Guard cell-specific inhibition of *Arabidopsis MPK3* expression causes abnormal stomatal responses to abscisic acid and hydrogen peroxide

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Summary

• MAP kinases have been linked to guard cell signalling. *Arabidopsis thaliana* MAP Kinase 3 (MPK3) is known to be activated by abscisic acid (ABA) and hydrogen peroxide (H_2O_2), which also control stomatal movements.

• We therefore studied the possible role of MPK3 in guard cell signalling through guard cell-specific antisense inhibition of *MPK3* expression.

• Such transgenic plants contained reduced levels of *MPK3* mRNA in the guard cells and displayed partial insensitivity to ABA in inhibition of stomatal opening, but responded normally to this hormone in stomatal closure. However, ABA-induced stomatal closure was reduced compared with controls when cytoplasmic alkalinization was prevented with sodium butyrate. *MPK3* antisense plants were less sensitive to exogenous H_2O_2, both in inhibition of stomatal opening and in promotion of stomatal closure, thus MPK3 is required for the signalling of this compound. ABA-induced H_2O_2 synthesis was normal in these plants, indicating that MPK3 probably acts in signalling downstream of H_2O_2.

• These results provide clear evidence for the important role of MPK3 in the perception of ABA and H_2O_2 in guard cells.

Key words: abscisic acid (ABA), *Arabidopsis thaliana*, hydrogen peroxide (H_2O_2), MAP kinase, MPK3, stomata.

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Introduction

Plants exchange CO_2 and water vapour with the atmosphere through stomatal pores. The aperture of stomatal pores is finely regulated by environmental conditions and hormonal balance, as the control of gas exchange, especially water loss, is an important element in the adaptation of plants to reduced soil water availability. Swelling of the two guard cells that surround the stomatal pore causes an increase in stomatal aperture, whereas a reduction in stomatal aperture induced by abscisic acid (ABA), for example, is brought about by a reduction in guard cell turgor (reviewed by Hetherington & Woodward, 2003; Fan *et al.*, 2004; Roelfsema & Hedrich, 2005).

Reactive oxygen species (ROS) are important signals mediating stomatal movements and other physiological processes in plants (Apel & Hirt, 2004; Desikan *et al.*, 2004a; Mittler *et al.*, 2004; Foyer & Noctor, 2005). Hydrogen peroxide (H_2O_2) will induce stomatal closure and cytosolic calcium elevations in *Commelina communis* guard cells (McAinsh *et al.*, 1996). Additionally, H_2O_2 is produced in guard cells in response to numerous stimuli, including pathogen elicitors (Lee *et al.*, 1999), ABA (Pei *et al.*, 2000; Zhang *et al.*, 2001), extracellular calmodulin

(Chen *et al.*, 2004), methyl jasmonate (Suhita *et al.*, 2004), darkness (Desikan *et al.*, 2004b; She *et al.*, 2004) and ozone (Joo *et al.*, 2005). H_2O_2 is necessary for the activation of plasma membrane calcium channels in ABA-induced stomatal closure (Pei *et al.*, 2000). Plasma membrane NADPH oxidases have been identified as a source of ROS necessary for stomatal closure, as the double mutant *atrbohD/F* is partially impaired in ABA-induced stomatal closure, and in the activation of calcium channels (Kwak *et al.*, 2003).

ABA-insensitive *Arabidopsis* mutants show reduced production of ROS in guard cells, but will respond normally to exogenous H_2O_2, indicating that genes affected by these mutations are involved in signalling upstream of H_2O_2 generation. The *abi1* PP2C phosphatase (Murata *et al.*, 2001) and *ost1* kinase (Mustilli *et al.*, 2002) mutants do not produce H_2O_2 in response to ABA, but respond normally to exogenously added H_2O_2. The ABA-insensitive *gpa1* G protein alpha subunit mutant (Wang *et al.*, 2001) shows a reduced production of H_2O_2 in guard cells in response to extracellular calmodulin (Chen *et al.*, 2004) and ozone (Joo *et al.*, 2005).

Mitogen-activated protein kinase (MAPK) cascades in plants are associated with the signalling of hormones, biotic and abiotic stresses, and in particular with ROS and stomatal movements (reviewed by Morris, 2001; Jonak *et al.*, 2002; Nakagami *et al.*, 2005; Pitzschke & Hirt, 2006). In the context of stomatal signalling, Mori & Muto (1997) found an ABA-inducible myelin basic protein (MBP) kinase activity in guard cell protoplasts from *Vicia faba*, while Burnett *et al.* (2000) found that a MAPK/ERK kinase (MEK) inhibitor (PD98059) blocks ABA action on guard cell movements in pea. More recently, Jiang *et al.* (2003) found that the same inhibitor prevents ABA-induced H_2O_2 generation in guard cells. MAPK genes are known to be expressed in guard cells; Kwak *et al.* (1997) found two expressed sequence tags (ESTs) with 100% identity at the amino acid level to the *MPK3* and *MPK4* genes of *Arabidopsis* in a *Brassica campestris* guard-cell library; and a cDNA very similar to *WIPK* (the tobacco orthologue of *Arabidopsis MPK3*) was isolated from a *Nicotiana rustica* guard cell library (C. Leckie *et al.*, unpublished).

