Brassinosteroid-regulated GSK3 kinases phosphorylate MAP kinase kinases, which control stomata development in Arabidopsis thaliana.

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*Running title: Brassinosteroid-regulated GSK3 kinases phosphorylate MAPKK

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Background: brassinosteroids (BRs) are plant steroids, which signal through the inhibition of GSK3/shaggy-like kinases such as BIN2.

Results: We here show that BIN2 phosphorylates MKK4, which inhibits its activity against MPK6, in a MAPK module that controls stomata patterning.

Conclusion: BRs control cellular patterning via BIN2-mediated suppression of MKK4 activity.

Significance: Novel cross-talk of GSK3 and MAPK signaling is revealed.

Brassinosteroids (BRs) are steroid hormones, which coordinate fundamental developmental programs in plants. In this study we show that in addition to the well-established roles of BRs in regulating cell elongation and cell division events, BRs also govern cell fate decisions during stomata development in Arabidopsis thaliana (arabidopsis). In wild-type arabidopsis stomatal distribution follows the one-cell spacing rule, that is adjacent stomata are spaced by at least one intervening pavement cell. This rule is interrupted in BR-deficient and BR signaling-deficient arabidopsis mutants resulting in clustered stomata. We demonstrate that BIN2 and its homologues, GSK3/shaggy-like kinases involved in BR signaling, can phosphorylate the MAPK kinases MKK4 and MKK5, which are members of the MAPK module YODA-MKK4/5-MPK3/6 that controls stomata development and patterning. BIN2 phosphorylates a GSK3/shaggy-like kinase recognition motif in MKK4, which reduces MKK4 activity against its substrate MPK6 in vitro. In vivo we show that MKK4 and MKK5 act down-stream of BR signaling, since their over-expression rescued stomata patterning defects in BR-deficient plants. A model is proposed in which GSK3-mediated phosphorylation of MKK4 and MKK5 enables for a dynamic integration of endogenous or environmental cues signaled by BRs, into cell fate decisions governed by the YODA-MKK4/5-MPK3/6 module.
One of the major challenges in cell and developmental biology is to understand how cells integrate multiple signaling pathways to regulate specific cell differentiation events. Intercellular communication is essential in the coordination of cell fate specification and patterning, and especially in sessile organisms such as plants, cell fate decisions are made to integrate environmental signals with internal developmental programs (1-3).

Plant developmental plasticity is governed by plant hormones, which also play decisive roles in the regulation of cellular patterning. One class of plant hormones that are considered necessary for the regulation of adaptive growth and development are the brassinosteroids (BRs). BRs are polyhydroxylated steroid hormones similar in their structure to mammalian steroid hormones and ecdysteroid from insects (4). BRs are synthesized from sterols and in Arabidopsis thaliana (arabidopsis) signal through a phosphorylation-dependent signal transduction cascade that is initiated by perception of the biologically active BRs brassinolide (BL) or castasterone (CS) by a cell surface receptor complex containing the receptor-like kinase BRI1 (brassinosteroid insensitive 1). In response to BR-binding BRI1 autophosphorylates, interacts with BAK1 (BRI1-associated receptor kinase 1) and facilitates an interaction of BSK1 (BR signaling kinase 1) a receptor-like cytoplasmic kinase with BSU1 a serine/threonine phosphatase. BSU1 mediates dephosphorylation and thereby inactivation of the GSK3 (glycogen synthase kinase 3)/shaggy-like kinase BIN2 (brassinosteroid-insensitive 2) and its redundantly acting homologues such as ASK0 (arabidopsis shaggy-like kinase 0) that act to regulate transcription factors of the BES1/BZR1 family and likely also of a set of bHLH transcription factors including CESTA, BEE1 and BEE3 (5), which in turn control the expression of BR target genes. Thus, at present it is thought that BRs confer their effect through the inhibition of GSK3/shaggy-like kinases, which phosphorylate transcription factors to alter their activity on BR-responsive promoters (6-7).

BRs play well-defined roles in cell division and cell elongation (8-9). In addition it is becoming increasingly evident that BRs also control the balance between proliferation and cell fate specification; however these roles are not well established and the underlying molecular mechanisms have remained ill defined. The observation that inhibition of BR biosynthesis prevented differentiation of mesophyll cells into tracheary elements (10-11), while application of BL had a promotive effect (12) provided first evidence for a role of BRs in xylem differentiation. Recently BRs were shown to participate in controlling root meristem size (13-14) and to impact on the specification of cell fate and patterning in the root epidermis (15). In the leaf epidermis stomatal distribution follows the one-cell spacing rule, that is, adjacent stomata are spaced by at least one intervening pavement cell (16-17). Interestingly, this rule is disrupted in the BR biosynthesis mutant cpd where stomatal duplications were reported (18), indicating that BRs may also impact on cellular patterning in stomata development.

