells of the *fia* mutant using H_2DCF-DA and H_2O_2-induced stomatal closure in the *fia* mutant was examined.

Application of 10 μM ABA elicited ROS production in guard cells of the wild type but ABA failed to elicit ROS production in guard cells of the *fia* mutant (Fig. 2A). Moreover, exogenous H_2O_2 induced stomatal closure in the wild type and the *fia* mutant (Fig. 2B). These results suggest that FIA functions upstream of ROS production in ABA signalling and that signal components downstream of ROS production are intact in the *fia* mutants.

NO production and NO-induced stomatal closure in fia *mutants*

ABA induces NO production to induce stomatal closure and exogenous NO induces stomatal closure (Neill *et al.*, 2002; Desikan *et al.*, 2002; Bright *et al.*, 2006). NO production in guard cells of the *fia* mutant was measured using DAF-2DA and NO-induced stomatal closure in the *fia* mutant was investigated.

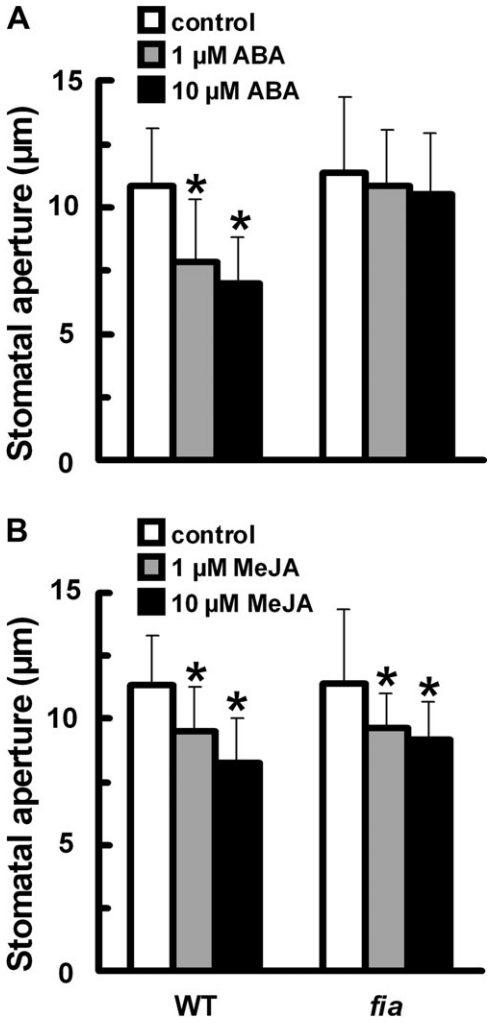

Fig. 1. Stomatal movement in *fia* (fava bean impaired in ABA-induced stomatal closure) mutant. Epidermal tissues of the wild-type (WT) plants and the *fia* plants were treated with (A) 1 and 10 μM of abscisic acid (ABA) and (B) 1 and 10 μM of methyl jasmonate (MeJA) for 1 h. As a control for ABA and MeJA treatments, 0.1% (v/v) ethanol was used. After incubation, stomatal apertures were measured. Averages from three independent experiments (20 total stomata each) are shown. Error bars represent standard deviations. Asterisks denote significant differences ($P < 0.05$).

Application of 10 μM ABA induced NO production in guard cells of the wild type but not in guard cells of the *fia* mutant (Fig. 2C), and application of an NO donor, *S*-nitroso-*N*-acetyl-penicillamine (SNAP), induced stomatal closure in both the wild type and the *fia* mutant plants (Fig. 2D). These results suggest that FIA functions upstream of NO production in ABA signalling and that signal components downstream of NO production are intact in the *fia* mutant.