MPK3 and MPK4 are thus candidates for ABA signal transduction in *Arabidopsis* guard cells. However, inactivation of the *MPK4* gene does not result in impaired physiological responses to abiotic responses such as high temperature and high salinity, but rather in enhanced disease resistance and constitutive activation of defence responses (Petersen *et al.*, 2000). In contrast, MPK3 appears to be involved in both biotic and abiotic stress responses. MPK3 probably has an important role in defence signalling, as it is part of a cascade that appears to function downstream of the bacterial elicitor flagellin receptor FLS2 (Asai *et al.*, 2002). In addition, *MPK3* gene transcription and/or kinase activity is activated upon interaction with fungi or with the fungal elicitor chitin (Wang *et al.*, 2001; Schenk *et al.*, 2003). MPK3 also appears to play an important role in the response to environmental stresses, as *MPK3* transcription is rapidly induced in *Arabidopsis* plants treated with touch, low-temperature or salinity stress (Mizoguchi *et al.*, 1996). Kovtun *et al.* (2000) found strong activation of MPK3 enzyme activity in leaf mesophyll protoplasts by H_2O_2 but not by ABA treatments. In contrast, Lu *et al.* (2002) found that this kinase is activated by both ABA and H_2O_2 in *Arabidopsis* seedlings. The discrepancy between the latter two reports in activation of MPK3 by ABA might possibly be accounted for by the material used. In addition, Lu *et al.* (2002) showed that overexpression of the *MPK3* gene increases ABA sensitivity in ABA-induced postgermination arrest of growth, suggesting that the ABA signal is transmitted through MPK3 in this system. Other sources of abiotic stress also activate MPK3, for example, subjecting an *Arabidopsis* cell suspension to hyperosmotic conditions (Droillard *et al.*, 2000). Ozone, an ROS-generating atmospheric pollutant, also causes MPK3 activation and its translocation to the nucleus (Ahlfors *et al.*, 2004)

In this work we focused our attention on the possible role of MPK3 in guard cell signalling. In order to avoid complications arising through downregulation of MPK3 in other tissues, we have produced guard cell-specific *MPK3* antisense *Arabidopsis* plants by using a deleted version of the potato *KST1* promoter (Plesch *et al.*, 2001) to drive expression of a 3′ sequence of the *MPK3* cDNA in inverse orientation. Plants expressing this construct display a partial insensitivity to ABA in inhibition of stomatal opening, but respond normally to this hormone in stomatal closure. However, like *gpa1* mutants (Wang *et al.*, 2001), they respond less to ABA in stomatal closure when cytoplasmic alkalinization by ABA is prevented with sodium butyrate. *MPK3* antisense plants are less sensitive to exogenous H_2O_2 both in inhibition of stomatal opening and in promotion of closure, indicating that MPK3 is also required for the signalling of this compound. These results provide evidence that MPK3 plays a critical role in the control of stomatal movements.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh. ecotype Columbia 1 (Nottingham Arabidopsis Stock Centre) was used as the wild type. Seeds were surface-sterilized in 10% (v/v) commercial bleach with 0.01% (v/v) Tween 20 for 10 min and rinsed four times in sterile water. Seeds were plated in Petri dishes containing half-strength Murashige and Skoog medium (Invitrogen, Carlsbad, CA, USA) with 1% (w/v) sucrose and 0.6% (w/v) agar. Seeds on plates were maintained in the dark for 2–3 d at 4°C to break dormancy, before being transferred to a growth room at 22–23°C, with a 16-h light photoperiod. Illumination was provided with Gro Lux fluorescent tubes (Sylvania Corps, Danvers, MA, USA) (photon fluency rate approx. 45 μmol m^{-2} s^{-1}). After 5 or 6 d, seedlings were transferred to pots containing a mixture of vermiculite, peat and perlite (1 : 1 : 1) and fertilized every 2 d.

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MPK3 antisense construct and plant transformation

An MPK3 EST (accession 96H23T7, Newman *et al.*, 1994) was obtained from the Ohio Arabidopsis Stock Centre. An *Hae*III–*Sfc*I 422-bp fragment from the *MPK3* cDNA was subcloned into the compatible *Sma*I and *Sal*I sites of the polylinker of BinK, a binary vector with a 642-bp-long fragment of the potato *kst1* promoter (Plesch *et al.*, 2001) inserted in the polylinker. BinK was a kind gift from Dr Bernd Müller-Röber (Max-Planck-Institute of Molecular Plant Physiology, Germany). In the resulting construct, the fragment of the *MPK3* gene is oriented antisense relative to the *KST1* promoter. The antisense construct was introduced in plants by *Agrobacterium tumefaciens*-mediated transformation using the floral-dip method (Clough & Bent, 1998). Control plants were transformed with the unmodified BinK vector. T_1 transgenic seeds were selected on kanamycin, and T_2 plants homozygous for a single copy of the insertion locus (as determined by segregation of kanamycin resistance) were used for further experiments.

Genetic analysis of *MPK3* antisense plants

The presence of the transgene in T_2 plants was confirmed by PCR analysis of genomic DNA using primers MP3 forward (5′-ACGTTTGACCCCAACAGAAG-3′) and MP3 reverse (5′-GGCTTTTGACAGATTGGCTC-3′). These primers comprise a region of the MPK3 cDNA used for the antisense construct, and span an intron of MPK3 genomic sequence. Therefore PCR amplification yields two bands in *MPK3* antisense plants and one band in wild-type and transgenic plants transformed with the empty vector.