Correct stomata patterning in arabidopsis requires the YODA-MKK4/5-MPK3/6 MAP kinase cascade (19-21). In this cascade YODA (YDA) a mitogen-activated protein (MAP) kinase kinase kinase (MAPKKK), phosphorylates the MAPKKs MKK4/MKK5, which in turn control the activity of the MAP kinases MPK3/MPK6 (19-22). Three bHLH transcription factors, SPCH (SPEECHLESS), MUTE and FAMA, act directly downstream of MAP kinases, to regulate specific events in the entry into and progression through the stomata development pathway (20,22). MAPK signaling networks are found in all eukaryotic organisms and regulate fundamental aspects of biology including but not limited to cell division, initiation of developmental pathways and responses to abiotic and biotic stresses (23-25). Via a phosphorelay mechanism these cascades, minimally composed of a MAPKKK, a MAPK and a MAPK link upstream receptors to downstream targets (23). MAPK pathways usually signal multiple stimuli and also the YODA-MKK4/5-MPK3/6 module not only participates in cellular patterning but also transmits abiotic and biotic stress signals perceived by the receptor kinase flagellin sensing 2 (FLS2) (24).

In the present study we demonstrate that BR signaling governs cell fate specification in arabidopsis stomata patterning. Our results show that BIN2 phosphorylates MKK4 in its
activation loop and that BIN2-mediated phosphorylation severely reduces MKK4 activity against MPK6 in vitro. An over-expression of MKK4 and its homologue MKK5 rescued stomata patterning defects of BR-deficient plants providing in planta evidence for a function of BIN2 upstream of MKK4/5 in stomata development. A model in which BR signaling impacts on stomata patterning by suppressing BIN2-mediated phosphorylation of MKK4/5 at T234 and thus activating the YDA-MKK4/5-MPK3/6 module is presented and discussed.

**EXPERIMENTAL PROCEDURES**

Plant growth conditions and treatments - Arabidopsis thaliana (arabidopsis) ecotype Columbia-0 (Col-0) was the wild-type used for generation of all plant material described in this study. The seeds were sterilized using the chloroform vapor method (26) and plated on arabidopsis thaliana salts (ATS) media (27). After stratification at 4°C for 2 days, seeds were placed in a growth chamber at 21±2°C and incubated in long-day growth conditions (16 hrs 80 μmol·m-2·s-1 cool white light/ 8 hrs dark). For analyzing stomata patterning, seeds of Col-0 and other lines were grown vertically on ATS medium without or with 1 mM Brassinazole (Brz) (TCI Europe, Eschborn, Germany) for 10 days.

Molecular cloning and generation of transgenic plant lines - For generation of plants over-expressing MKK4-YFP or MKK5-YFP, the coding sequence of the genes were amplified with primers and inserted as NcoI+NotI fragments into the corresponding cloning sites of the pGreen derivative 0029 (28) down-stream of the constitutive Cauliflower Mosaic Virus 35S promoter. A YFP (yellow fluorescent protein) tag was subsequently inserted in frame as a NotI+NotI fragment. For the 35S:MKK2-MYC construct the coding region of MKK2 was PCR amplified and introduced ApaI+NotI also into pGreen0029. The MYC tag was cloned into the NotI restriction site. For the 35S:MKK4-234D-YFP construct a T234A point mutation was introduced into MKK4 by site directed mutagenesis and was cloned NcoI+NotI into the binary plant expression vector pGWR8 (29) down-stream of the constitutive Cauliflower Mosaic Virus 35S promoter. The YFP tag was inserted as a NotI+NotI fragment. The integrity of the constructs was verified by sequencing. The resulting plasmids were transformed into Agrobacterium tumefaciens GV3101 carrying the Ti helper plasmid pSOUP. Transgenic plants were generated using the floral dip method (26). Homozygous lines expressing the 35S:MKK4-YFP, the 35S:MKK5-YFP or the 35S:MKK2-MYC construct to high levels where used in bikinin response experiments described.

To analyze MKK4-YFP localization under its own promoter, the promoter and coding sequence of MKK4 was PCR amplified and cloned XhoI+BamHI into the vector described above, to replace the 35S promoter, yielding pMKK4:MKK4-YFP. For cloning of 35S:BIN2-YFP the coding sequence of BIN2 was amplified by PCR, cloned as a NcoI+NotI fragment into pGWR8 and tagged C-terminally with YFP. For ASK0 and ASKβ published 35S promoter-driven constructs were used (30-31) and a YFP or cYFP-tag was inserted using the NotI sites.

For protein expression the coding region of MKK2 (32), MKK4, MKK5, MKK7 or MKK4234D were cloned in frame with the GST coding sequence into pGEX-4T2 (GE Healthcare, Buckinghamshire, UK). To obtain a HIS-MYC-tagged version of MKK4 the coding region was cloned as a NcoI+NotI fragment into the pMAL vector (New England BioLabs, Frankfurt am Main, Germany), in which a MYC tag was introduced with specific primers in frame in front of the HIS tag. The S6A, S230A, S231A, T234D and T234E point mutations were introduced into the pGEX-MKK4 construct by site directed mutagenesis. Similarly, the K69R loss of function mutation of BIN2 (BIN2lof) was introduced into pGEX5X3-BIN2 (33) by in vitro mutagenesis. The coding sequence of MPK6 was amplified from cDNA by using gene specific primers and cloned as a NcoI+NotI fragment into the Gateway-intermediate vector pENTR™ (Invitrogen, Darmstadt, Germany). The entry clone was then introduced into the pDEST™17 (Invitrogen, Darmstadt, Germany) vector by site-specific recombination.