Potassium channel currents in fia *mutants*

Abscisic acid suppresses plasma membrane K_{in} channel activity in guard cells, which is favourable to ABA-induced stomatal closure (Schroeder and Hagiwara, 1989). A whole-cell

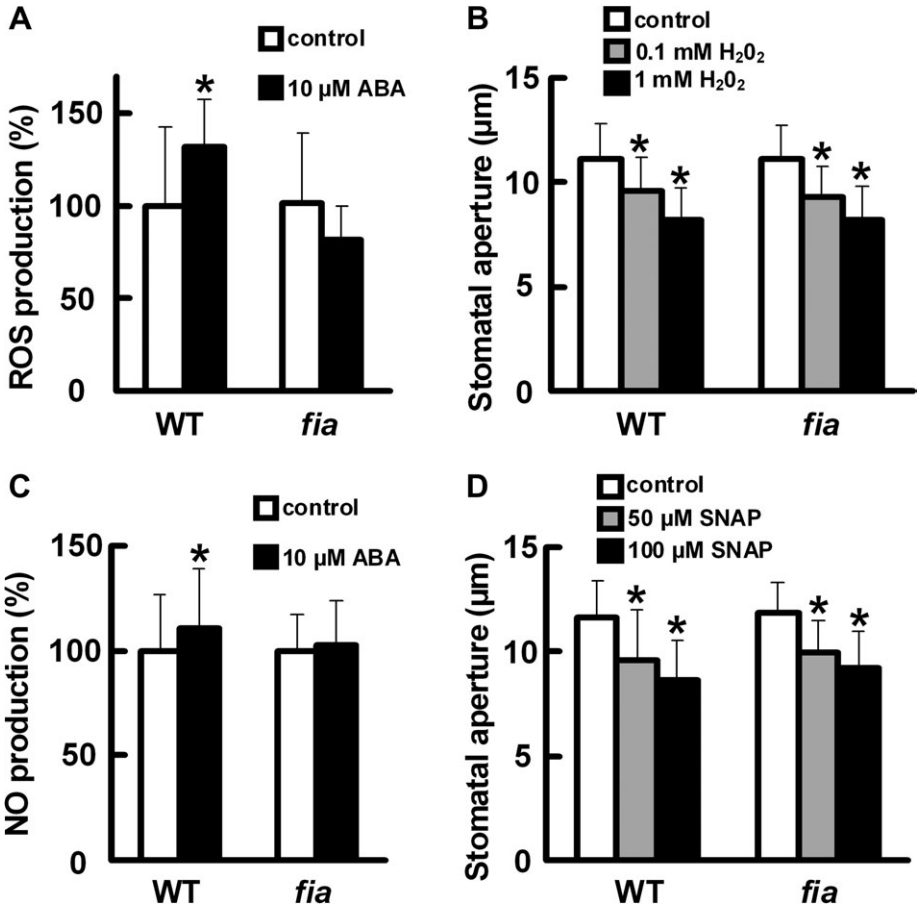

Fig. 2. Reactive oxygen species (ROS) and nitric oxide (NO) production in guard cells of *fia* (fava bean impaired in ABA-induced stomatal closure) mutant. (A) The *fia* mutation impairs ROS production induced by abscisic acid (ABA) treatments. After pretreatment with 2′,7′-dichlorodihydrofluorescein diacetate, epidermal tissues of the wild-type plant and the *fia* plant were treated with 0.1% ethanol or 10 μM ABA for 15 min, and then fluorescent intensities were measured. (B) Exogenous H_2O_2 induces stomatal closing in *fia* plants. Epidermal tissues of the wild-type plant and *fia* plant were treated with 0 to 1 mM H_2O_2 for 1 h. After incubation, stomatal apertures were measured. Averages from three independent experiments (20 stomata each) are shown. (C) The *fia* mutation impairs NO production induced by ABA treatments. Epidermal tissues were treated with 0.1% (v/v) ethanol or 10 μM ABA for 40 min, and then fluorescent intensity were measured. (D) An NO donor, *S*-nitroso-*N*-acetyl-penicillamine (SNAP) induces stomatal closing in the *fia* mutant plants. After pretreatment with 4,5-diaminofluorescein-2 diacetate, epidermal tissues were treated with 0 to 100 μM SNAP for 1 h. Averages from three independent experiments (20 stomata each) are shown. The vertical scales of (A) and (C) represent the percentages of fluorescence levels when fluorescent intensity of ABA-treated cells was normalized to control value taken as 100% for each experiment. Each datum was obtained from >60 total guard cells. Error bars represent standard deviations. Asterisks denote significant differences ($P < 0.05$).

patch-clamp technique was used to measure K_{in} channel currents of ABA-treated *fia* GCPs.