Isolation of guard cell-enriched epidermis

Abaxial epidermis was isolated from the four youngest fully developed leaves of plants at the same developmental stage as used for stomatal bioassays. For each RNA extraction, 16 leaves in total were used. Leaves were detached and stuck by their abaxial surface to 3M 810 tape (3M, St Paul, MN, USA). Using batches of eight leaves, first petioles and central nerves were removed with a razor blade. The remaining leaf tissue was then scraped with a razor blade so that only abaxial epidermis remained attached to the tape. The tape with adhered epidermis was placed in a 15-ml plastic tube containing 10 ml 10 : 50 buffer (see below), and sonicated with a Virsonic sonicator (Virtis, Gardiner, NY, USA) for 5 s using the microtip provided (power set at 5). After draining off excess liquid, the tape was placed on a clean glass with the adhesive side upwards, and 150 µl 2-propanol added. The residual epidermis and adhesive was subsequently scraped off with a razor blade. The resulting material was transferred with forceps into a 2-ml screw-cap tube containing approx. 200 µl 0.5-µm acid-washed glass beads (Sigma, Poole, UK). The tube was immediately frozen in liquid nitrogen. Viability of guard cells in sonicated epidermis was checked with fluorescein diacetate. More than 99% fluorescing cells in the resulting epidermis corresponded to guard cells.

RNA extraction

750 µl Trizol (Invitrogen) was added to the tubes containing epidermis and glass beads. Tubes were subsequently homogenized three times in a Minibeadbeater-1 homogenizer (Biospec Products, Bartles, OK, USA) at 4600 rpm for 30 s, alternating with incubations in ice to avoid overheating. Subsequent RNA extraction was performed as indicated by the manufacturer. Tubes were centrifuged at 12 000 ***g*** for 10 min at 4°C and the liquid phase was transferred to a new microfuge tube. The adhesive layer of the tape – an acrylic polymer – is insoluble in Trizol and floated after centrifugation. This centrifugation step was repeated if necessary. Then 150 µl chloroform was added, and samples were centrifuged at 12 000 ***g*** for 15 min at 4°C. The upper aqueous phase was transferred to another tube in which RNA was precipitated for 1 h at −20°C with 375 µl isopropanol in the presence 5 µg glycogen (Invitrogen). After centrifuging at 12 000 ***g*** for 10 min at 4°C, the pellet was washed once with 75% ethanol. The pellet was air-dried and resuspended in 20 µl water.

Analysis of *MPK3* and *MPK4* expression

Before performing RT–PCR, 10 µl of the sample was treated with 1 U RQ1 DNAse (Promega, Madison, WI, USA), which was subsequently heat-inactivated as indicated by the manufacturer. Effectiveness of DNAse treatment was checked in all samples by using 1 µl DNAse-treated RNA as template in a PCR reaction with EF1α primers (see below). In all cases, no product was detected after 35 cycles of amplification. Reverse transcription with 50 U Mouse Moloney Murine Leukemia virus (MMLV)–RT (Invitrogen) was performed at 37°C as indicated by the manufacturer. The specific primers used for RT reactions were EF1c reverse (for standard control, EF1α) (*EF1*), MAPK3B reverse (for *MPK3*) or MAPK4G (for *MPK4*) (see primer sequence below). 5 µl DNAse-treated total RNA was used as template. Quantification of EF1α and MPK3 cDNA was performed by quantitative PCR using SYBR Green I, using an Opticon cycler (MJ Research, Reno, NV, USA). Primers used to amplify the *EF1α* gene were EF1c forward (5′-AGCACGCTCTTCTTGCTTTC-3′) and EF1c reverse (5′-GGGTTGTATCCGACCTTCTTC-3′), *MPK3* was amplified with MAPK3B forward (5′-GACAGAGTTGCTTGGCACAC-3′) and MAPK3B reverse (5′-CCTCATCCAGAGGCTGTTGT-3′), and *MPK4* was amplified with MAPK4G forward (5′-ACTTTGCTGCTA-GATTCCC-3′) and MAPK4G reverse (5′-GCTCCTTAATG-TTCTCTTCTG-3′). The MAPK3B forward primer anneals to a region of the *MPK3* gene outside the fragment used for

the antisense construct. Amplifications were done in duplicate reactions using 5% of the volume from the RT reaction as template. The PCR reaction mixture contained Taq Polymerase buffer (Invitrogen), 3 mM $MgCl_2$, 0.2 mM dTNPs, 400 nM of each primer, SYBR Green I 1/62 500 from a 10 000× stock (Roche, Mannheim, Germany), 5% dimethyl sulfoxide (DMSO) and 0.625 U Taq Polymerase (Invitrogen). Tubes were incubated for 2.5 min at 95°C followed by 35 cycles each consisting of 30 s at 92°C, 30 s at 58°C, 20 s at 72°C. Fluorescence was recorded at every cycle after the extension step. Template abundance in the samples was quantified through a standard curve, constructed using as the PCR reaction template dilutions of plasmids containing fragments of *EF1α* and *MPK3* genes, and the complete cDNA of *MPK4*. After each reaction, a 'melting' curve of the amplification products was performed to assure the presence of a single amplification product, which was also confirmed by agarose gel electrophoresis.