Recombinant protein purification from E. coli - GST and HIS tagged proteins were expressed in E. coli BL21 and purified using glutathione-sepharose beads (GE, Healthcare, Buckinghamshire, UK) or nickel-cellulose beads (Roth, Karlsruhe, Germany) as recommended by the suppliers.
Western blot - Western blot analysis was performed as described previously (5). Membranes were probed with either a mouse anti-GFP antibody (Roche Diagnostics, Indianapolis, IN, USA) or a mouse anti-cMYC antibody (Santa Cruz Biotechnology, CA, USA). Alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, Steinheim, Germany) was used as secondary antibody and detected by enhanced chemiluminescence using CDP-Star reagent (Amersham Biosciences, USA).

Microscopy - For investigation of stomata clustering leaves of 10-day-old seedlings were fixed on a metal support rack with Tissue-Tek (Sakura Finetek, Torrance, USA), shock frozen in liquid nitrogen and leaf epidermal cells were subsequently visualized with a Hitachi T-1000 scanning electron microscope (Hitachi High-Tech, Tokyo, Japan). Alternatively leaves were incubated in clearing solution (20 g chloral hydrate, 4.6 ml water, 2 ml glycerol 87%) at room temperature prior investigation by optical bright field microscopy. Leaves of stably transformed Arabidopsis seedlings expressing the 35S:MKK4-YFP construct were investigated with a Zeiss LSM Meta confocal microscope for YFP reporter expression. The images were assembled using the Zeiss LSM image browser software version 4.2.0.121.

For the analysis of YFP reporter constructs, plasmid DNA of pMKK4:MKK4-YFP, 35S:MKK4-YFP, 35S:BIN2-YFP, 35S:ASKθ-YFP or 35S:ASKα-YFP were transiently transformed into Arabidopsis protoplasts using a PEG mediated transformation protocol (34). Fluorescence was investigated using an Olympus Bx61 confocal microscope.

For split-YFP analysis cDNAs of M KK 4 and ASKθ were cloned in pGWR8 downstream of the 35S promoter and subsequently tagged with the N-terminal or C-terminal part of YFP (35). The sequenced constructs were used for transient transfection of Arabidopsis protoplasts (34) and bimolecular fluorescence investigated using an Olympus Bx61 confocal microscope.

GST pull-down and in vitro kinase assays - Two μg of GST-BIN2 and MKK4-HIS-MYC proteins were incubated in 500 μl of 1x PBS buffer for 1 h on ice. Then 20 μl of glutathione-sepharose beads (GE, Healthcare, Buckinghamshire, UK) were added and incubated further for 2 h at 4°C on a rotating wheel. After centrifugation at 1000 rpm for 1 minute, the beads were washed with PBS containing 0.05% Tween 20. 4x SDS loading buffer (200 mM TRIS/HCI pH 6.8, 400 mM DTT, 8% SDS, 40% glycerol and 0.01% bromophenol blue) was added to a final concentration of 1x and western blotting was performed using anti-Myc tag antibody (Santa Cruz Biotechnology, CA, USA). The in vitro kinase assays were performed as described previously (31). Briefly, 0.5 μg (unless otherwise stated) of purified recombinant proteins were mixed together, the reaction was started by addition of reaction mixture containing the kinase buffer (20 mM HEPES pH=7.4; 15 mM MgCl2; 5 mM EGTA; 1 mM DTT), 1 μM ATP and 10 μCi γ-32-ATP (unless otherwise stated). The reaction was performed in a total volume of 20 μl at RT for 30 min. The reaction was stopped by adding 6 μl of 4x SDS loading buffer (67 mM TRIS pH 6.8, 133 mM DTT, 2.7% SDS, 13% glycerol, 0.01% bromophenol blue). Kinase activities were analyzed by SDS-PAGE followed by autoradiography.

Mass spectrometry - After coomassie staining of the SDS-PAGE gel, protein bands corresponding to GST-BIN2 and GST-MKK4 were cut in 1-2 mm3 pieces and subsequently washed in consecutive baths of acetone, ultra-pure water and again in acetone to remove SDS-PAGE buffer and Coomassie dye and proceeded further according to the protocol given in detail in the Supplementary data.

RESULTS

BR signaling deficiency induces stomata patterning defects in Arabidopsis - To investigate if BRs impact on cell patterning during stomata differentiation we analyzed the abaxial epidermis of the first leaf pair of BR deficient and BR signaling deficient mutants by scanning electron microscopy. First we examined cpd (constitutive photomorphogenesis and dwarfism), a BR biosynthetic mutant, which has previously been reported to display duplicated guard cells (18). Fig. 1 shows that, whereas wild-type Arabidopsis seedlings exhibited the characteristic one-cell spacing patterning in stomatal distribution (1,36), stomata in cpd clustered at a strongly increased rate (of 9.1%). To verify this results wild-type seedlings were grown for 10 days on media containing
Brassinazole (Brz), an inhibitor of BR biosynthesis (37). As shown in Fig. 1 Brz treatment had the same effects on stomata distribution: it induced stomatal patterning defects; stomata clustering occurred at a rate of 14.0%. To investigate if this was a phenotype induced by BR deficiency or if it was due to defective BR signaling, we analyzed the BR signaling deficient mutants bri1-1 (38) and bin2-1 (39), as well as plants over-expressing ASK0, an arabidopsis GSK3/shaggy-like kinase that acts redundantly with BIN2 in BR signaling (31). Interestingly, in plants, in which BR downstream responses are constitutively impaired, a high frequency of clustered stomata (bri1-1: 14.9%; bin2-1: 11.1%; ASK0oe: 33%) was observed. These increases were statistically highly significant as compared to wild-type, as indicated by the low P-values derived of a two-tailed $\chi^2$-test (Fig. 1B).