Treatment with ABA reduced the steady-state K_{in} currents in the wild-type GCPs (Fig. 3A, C) but not in the *fia* mutant GCPs (Fig. 3B, D). The steady-state outward-rectifying K^+ (K_{out}) currents were not significantly affected by ABA treatment in either the wild-type GCPs (Fig. 3A, C) or the *fia* mutant GCPs (Fig. 3B, D). These results suggest that FIA is involved in suppression of K_{in} channel currents by ABA.

Activation of AAPK in fia *mutants*

It has been found that AAPK is an ABA-responsible protein kinase (Li and Assmann, 1996; Mori and Muto, 1997). To confirm that FIA functions in earlier ABA signalling of guard cells, activation of AAPK in guard cells of the wild type and the *fia* mutant in response to ABA was examined using an in-gel protein kinase assay. Treatment with 10 μM ABA for 5 min activated a 48-kDa protein kinase in guard cells of the wild type (Fig. 4A, upper panel), where the 48-kDa protein was confirmed as AAPK by immunoblot analysis using anti-AAPK antiserum raised against AAPK of fava bean (Fig. 4A, lower panel), in agreement with our previous result (Mori and Muto, 1997). As previously reported (Mori and Muto, 1997), the activities of two other kinases (46 kDa and 49 kDa) were also detected but these activations were constitutive and independent of ABA and these bands did not react with the anti-AAPK antibody (Fig. 4A, lower panel). In the *fia* mutant, ABA did not activate AAPK (Fig. 4A, upper panel) or affect expression of AAPK proteins in guard cells

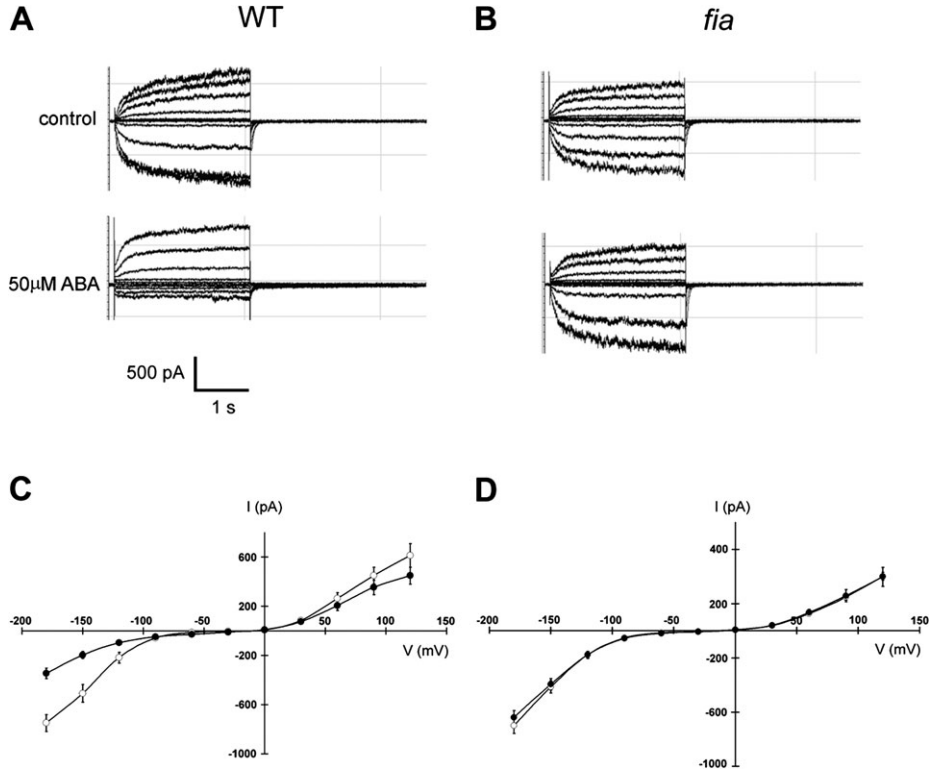

Fig. 3. K_{in} currents in guard cells of *fia* (fava bean impaired in ABA-induced stomatal closure) mutant. Whole cell recordings of steady-state currents in the wild-type (WT) guard cell protoplasts (GCPs) (A) and the *fia* mutant GCPs (B) treated with 50 μM abscisic acid (ABA) or 0.1% (v/v) ethanol, as a control. Seal resistances were >1 GΩ. Steady-state whole cell currents (I) were plotted against voltage (mV) for the wild-type GCPs (C) and *fia* GCPs (D). GCPs were treated with 0.1% (v/v) ethanol (open circles) or 50 μM ABA (closed circles). Each datum was obtained from at least seven GCPs. Bars represent standard deviations.