Stomatal aperture bioassays

For all experiments, the two youngest fully expanded leaves from 16- to 19-d-old, unbolted plants were used. To measure promotion of stomatal closure by ABA, detached leaves were floated in 10 : 50 buffer (10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES)-KOH pH 6.15 and 50 mM KCl) with the addition of 0.05 mM $CaCl_2$ for 2 h in the light (under the same conditions as used previously for plant growth). After this incubation, ABA (mixed isomers, Sigma) was added as indicated to a final concentration of 20 μM from a 50-mM stock solution in ethanol, and leaves were incubated for a further 2 h before measuring apertures. When indicated, sodium butyrate (Sigma) was added to a final concentration of 1 mM from a 100-mM stock solution in water, together with ABA, to both treated and control samples. Stomatal closure by H_2O_2 was measured in a similar way as with ABA, except for the presence of 0.1 mM EGTA (Pei *et al.*, 2000) and the absence of $CaCl_2$ in 10 : 50 buffer during stomatal opening. After the initial 2-h incubation, $CaCl_2$ was added to a final concentration of 0.2 mM, to both treated samples and controls. H_2O_2 was used when indicated to a final concentration of 100 μM from a 30% v/v stock (JT Baker, Phillipsburg, NJ, USA). To measure inhibition of stomatal opening by ABA, detached leaves were floated in the dark in 10 : 0 buffer (10 mM MES-KOH pH 6.15) for 2 h to promote stomatal closure. The leaves were transferred to 10 : 50 buffer, containing ABA at a concentration of 20 μM when indicated, and were incubated in the light for an additional 2-h period. Inhibition of opening by H_2O_2 was measured in a similar way, except that 0.1 mM EGTA was added to 10 : 0 buffer. Leaves were then transferred to 10 : 50 buffer containing 0.2 mM $CaCl_2$, and H_2O_2 was added as indicated to a final concentration of 100 μM.

After incubation, stomatal apertures were measured in epidermal strips prepared as follows: leaves were briefly rinsed on a paper towel and stuck through their abaxial surface to one of the two adhesive sides of a tape (the other side of the tape had previously been stuck to a cover slip). Using a razor blade, all leaf tissues except the abaxial epidermis were rapidly removed. The epidermis layers were moistened with a few drops of the incubation solution, and the cover slip was subsequently placed on a microscope slide. Apertures of 40 stomata from each experiment were measured in a Carl Zeiss microscope (×400) with the aid of an eyepiece micrometer. Data are presented as the average from 120 aperture measurements per treatment, collected from three independent experiments.

Hydrogen peroxide production in guard cells of *Arabidopsis* was monitored using dichlorofluorescin diacetate (H_2DCF-DA; Sigma) as described by (Murata *et al.*, 2001). Epidermal leaf peels were mounted using double-sided adhesive tape on a cover slip. Epidermal tissues were incubated for 2 h in 10 : 30 buffer (30 mM KCl, 10 mM MES-KOH pH 6.5). H_2DCF-DA was then added to the incubation medium from a 100-mM stock in DMSO to a final concentration of 50 μM. After 20 min, cover slips with attached epidermis were washed briefly with distilled water and transferred to 10 : 30 buffer with or without 50 μM ABA. After 20 min, fluorescence was quantified using an Olympus FV300 laser scanning confocal microscope (Olympus America, Melville, NY, USA) with the following settings: excitation 488 nm; emission 530 nm. Olympus FV300 software was used for image analysis.

Results

Production and analysis of transgenic plants for *MPK3* expression

To gain some insight into the possible role of MPK3 in guard cell signalling, we expressed in *Arabidopsis* a 422-bp fragment of *MPK3* cDNA in antisense orientation under the control of a deleted version of the potato *KST1* promoter, which confers guard cell-specific expression. This promoter is expressed only in mature guard cells (Plesch *et al.*, 2001). The cDNA fragment used corresponds to the 3′ end of the coding region of the *MPK3* gene (a region with relatively low identity at the nucleotide level when compared with the other *Arabidopsis* MAPK genes). In initial experiments, T_2 plants that were homozygous for kanamycin resistance were screened for an abnormal response to inhibition of stomatal opening by ABA. Four out of eight *MPK3* antisense lines showed partially reduced inhibition of opening, while three lines transformed with the empty vector displayed the same response to ABA as wild-type plants (data not shown). We selected three antisense transgenic lines (AS3a, AS3b and AS3c) and a vector-transformed control line (BK) to carry out in-depth characterization of stomatal responses. Insertion of the antisense construct in the genome of these lines was confirmed by PCR (Fig. 1a). RT–PCR analysis showed that the expression of *MPK3* in