Thus in summary BR deficiency and more specifically defects in BR signaling disrupt the coordinated cell fate determination of stomata versus pavement cells.

**BIN2 and its homologues can phosphorylate MKK4 in vitro** - The stomata patterning defects of bin2-1 and ASK0oe plants suggested that a cross-talk of BR signaling with factors controlling stomata cell fate specification occurred either at the level or downstream of BIN2 and redundantly acting GSK3/shaggy-like kinases. In a candidate gene approach we thus tested, if GSK3/shaggy-like kinases may directly phosphorylate players of the YDA-MKK4/5-MPK3/6 module. In vitro kinase assays were performed using recombinant MPK3, MPK6 and MKK4 and the GSK3/shaggy-like kinases BIN2, ASK0 and ASKα. While MPK3 and MPK6 could not be phosphorylated (data not shown & Fig. 4, lane 1), GST-BIN2 and its two homologues were able to efficiently phosphorylate GST-MKK4 (Fig. 2A, lanes 5, 6 and 7). To further verify this result a BIN2 loss-of-function (GST-BIN2lof) protein, generated by a point mutation of lysine 69 to arginine, was tested for its ability to phosphorylate MKK4. Fig. 2B illustrates the results of these kinase assays and shows that whereas wild-type BIN2 exhibited strong phosphorylation activity against MKK4, the BIN2-lof protein was unable to catalyze MKK4 phosphorylation. Moreover in vitro pull-down assays were performed which showed that recombinant BIN2, expressed and purified as a GST fusion protein from *E. coli*, was able to interact with recombinant cMYC-tagged MKK4 as detected by pull-downs using GST beads and western blot analysis (Fig. 2C).

To investigate if GSK3/shaggy-like kinases can interact with MKK4 in planta bimolecular fluorescence complementation assays (35) were carried out in arabidopsis protoplasts. For this purpose, ASK0-YFP was chosen, since it is expressed to comparably high levels as MKK4-YFP in protoplasts (whereas BIN2 is expressed at very low levels in this system; Suppl. Fig 1). ASK0 fused to the C-terminal portion of YFP and MKK4 fused to the N-terminal portion of YFP were co-expressed in protoplasts. As shown in Fig. 2D yellow fluorescence was observed diffusely in the nucleus when protoplasts were co-transformed with ASK0 and MKK4. In contrast the controls (protoplast transformed with one of the constructs only) did not emit detectable fluorescence (data not shown).

**BIN2 specifically phosphorylates MKK4 and MKK5 in vitro** - MKK4 acts redundantly with MKK5 in controlling MPK3 and MPK6 activity to negatively regulate stomata development (20). Thus the question arose whether MKK5 may also be a substrate of BIN2 and whether MKK4- or MKK5-unrelated MKKs would also be phosphorylated. For this analysis two additional MKKs were chosen: MKK7, which has recently been shown to play a role in stomata development albeit by presently unknown means (21), and MKK2, which is one of the most distant relatives of MKK4 in sequence similarity (40). In vitro kinase assays were performed in which the activity of BIN2 was investigated against recombinant GST-tagged versions of MKK2, MKK4, MKK5 and MKK7. Interestingly BIN2 exhibited strong activity against MKK4 and MKK5, whereas its activity against MKK2 or MKK7 was neglectable (Fig. 3).

Therefore BIN2 interacts with and phosphorylates MKK4 and its closest homologue MKK5, but does not accept MKK2 and MKK7 as substrates in vitro.

**BIN2 phosphorylation of MKK4 negatively regulates its activity against MPK6 in vitro** - The fact that in the dominant bin2-1 mutant and in ASK0oe plants stomata clustering phenotypes occurred, which are reminiscent of plants in...
which \textit{MKK4} and/or \textit{MKK5} expression is silenced (20), suggested a model in which GSK3-mediated phosphorylation inhibits MKK4. To test this hypothesis, \textit{in vitro} kinase assays were performed in which the activity of GST-MKK4 was investigated using recombinant HIS-MPK6 as a substrate in the presence or absence of GST-BIN2. In the absence of BIN2, MKK4 phosphorylated MPK6 as expected whereas this phosphorylation was abolished when BIN2 was added to the reaction (Fig. 4, lanes 2 & 5). When an inactive BIN2 variant (BINlof) was used (lane 6) or when BIN2 was inhibited by adding the GSK3/shaggy-like kinase inhibitor bikinin (33) to the reaction (lane 8), phosphorylation of MPK6 by MKK4 was restored, proofing that BIN2 phosphorylation negatively regulates MKK4 activity \textit{in vitro}.