(Fig. 4A, lower panel). Treatment with ABA did not affect the activities of the other two kinases in the wild type and the *fia* mutant (Fig. 4A, upper panel). These results indicate that the *fia* mutation impairs AAPK activation in response to ABA.

To analyse the amino acid sequence of AAPK in the *fia* mutant, *AAPK* cDNA was isolated from the *fia* mutant and the DNA sequence was determined. Comparison of the amino acid sequences between the *fia* mutant and the wild type indicated that Ser is substituted with Phe at amino acid residue 96 (Fig. 4B). However, an ATP-binding site and an activation loop of AAPK, which are important for AAPK kinase activity, were fully conserved in the *fia* mutant (Fig. 4B).

Discussion

In this study, the aim was to elucidate the function of FIA in ABA signal transduction leading to stomatal closure in *Vicia faba*. The *fia* mutation impaired ABA-induced stomatal closure but not MeJA-induced stomatal closure (Fig. 1), indicating that FIA acts as a signal component of guard cell ABA signalling and that FIA functions upstream of the branching point of the MeJA and ABA signal pathways.

ABA did not induced ROS production in the guard cells of the *fia* mutant in contrast to the wild type (Fig. 2A). Exogenous H_2O_2 induced stomatal closure in both the *fia* mutant and the wild type (Fig. 2B), suggesting that FIA functions upstream of ROS production and that downstream of ROS production in the ABA signal pathway is intact in the *fia* mutant. The *fia* mutation suppressed ABA-induced NO production in guard cells (Fig. 2C) and the application of an NO donor, SNAP, induced stomatal closure in the *fia* mutant guard cells as well as the wild-type guard cells (Fig. 2D), suggesting that FIA functions upstream of NO production.

Production of ROS and NO is induced by variety of stimuli (Neill *et al.*, 2003; Apel and Hirt, 2004). ABA induces NO production leading to stomatal closure (Desikan *et al.*, 2002; García-Marta and Lamattina, 2002; Neill *et al.*, 2002), although Lozano-Juste and León (2010) have recently shown that NO acts as a negative regulator of ABA signalling. Moreover, Clarke *et al.* (2000) have shown that production of ROS and NO are induced in parallel and Bright *et al.* (2006) have demonstrated that ABA-induced NO production in guard cells depends on ABA-induced ROS production. Therefore, the involvement of NO to ABA signalling leading to stomatal closure is still controversial.

ABA activates S-type anion channels and suppresses K_{in} channels (Schroeder and Hagiwara, 1989; Schmidt *et al.*, 1995). Steady-state K_{in} currents in the *fia* mutant GCPs were not suppressed by treatment with ABA (Fig. 3B, D) in