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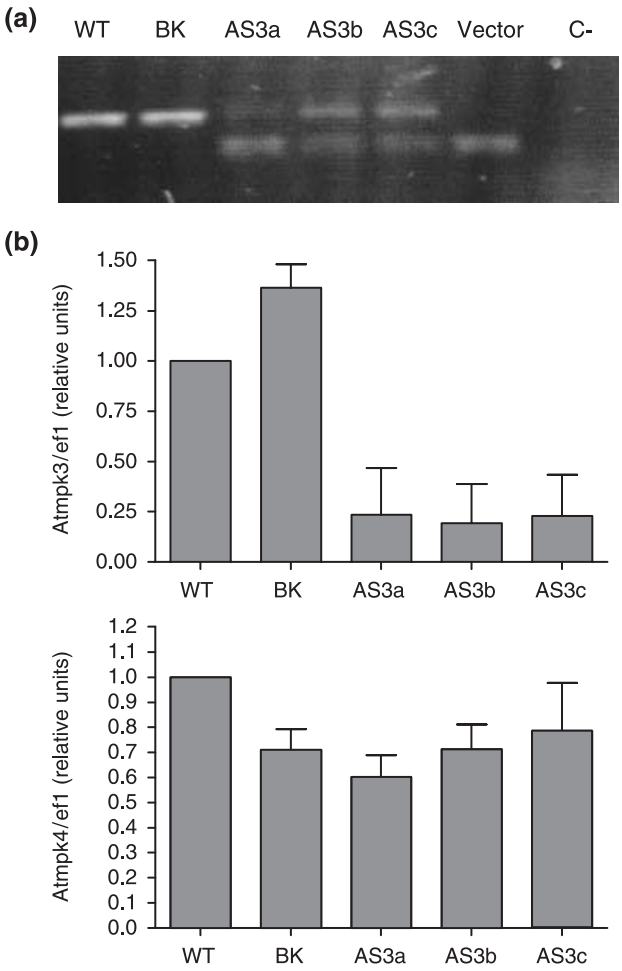

Fig. 1 Analysis of the *Arabidopsis MPK3* gene and its expression in antisense lines. (a) PCR amplification of the *MPK3* gene with the MP3 primers. The upper band corresponds to the product of endogenous genomic amplification (spanning an intron); the lower band to amplification of the introduced expressed sequence used for the antisense construct. WT, wild type; BK, empty vector control; AS3a, AS3b, AS3c, antisense lines; Vector, plasmid DNA used to transform the plants; C-, water control. (b) Expression of *MPK3* gene relative to *elongation factor 1α* in guard cell enriched epidermis. Bars = SE ($n = 2$). Genotypes as in (a). (c) Expression of the *MPK4* gene relative to *elongation factor 1α* in epidermis. Bars = SE ($n = 2$). Genotypes as in (a).

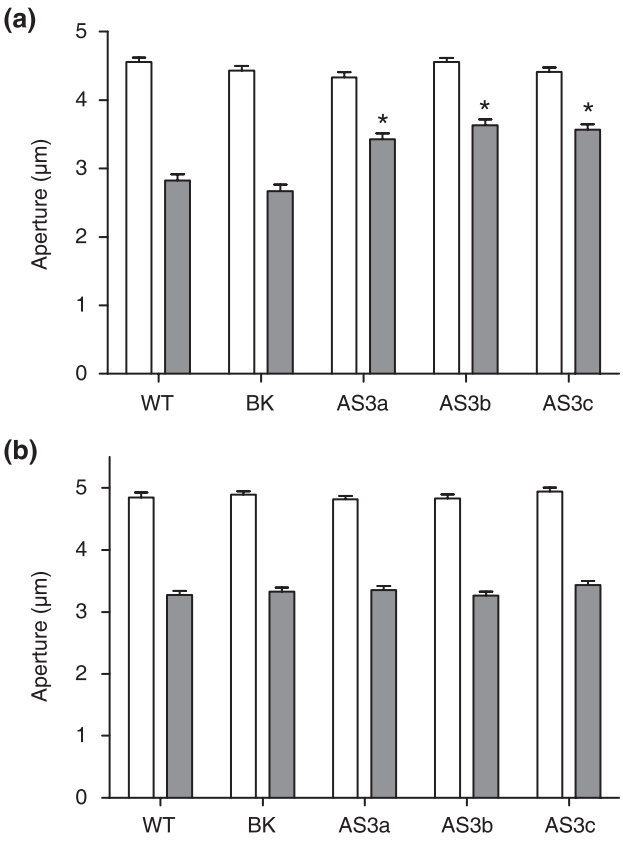

Fig. 2 *Arabidopsis MPK3* antisense plants have reduced sensitivity to abscisic acid (ABA) in inhibition of stomatal opening but respond normally to the hormone in promoting closure. (a) Inhibition of stomatal opening; (b) promotion of stomatal closure by 20 μM ABA (closed bars) or no treatment (open bars). WT, wild type; BK, empty vector control; AS3a, AS3b, AS3c, antisense lines. Error bars, SE from three independent trials; $n = 40$ per trial. Antisense lines displayed significantly less sensitivity to ABA in inhibition of opening experiments. *, Significant differences ($P < 0.01$) compared with wild-type control treated with ABA (Student's *t*-test).

guard cell-enriched fractions from the abaxial epidermis from these plants is reduced (Fig. 1b). However, *MPK3* transcript abundance was similar to that in the wild type in experiments performed with RNA extracted from whole leaves of antisense plants (data not shown), indicating that *MPK3* is expressed in different kinds of leaf cells, but the antisense construct inhibits *MPK3* expression primarily in guard cells. The expression of *MPK4*, which is also expressed in *Arabidopsis* guard cells (Petersen *et al.*, 2000), is not affected in *MPK3* antisense plants (Fig. 1c), demonstrating that the antisense construct used is specifically targeted against *MPK3*.