Identification of BIN2 phosphorylation sites in MKK4 by mass spectrometry analysis - In order to identify putative BIN2 phosphorylation sites in MKK4 a mass spectrometric analysis was performed. Purified GST-MKK4 fusion protein was incubated with GST-BIN2 in the presence of ATP. The reaction products were separated by SDS-PAGE and a band corresponding to GST-MKK4 was isolated from the gel and digested with trypsin. The resulting peptides were subsequently subjected to mass spectrometry (MS) analysis. Phosphopeptides that increased in abundance following phosphorylation by BIN2 contained phosphoserine at position 6, phosphothreonine at position 234 and phosphoserine at position 230 or 231, which are located in the activation loop (MS analysis did not allow to distinguish which of both serines was phosphorylated) (for mass spectra see Suppl. Fig 2). These sites were mutated to alanine (A) to create single mutants and kinase assays were performed with the mutants. While BIN2-mediated phosphorylation of the S6A mutant (Suppl. Fig. 3) and a S231A variant was similar to wild-type (Fig. 5A, Lane 8), phosphorylation of the S230A and T234A mutants was strongly reduced (Fig. 5A, Lane 7 and 9). Thus, S230 and T234 are essential for phosphorylation of MKK4 by BIN2 \textit{in vitro}. Interestingly mutation of T234 also to other amino acids such as E or D abolished activity of MKK4 against MPK6 \textit{in vitro} (Suppl. Fig. 4) showing that a T at this site is of high importance for MKK4 activity \textit{in vitro}.

Over-expression of T234A induces phenotypes reminiscent of plants in which activities of MKK4 and MKK5 are lost - In an aim to investigate the effect of modification of T234 on MKK4 activity \textit{in planta} we generated a \textit{MKK4}^{T234A}\textit{-YFP} fusion construct and over-expressed it under control of the constitutive 35S promoter in 	extit{arabidopsis} plants. T1 plants were selected and were analyzed for MKK4^{T234A}\textit{-YFP} expression by western blotting. Very interestingly, representative plants, which expressed MKK4^{T234A}\textit{-YFP} to high levels, were characterized by striking developmental defects, such as severe dwarfism, round leaves, sterility and premature death (Fig. 5B). Since these phenotypes were in clear resemblance to phenotypes displayed by plants in which MKK4 and MKK5 is silenced (20) we assessed if 35S:\textit{MKK4}^{T234A}\textit{-YFP} lines may also be affected in stomata distribution by scanning electron microscopy. The result is shown in Fig. 5C and illustrates that MKK4^{T234A}\textit{-YFP} over-expression resulted in drastic stomata patterning defects, with the epidermis of cotyledons almost solely composed of stomata.

\textit{MKK4} expression at the subcellular level - To gain further insight into the role of MKK4, its subcellular localization was investigated. A YFP fusion to the C-terminus of full-length MKK4 was expressed under control of the 35S promoter (35S:\textit{MKK4}\textit{-YFP}) and plant lines stably expressing this construct were generated (Fig. 6A). A subsequent analysis of MKK4-YFP subcellular expression revealed that MKK4 is localized in the cytoplasm as well as in the nucleus, both when driven by the 35S promoter (analyzed \textit{in planta}: Fig. 6B) as well as when driven by the endogenous \textit{MKK4} promoter (analyzed in protoplasts: Suppl. Fig. 5). BR treatment or treatment with Brz did not alter MKK4 subcellular localization, when analyzed in protoplasts (Suppl. Fig. 5).

MKK4 or MKK5 over-expression suppresses stomata clustering in BR-deficient \textit{plants} - The data presented above suggested a model in which, in a BR-deficient situation, BIN2 and redundantly acting GSK3/shaggy-like kinases would phosphorylate MKK4/MKK5 to reduce their activity and thereby promote stomata clustering. Therefore an increase in the level of MKK4 should (partially) rescue the stomata clustering phenotype of BR-deficient
plants. To investigate this hypothesis we treated wild-type Col-0 and a representative MKK4-YFP over-expressing plant line with Brz. As shown in Fig. 6C and D wild-type and 35S:MKK4-YFP plants reacted to Brz treatment as expected: a BR-deficient phenotype was induced, characterized among others by shortened hypocotyls and dark green, downwardly curled leaves. However, importantly, when the frequency of stomata clustering was determined, a statistically highly significant difference was found between the two lines. While 14.0% of the stomata of Brz-treated wild-type seedlings were present in clusters, only 7.6% of the stomata of 35S:MKK4-YFP plants grown under the same conditions were clustered (P-value of a χ²-test: 0.0035). In the absence of Brz both lines showed a very low clustering frequency of less than 1.0% (Fig. 6E). 35S:MKK5-YFP expressing plants similarly showed an increased resistance to Brz application in regard to stomata clustering (6.4 %; P-value of a χ²-test: 0.0061), whereas 35S:MKK2-MYC expressing plants behaved like wild-type in this assays (Suppl. Fig. 6).

This result shows that MKK4-YFP and MKK5-YFP over-expression suppresses stomata clustering in BR-deficient plants.