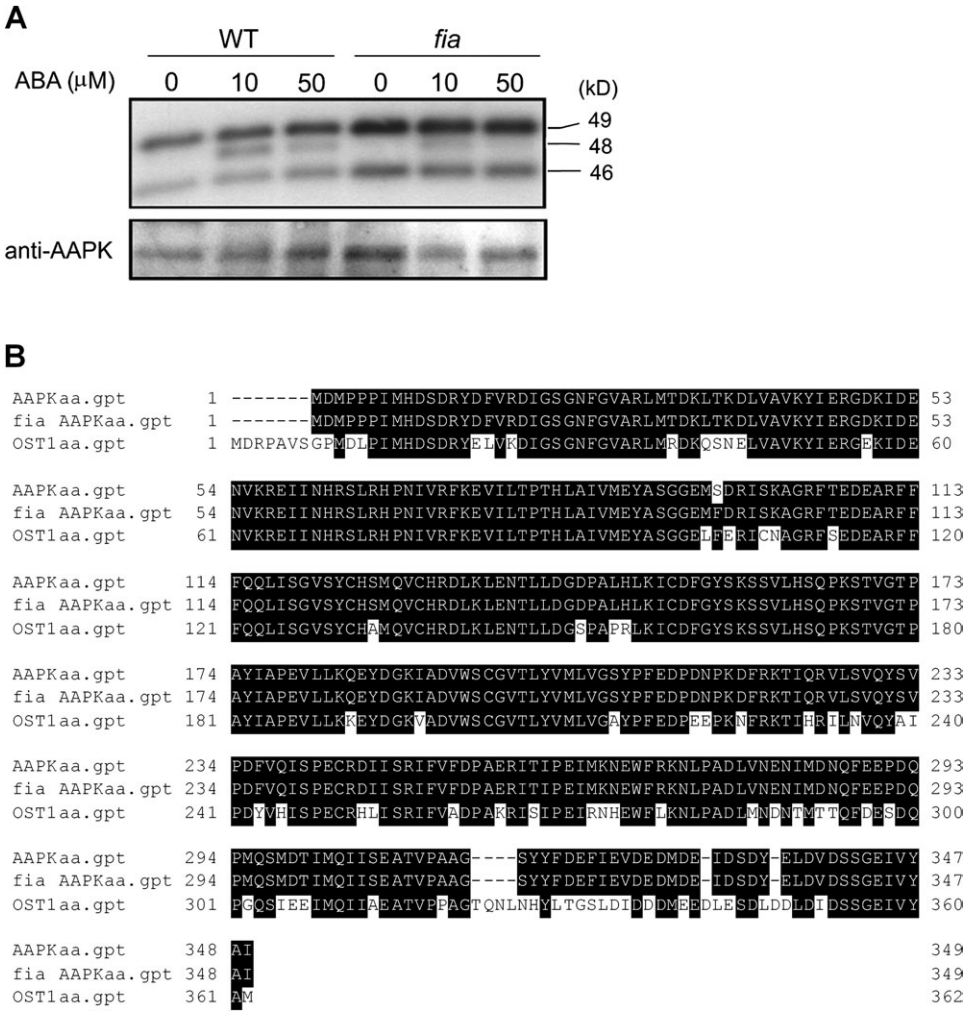

Fig. 4. ABA-activated protein kinase (AAPK) activation and amino acid sequences of AAPK. (A) Guard cell protoplasts (GCPs) from the wild-type (WT) plants and the *fia* (fava bean impaired in ABA-induced stomatal closure) plants were incubated with indicated concentration of abscisic acid (ABA) for 5 min. Each sample contained proteins isolated from 4×10^4 GCPs. The protein kinase assay was performed with a substrate, MBP, as described in Materials and methods. The amount of AAPK protein in each sample was confirmed by the immunoblot analysis (lower panel). Equal amount of protein extracts from GCPs were fractionated by SDS-PAGE, and followed by immunoblot analysis using anti-AAPK antibody. (B) Alignment of the amino acid sequences of AAPK in fava bean wild type (accession no. AF186020) and in *fia* mutant (in this study) and OST1 in *Arabidopsis* (accession no. AJ316009). The AAPK sequences were highly conserved between the *fia* mutant and the wild type. Ser was substituted with Phe at amino acid residue 96 in the *fia* mutant. Sequences were aligned by GENETIX ver. 8.0 software (GENETIX, Tokyo, Japan).

contrast to results in the wild-type GCPs (Fig. 3A, C), indicating that FIA functions in the early stages of ABA signalling in guard cells.

The results of in-gel kinase assay showed that the *fia* mutation disrupted activation of AAPK by ABA (Fig. 4). Orthologues of AAPK in *Arabidopsis*, Snf1-related kinase 2 (SnRK2) proteins, including OST1, had been identified as positive regulators in ABA signalling (Yoshida *et al.*, 2002; Mustilli *et al.*, 2002). SnRK2 proteins are implicated in phosphorylation of ABA response element binding factors, which regulate transcription of ABA-responsible genes (Fujii *et al.*, 2007). In faba bean, AAPK is autophosphorylated in the presence of ABA (Li and Assmann, 1996) and in *Arabidopsis*, autophosphorylation sites of OST1 are critical for OST1 functions as a protein kinase and as a signal component leading to stomatal closure (Belin *et al.*, 2006).