Effects of ABA on stomatal movement in antisense plants

Subsequently, we investigated in more detail the stomatal behaviour of *MPK3* antisense lines in response to ABA. While there was partial reduction of ABA-induced inhibition of stomatal opening in the antisense lines (Fig. 2a), these plants exhibited a wild-type response in a bioassay designed to investigate ABA-induced stomatal closure (Fig. 2b). These results suggest that MPK3 is involved in inhibition of stomatal opening by ABA, but either it does not take part in the pathway by which ABA induces stomatal closure, or its loss is compensated for by other signalling components specifically in promotion of closure.

The *Arabidopsis* G protein alpha subunit mutant *gpa1* shows a similar phenotype: it is also affected in the response to ABA

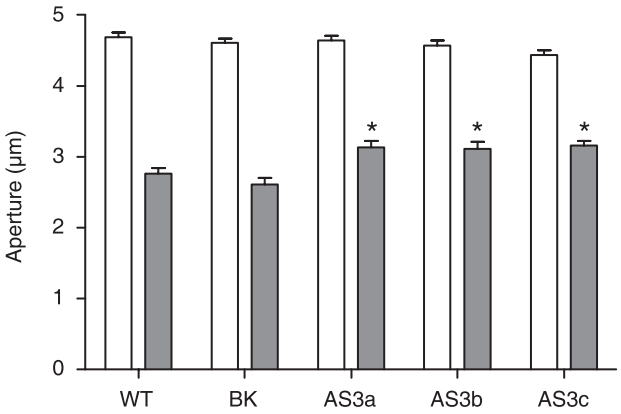

Fig. 3 Imposition of a cytosolic pH clamp with butyrate reduces abscisic acid (ABA) sensitivity in promotion of stomatal closure of *Arabidopsis MPK3* antisense plants. Promotion of stomatal closure in the presence of 1 mM sodium butyrate with (closed bars) or without (open bars) 20 μM ABA. WT, wild type; BK, empty vector control; AS3a, AS3b, AS3c, antisense lines. Error bars, SE from three independent trials; $n = 40$ per trial. Antisense lines displayed significantly less sensitivity to ABA than controls when sodium butyrate was present during the incubation. *, Significant differences ($P < 0.01$) compared with wild-type control treated with ABA (Student's *t*-test).

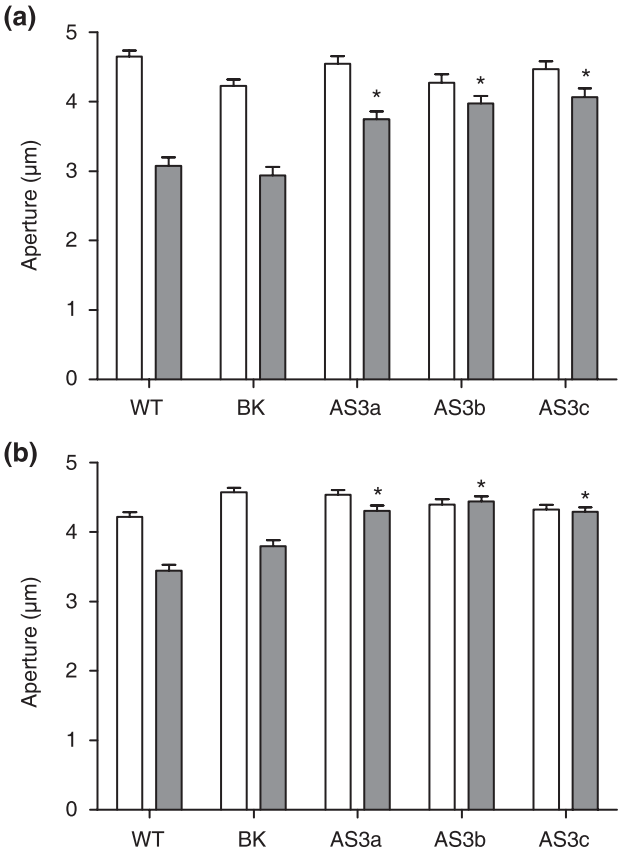

Fig. 4 *Arabidopsis MPK3* antisense plants are less sensitive to hydrogen peroxide (H_2O_2) in inhibition of stomatal opening and promotion of closure. (a) Inhibition of stomatal opening by 100 μM H_2O_2 (closed bars) or no treatment (open bars); (b) promotion of stomatal closure by 100 μM H_2O_2 (closed bars) or no treatment (open bars). WT, wild type; BK, empty vector control; AS3a, AS3b, AS3c, antisense lines. Error bars, SE from three independent trials; $n = 40$ per trial. Antisense lines displayed significantly less sensitivity to H_2O_2 in experiments on inhibition of opening and promotion of closure. *, Significant differences ($P < 0.01$) compared with wild-type controls treated with H_2O_2 (Student's *t*-test).

in inhibition of stomatal opening, while showing a wild-type promotion of closure response by this hormone (Wang *et al.*, 2001). However, these authors found that when ABA-induced cytosolic alkalinization is prevented by imposing a pH clamp with the membrane-permeant weak acid butyrate, the *gpa1* mutant shows reduced promotion of stomatal closure by ABA relative to wild-type plants. Thus we measured promotion of closure by ABA in antisense *MPK3* mutants in the presence of 1 mM sodium butyrate. As shown in Fig. 3, all three antisense lines showed a small but statistically significant reduction (relative to controls) in the response to ABA, suggesting that the ABA-induced pH increase compensates for the reduction of MPK3 in antisense lines in the ABA-induced promotion of closure.