DISCUSSION

Stomata are cellular epidermal valves, which plants have evolved to control water and gas exchange (41-42). In Arabidopsis stomata development is preceded by asymmetric cell divisions and stomata distribution follows the one-cell spacing rule, reflecting the coordination of cell fate specification. Stomatal patterning is considered to involve the transmission of spatial cues from the stomata to the adjacent cell, which are required to correctly orient the plane of the spacing division (41). Thus spacing is thought to result from cell-cell signaling and it is known that the MAPK signaling module YDA-MKK4/5-MPK3/6 is necessary for transducing signals to control cell division and cell fate decisions during stomata development and in embryogenesis (20,21,43-44).

BRs are essential plant hormones that regulate diverse aspects of growth and development, including cell elongation, cell division and cell differentiation (12,45-46). Previous findings suggested that BRs also participate in cellular patterning (15) and here further evidence is provided, which supports this role. In addition our results also provide a possible mechanistic explanation how BRs may influence stomata patterning. We show that BIN2 directly interacts with and phosphorylates MKK4 to negatively regulate its activity in phosphorylating MPK6 in vitro. Interestingly a regulatory effect of BIN2 on the YDA-MKK4/5-MPK3/6 module was described very recently in a different study. The authors showed that BIN2 phosphorylates YDA to reduce its activity (47). Together these results indicate that BIN2 targets both MAPKKK and MAPKK in the MPK3/6 module for suppression, which would allow for a dynamic cellular re-patterning in response to developmental or environmental cues perceived and transduced by BRs.

The BR response pathway signals through the inhibition of GSK3/shaggy-like kinase activities including BIN2, and here we provide evidence that BIN2 and homologues control the activity of MAPKKs. GSK3/shaggy-like kinases are a group of highly conserved, constitutively active S/T kinases implicated in numerous signaling pathways and controlling metabolism, cell fate determination and tissue patterning in various organisms (48-50). In mammalian systems substrates of GSK3s include glycogen synthase, β-catenin, cyclin D1, cJun and Smad1 (48-50). This work is the first across all biological kingdoms to report that a GSK3/shaggy-like kinase can phosphorylate a MAPKK. BIN2 phosphorylates MKK4 at S230 and T234 in vitro, which form a classical GSK3 phosphorylation motif (S/TxxxS/T; (51)). Since mutation of T234 completely abrogates BIN2 phosphorylation of MKK4 it is possible that T234 is the main phosphorylation site and S230 may be less important. Our results show that phosphorylation of T234 by BIN2 rendered MKK4 inactive. Furthermore, a point mutation of this site to other amino acids resulted in a kinase-dead protein (Suppl. Fig. 4), highlighting the importance of a T at position 234. Indeed T234 is highly conserved in eukaryotic MAPKKs, suggesting a functional relevance in controlling MAPKK activities (Fig. 7). S230 is part of the activation loop of MKK4, which is suggested to be phosphorylated by the upstream kinases MEK1 and YDA to activate it in stress signaling and stomatal patterning pathways, respectively (20,52). It seems possible that
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GSK3-mediated phosphorylation of T234 may sterically hinder phosphorylation of the active loop by upstream MAPKKK. In a different scenario a competition for phosphorylation at S230 between upstream MAPKKK and BR-regulated GSK3/shaggy-like kinases could take place. It will be interesting to investigate if MKK4 and/or MKK5 phosphorylation by BIN2 at T234 over-rides upstream regulatory events in this MAPK module.

An over-expression of MKK4 T234A, which is inactive against MPK6 in vitro, resulted in phenotypes reminiscent of plants in which the activities of either MKK4+MKK5 or MPK3+MPK6 are lost (20). This suggests that also in plants MKK4 T234A is inactive, since in a situation of constitutive over-expression it may out competed endogenous, active MKK4+MKK5 in binding to MPK3+MPK6, which would interfere with their activation and result in the observed developmental defects. It will be interesting to determine the physiological significance of BIN2-mediated S230 and/or T234 phosphorylation events and investigate, if modifications at these sites may allow for a dynamic regulation of MKK4 and MKK5 activity by the BR and YDA-MKK4/5-MPK3/6 signaling pathways in planta.

Cross-talk of BR signaling with the YDA-MKK4/5-MPK3/6 cascade at the receptor level has previously been reported with BAK1 playing a dual role as a co-receptor of both BRI1 and FLS2 (53-55). Moreover very recently BIN2 was shown to also directly control SPCH activity (56). Our study now provides further indications for a cross-communication of the two pathways, opening up the possibility of a web of interactions, which enables BRs to dictate cellular patterning and other responses signaled by the YDA-MKK4/5-MPK3/6 MAPK module.