AAPK conserves the SnRK2-specific domain required for kinase activity of OST1 (Mustilli *et al.*, 2002; Belin *et al.*, 2006). AAPK phosphorylates AAPK-interacting protein 1, which is an RNA-binding protein A/B (Li *et al.*, 2002). AAPK also phosphorylates the recombinant peptide of the C-terminal region of KAT1 in a fava bean protoplast system (Mori *et al.*, 2000). Recently, the related phosphorylation site of KAT1 has been identified (Sato *et al.*, 2009). The comparison of sequences of AAPK between the *fia* and the wild-type plant indicates that only one Ser residue is substituted with Phe at amino acid residue 96 (Fig. 4B) and that the substituted residue is not located either on the autophosphorylation site of OST1 or on a domain

conserved among SnRK2 proteins. These results suggest that FIA is not AAPK itself but a signal component that functions upstream of AAPK in ABA signalling.

Recently, PYR/PYL/RCARs have been identified as ABA receptors in *Arabidopsis* (Ma *et al.*, 2009; Park *et al.*, 2009). PYR/PYL/RCAR proteins bind to ABA and ABA-bound PYR/PYL/RCAR proteins interact with type 2C protein phosphatases (PP2Cs), including ABI1 and ABI2, resulting in suppression of their phosphatase activities. The detailed mechanism of ABA sensing via PYR/PYL/RCAR proteins has been clarified by crystal structure analysis (Melcher *et al.*, 2009; Miyazono *et al.*, 2009; Nishimura *et al.*, 2009). However, FIA is unlikely to function as an ABA receptor because PYR/PYL/RCARs redundantly function in ABA sensing in *Arabidopsis* (Ma *et al.*, 2009; Park *et al.*, 2009).

Furthermore, *Arabidopsis* novel GPCR-type G proteins, GTG1 and GTG2, also bind to ABA, mediating ABA signalling (Pandey *et al.*, 2009) and indicating the redundancy and diversity of ABA perception. Hence, it is not likely that the ABA insensitivity of the *fia* mutants is caused by disruption of ABA perception which involves PYR/PYL/RCAR proteins or GPCR-type G proteins. The detailed mechanism of signalling between the perception of ABA and AAPK activation remains to be clarified. Furuichi *et al.* (2005) indicated that recombinant AAPK protein was phosphorylated only with the extract from ABA-treated guard cells, suggesting the presence of a kinase functioning upstream of AAPK.

In conclusion, our results suggest that FIA functions as an early signal component upstream of AAPK activation in ABA signalling but does not function in MeJA signalling in guard cells of *Vicia faba* (Fig. 5). To clarify whether FIA functions as a positive regulator of AAPK after ABA perception, further investigations are required.

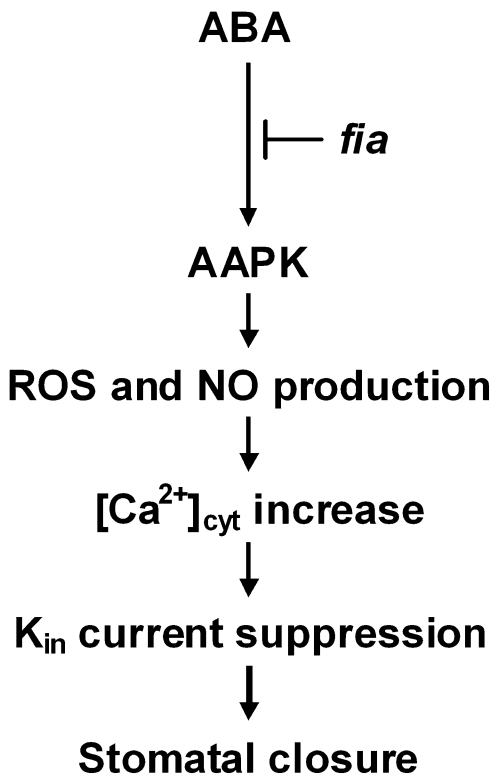

Fig. 5. Simple model of abscisic acid (ABA) signalling in guard cells. FIA (fava bean impaired in ABA-induced stomatal closure) functions as an early component factor upstream of ABA-activated protein kinase (AAPK) activation in ABA signalling leading to stomatal closure although FIA does not mediate methyl jasmonate signalling. NO, nitric oxide; ROS, reactive oxygen species.

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