Effects of H_2O_2 on stomatal movement in antisense plants

As MPK3 is reported to be activated by exogenous H_2O_2 (Kovtun *et al.*, 2000; Lu *et al.*, 2002), we investigated whether stomatal responses to H_2O_2 are affected in *MPK3*-silenced plants. Our results show that these plants are less responsive than controls to this compound, both in promotion of closure (Fig. 4a), and in experiments on inhibition of opening (Fig. 4b). These results indicate that MPK3 is required for signalling of H_2O_2-induced stomatal movements, and that its role cannot be fully compensated for by other signalling components, as might be the case in ABA-induced promotion of closure. Further, these results are consistent with a role for MPK3 in the transduction of ABA-generated H_2O_2 in guard cells. Given that H_2O_2 is involved in stomatal closure by darkness (Desikan *et al.*, 2004b; She *et al.*, 2004), we investigated if stomatal closure by darkness (used in experiments on inhibition of opening by ABA and H_2O_2) is also affected in antisense plants. We found that stomata of antisense plants incubated for 2 h in the dark in 10 : 0 buffer close to an extent similar to those of control genotypes (data not shown). This indicates that the reduction in sensitivity of antisense plants to the above-mentioned compounds is not an artefact due abnormal promotion of closure in the dark.

ABA-induced H_2O_2 synthesis is not significantly affected in MPK3 antisense plants

MPK3 kinase activity increases in response to H_2O_2 in mesophyll protoplasts (Kovtun *et al.*, 2000), arguing that this

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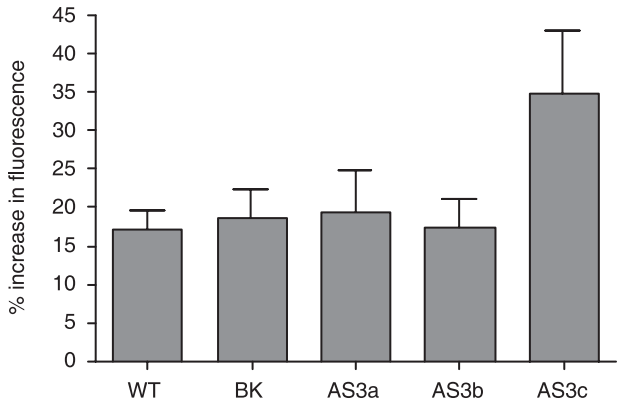

Fig. 5 Abscisic acid (ABA)-induced hydrogen peroxide (H_2O_2) synthesis in response to ABA. *Arabidopsis* epidermal peels were loaded for 20 min with 50 μM H_2DCFDA for 20 min, washed, incubated in buffer with or without 50 μM ABA for a further 20 min, and evaluated by confocal microscopy. Bars, mean percentage increase in fluorescence in ABA-treated guard cells compared with controls. WT, wild type; BK, empty vector control; AS3a, AS3b, AS3c, antisense lines. Error bars, SE from three independent trials; $n = 25$ per trial. No significant differences were found when antisense lines were compared with wild-type control (Student's *t*-test).

kinase is involved in signalling downstream of this compound. But at the same time, there is evidence that an MAPK cascade acts upstream of H_2O_2 synthesis both in ABA-treated guard cells (Jiang *et al.*, 2003) and in pathogen-infected plants (Ren *et al.*, 2002; Yoshioka *et al.*, 2003). Therefore we investigated whether ABA-induced H_2O_2 synthesis is affected in MPK3 antisense plants. Figure 5 shows the percentage increase in fluorescence after ABA treatment. Lines AS3a and AS3b display a level of ABA-induced H_2O_2 synthesis similar to control genotypes, while line AS3c shows a higher but not statistically different ($P > 0.95$) response compared with controls. Thus we conclude that ABA-induced H_2O_2 synthesis is not substantially affected in the MPK3-silenced lines, which is consistent with the postulated role of this MAPK in signalling downstream of H_2O_2.

Discussion

MPK3 is activated by numerous factors, such as ABA, H_2O_2, abiotic stresses and pathogen elicitors, and this protein kinase is thought to have an important signalling function with respect to these stimuli, and in plant stress responses in general. As MPK3 is expressed in guard cells, and ABA and H_2O_2 are known to regulate stomatal aperture, we studied the effect of guard cell-specific antisense inhibition of *MPK3* expression on the control of stomatal movements. Guard cell-specific ablation of gene expression was used to avoid complications of interpretation arising from the general downregulation of *MPK3* expression.

Expression of the *MPK3* gene in guard cells was greatly reduced but probably not completely inhibited in these antisense plants. We found that the *MPK3* antisense plants are partially affected in their response to ABA in the inhibition of stomatal opening, yet they respond in the same way as wild-type plants to the promotion of stomatal closure elicited by this hormone. As only a partial reduction in ABA sensitivity in the inhibition of stomatal opening experiments was observed, this could be caused by residual MPK3 activity in the guard cells of the silenced plants. Alternatively, other (unsilenced) signalling components might be acting in parallel to MPK3 in these processes, and could account for the partial responses observed.