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FOOTNOTES

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2The abbreviations used are: GSK3, glycogen synthase kinase 3; MAPK, mitogen activated protein kinase; BRs, brassinosteroids; BIN2, brassinosteroid insensitive 2; MKK4/5, map kinase kinase 4/5; MPK3/6, mitogen-activated protein kinase 3/6; BL, brassinolide; CS, castasterone; BRI1, brassinosteroid insensitive 1; BAK1, BRI1-associated receptor kinase; BSK1, brassinosteroid signaling kinase 1; BSU1, BRI1 suppressor 1; ASK0/α, Arabidopsis shaggy-like kinase 0/α; BEE1/3, brassinosteroid enhanced expression 1/3; cpd, constitutive photomorphogenesis and dwarfism mutant; bHLH, basic helix loop helix; SPCH, speechless; YDA, YODA; FLS2, flagellin sensing 2; Col-0, Columbia-0; ATS, Arabidopsis thaliana salts; Brz, brassinozole; YFP, yellow florescent protein; GST, glutathion S-transferase; HIS, histidine 6x tag; GFP, green fluorescent protein; IgG, immunoglobulin G; DTT, Dithiothreitol; ASK9αe, Arabidopsis shaggy-like kinase 9 o-expressor mutant; lof, loss-of-function; ATP, adenosine triphosphate; MS, mass spectrometry; T, threonine; S, serine; A, alanine; SEM, scanning electron microscope; AR, autoradiography; CBB, coomassie brilliant blue.
REFERENCES


Brassinosteroid-regulated GSK3 kinases phosphorylate MAPKK

FIGURE LEGENDS

FIGURE 1. BR deficiency and BR signaling deficiency induces stomata patterning defects in Arabidopsis. A, Seedlings of wild-type Col-0, cpd, bril-1, bin2-1 and ASKllof were grown vertically on ATS plates for 10 days and stomatal distribution was analyzed by scanning electron microscopy (SEM). For Brz treatment 2 μM Brz was directly added to ATS media. B, The frequency of clustering events was calculated by counting at least 100 stomata per line or treatment. All lines showed statistically highly significantly increased stomata clustering compared to untreated wild-type as indicated by the low P-values of a χ²-test.

FIGURE 2. BIN2 interacts with and phosphorylates MKK4. A, In vitro kinase assays using GST-BIN2, GST-ASKl or GST-ASKa as well as MKK4 were performed. Proteins were incubated in kinase buffer in the presence of [γ-33P]-ATP as a co-substrate. Subsequently samples were separated by SDS-PAGE and subjected to autoradiography (AR). Lanes 1 to 4 show the autophosphorylation of the kinases. In lanes 5 to 7 phosphorylation of MKK4 by the ASKs is visible. B, In vitro kinase assays using MKK4 as substrate with wild-type and a loss-of-function version of GST-BIN2 (GST-BIN2 and GST-BIN2-lof, respectively).
Stainings with coomassie brilliant blue (CBB) are shown as loading controls. For this assay $[^{32}\text{P}]-\text{ATP}$ was used as a co-substrate. C, In vitro pull-down assays using GST-BIN2 and MKK4-HIS-MYC proteins were performed. Recombinant MKK4-HIS-MYC protein was incubated either alone, with GST or with GST-BIN2 in reaction buffer on ice and the proteins were then pulled-down with GST beads. MKK4-HIS-MYC (input) and pulled-down proteins were detected with anti-MYC antibody. D, Bimolecular fluorescence complementation assay showing a representative protoplast transformed with ASK0-\text{cYFP} and MKK4-\text{nYFP} constructs.

FIGURE 3. BIN2 specifically phosphorylates MKK4 and MKK5. In vitro kinase assays using GST-BIN2 were performed to analyze its activity against GST-MKK2, GST-MKK4, GST-MKK5 and GST-MKK7. Stainings with coomassie brilliant blue (CBB) are shown as loading controls in the lower panel.

FIGURE 4. BIN2 phosphorylation of MKK4 inhibits its activity against MPK6 in vitro. In vitro kinase assays were performed using recombinant GST-BIN2, GST-BIN2-lof, GST-MKK4 and HIS-MPK6 proteins. The proteins were incubated together in kinase reaction mixture containing 500 $\mu\text{M}$ ATP and 20 $\mu$Ci radioactively labeled $[^{32}\text{P}]-\text{ATP}$, samples were then separated by SDS-PAGE and subjected to autoradiography. Lanes 1 and 2 show phosphorylation of MPK6 by BIN2 and MKK4, respectively, while lane 3 and 4 show phosphorylation of MKK4 by BIN2 or BIN2-lof, respectively. In lane 5 and 6 BIN2 or BIN2-lof was pre-incubated with MKK4 in the kinase buffer containing 300 $\mu\text{M}$ ATP and 20 $\mu$Ci $[^{32}\text{P}]-\text{ATP}$ at RT for 30 min prior addition of MPK6. In lane 8 BIN2 and 30 $\mu\text{M}$ bikinin were incubated together for 10 min prior to addition of MKK4, MPK6 and the reaction mixture. Lane 7 is a control for bikinin specificity in which MKK4 was incubated with 30 $\mu\text{M}$ bikinin for 10 min prior to adding MPK6 and reaction mixture.

FIGURE 5. Effects of BIN2-target site mutations on MKK4 activity in vitro and in planta. A, In vitro kinase assays using recombinant GST-BIN2 and GST-tagged wild-type or mutant variants of MKK4. B, Top: Wild type plants and T1 plants expressing 35S:MKK4$^{T234A}$-\text{YFP}. Bottom: Western blot analysis of shown lines with anti-YFP antibody. C, SEM image of the abaxial cotyledon epidermis of a representative 35S:MKK4$^{T234A}$-\text{YFP} plant 12 days after germination. The scale bar represents 30 $\mu\text{m}$.