This latter possibility certainly seems to occur in the case of promotion of closure by ABA, as the *MPK3* antisense and control lines differ in their response to the hormone only when in the presence of sodium butyrate, which has the effect of preventing an ABA-induced cytosolic pH increase (Blatt & Armstrong, 1993). The difference in stomatal aperture seen in the presence of butyrate indicates that, when guard cells are treated with ABA, cytosolic alkalinization and MPK3 act in different signalling pathways to effect closure.

The finding that *MPK3* antisense lines are insensitive to ABA only in the inhibition of stomatal opening, but respond normally to the hormone in promotion of closure, lends support to the model in which the ABA signal is transduced through at least partially different signalling networks during opening and closure, as previously demonstrated by *Arabidopsis* G protein alpha subunit gene *GPA1* knockouts (Wang *et al.*, 2001) or by phospholipase C gene silencing in tobacco (Hunt *et al.*, 2003). The *gpa1* mutants also resemble *MPK3* antisense plants in that they, too, become partially unresponsive to ABA in the promotion of closure when ABA-induced cytoplasmic alkalinization is prevented with sodium butyrate. Thus it seems conceivable that MPK3 and GPA1 might participate in the same ABA-signalling pathway in guard cells. A recent report has further explored the bifurcation of ABA signalling in guard cells, as it was shown that phosphatidic acid can bind and inhibit the activity of ABI-1 (a negative regulator of ABA action), thus promoting stomatal closure, whereas phospholipase Dα1 (which liberates phosphatidic acid) itself can bind and inhibit GPA1 activity, resulting in reduced inhibition of opening by ABA (Mishra *et al.*, 2006).

Consistent with previous reports showing that MPK3 enzyme activity is induced by exogenous H_2O_2 (Kovtun *et al.*, 2000), stomatal movements in response to H_2O_2 turned out also to be affected in *MPK3* antisense lines. Inhibition of opening was partially affected, while promotion of closure was virtually abolished in the *MPK3*-silenced lines. The data indicate that here MPK3 is acting downstream of H_2O_2, and in contrast to ABA, the function of MPK3 in H_2O_2-induced promotion of closure signalling is not compensated for by other endogenous signalling components.

As there are reports suggesting that MAPK cascades may act upstream of H_2O_2 synthesis in ABA-treated guard cells

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(Jiang *et al.*, 2003) and in pathogen-infected plants (Ren *et al.*, 2002; Yoshioka *et al.*, 2003), we investigated whether ABA-induced H_2O_2 synthesis is normal in the *MPK3*-silenced plants. Two of the antisense lines showed a response to ABA identical to that of control genotypes, while line AS3c displayed a slightly higher (but not statistically significantly higher) level of ABA-induced H_2O_2 synthesis. These results are consistent with our previous evidence indicating that MPK3 acts downstream of H_2O_2 in ABA signalling, but the potential for MPK3 to be implicated in a feedback regulation of H_2O_2 synthesis should not be ignored; further work is needed to investigate this possibility.

Exogenous H_2O_2 promotes stomatal closure and activates plasma membrane calcium channels in the same way as ABA (Pei *et al.*, 2000). In agreement with this, *AtrbohD/AtrbohF* double mutants, defective in two plasma membrane NADPH oxidases, are impaired in ABA-induced H_2O_2 production and also partially unresponsive to ABA-induced promotion of stomatal closure (Kwak *et al.*, 2003). If MPK3 is involved in sensing this ABA-induced H_2O_2, one might expect that *MPK3*-silenced plants would also be affected in ABA-induced stomatal closure. However, as they respond normally to ABA, this again indicates that ABA functions through several pathways in the promotion of closure, one of which involves H_2O_2 production and requires MPK3.

The *gpa1* mutants, as well as showing a similar response to ABA to that of the *MPK3*-silenced plants, are known to be impaired in guard cell production of H_2O_2 in response to exogenously added calmodulin (Chen *et al.*, 2004) and ozone (Joo *et al.*, 2005), indicating that here GPA1 acts upstream of H_2O_2 generation. If this also holds true for ABA, it suggests a signal transduction pathway for ABA signalling in guard cells during the inhibition of opening in which GPA1 acts to increase endogenous H_2O_2 in response to ABA, and MPK3 responds to these enhanced H_2O_2 levels. An analysis of *gpa1*/*MPK3*-silenced plants would help resolve this possibility.

MPK3 may be a mediator of ABA and H_2O_2 signalling in general; previous reports have shown that ABA treatment can induce MAPK activity, H_2O_2 production and antioxidant gene expression in many different plant cells and tissues, not just in guard cells (Guan *et al.*, 2000; Pei *et al.*, 2000; Jiang *et al.*, 2003; Kwak *et al.*, 2003). In particular, MPK3 activity is induced by H_2O_2 and ABA (Kovtun *et al.*, 2000; Lu *et al.*, 2002), therefore this particular MAPK could be part of a signalling network mediating the response against oxidative stresses throughout the plant.

In summary, the results presented here provide clear evidence for the important role of MPK3 in ABA and H_2O_2 signalling in guard cells. These findings are in agreement with previous evidence showing that MPK3 is activated in response to H_2O_2 and ABA, and that MAP kinase signalling is central to the regulation of stomatal movements, thus shedding further light on a complex and important topic.

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