FIGURE 6. BIN2 acts upstream of MKK4 in stomata patterning. A, Top: Wild-type and homozygous lines expressing 35S:MKK4-\text{YFP}. Bottom: Western blot analysis of shown lines with anti-YFP antibody. B, Analysis of MKK4-\text{YFP} subcellular localization in arabidopsis plants stably expressing 35S:MKK4-\text{YFP}. C, Phenotypes of seedlings with or without 2 $\mu\text{M}$ Brz. D, Hypocotyl length of seedlings used for quantification of stomata clustering. E, The frequency of stomata clustering in wild-type and 35S:MKK4-\text{YFP} plants in the presence or absence of 2 $\mu\text{M}$ Brz as determined by light microscopy. The results are shown in %. P-values derived of a $\chi^2$-test are given.

FIGURE 7. Amino acid alignment of MAKKs from different eukaryotes. Dashes indicate gaps introduced to maximize alignment of conserved residues. Residues corresponding to T224, S230 and T234 are marked in red.
Khan et al. Fig 2

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AR
CBB
Khan et al. Fig 5
SUPPLEMENTARY MATERIAL AND METHODS

Mass spectrometry
Proteins were subjected to disulfide bond reduction by incubating at 60°C for 45 min in 40 µl of a solution containing 50 mM TEAB (triethylammonium bicarbonate) pH 8.5 and 10 mM TCEP (tris(2-carboxyethyl)phosphine hydrochloride). After acetonitrile washing, proteins were subjected to thiol group alkylation by incubating at RT for 30 min in 45 µl of a solution composed of 50 mM TEAB pH 8.5 and 20 mM MMTS (S-Methyl methanethiosulfonate). After acetonitrile washing, proteins were digested by incubating in 50 µl of a solution composed of 50 mM TEAB pH 8.5 and 0.4 µg trypsin at 37°C overnight. After digestion, the supernatant, containing peptides, was recovered. The peptides remaining in the pieces of gel were additionally extracted, consecutively, with 40 µl of 1% formic acid and 50 µl of acetonitrile and pooled with the previous supernatant.

The obtained peptide solutions (150 µl) each was split into two parts: A) 50 µl intended for direct mass spectrometry (MS) analysis and B) 100 µl intended for phosphopeptide enrichment using IMAC resin and MS analysis. The split samples were dried in a speed-vacuum centrifuge and peptides were resuspended in: A) 12 µl of 0.1% formic acid B) 50 µl of 1.5% acetic acid and 30% acetonitrile.

Ten µl IMAC resin (PHOS-Select™ Iron Affinity Gel, Sigma-Aldrich) resin were washed three times with 150 µl wash solution (1.5% acetic acid, 30% acetonitrile in water, v:v), prior addition of 50 µl of peptide solutions binding of phosphopeptide beads were performed on a rotating wheel at RT for 135 min. Subsequently, the IMAC resin was centrifuged for 30 s at 8,200 g and the supernatant was discarded. The IMAC resin was then washed twice with 100 µl washing solution and once with 100 µl ultra-pure water. The IMAC resin was finally incubated for 5 min at RT with gentle shaking in 50 µl of 400 mM ammonium hydroxide. After centrifugation (2 min at 8,200 g) the supernatant, containing the eluted phosphopeptides, was dried in a speed-vacuum centrifuge and phosphopeptides were finally resuspended in 18 µl of 0.1% formic acid.

NanoLC-MS/MS analyses were performed on a Dual Gradient Ultimate 3000 chromatographic system (Dionex) coupled to a LTQ-Orbitrap XL mass spectrometer (Thermo-Fisher Scientific). Approximately 2/3 of the A and B (IMAC eluates) split fractions were injected per LC-MS/MS analysis. Phosphopeptide samples were loaded onto a C18 pre-column (Acclaim PepMap C18, 5 mm length x 300 µm I.D., 5 µm particle size, 100 Å porosity, Dionex). After desalting for 5 min with buffer A (water/acetonitrile/formic acid, 98/2/0.1, v/v/v), peptide separation was carried out on a C18 capillary column (Acclaim PepMap C18, 15 cm length x 75 µm I.D. x 3 µm particle size, 100 Å porosity, Dionex) with a gradient starting at 100% solvent A, ramping to 70% solvent B (water/acetonitrile/formic acid, 20/80/0.1, v/v/v) over 70 min, then to 100% solvent B over 2 min (held 10 min), and finally decreased to 100% solvent A in 3 min. The
LC eluent was sprayed into the MS instrument with a glass emitter tip (Pico-tip, FS360-50-15-CE-20-C10.5, New Objective, Woburn, MA, USA). The mass spectrometer was operated in positive ionization mode. One FTMS scan was acquired over the mass range m/z [400, 1600] to reach a resolution of 30,000, and was followed by three pairs of MS2/MSA (MultiStage Activation) scans in the linear ion trap. Neutral loss masses specified for MSA fragmentation were 32.67 and 49 Da. Ionic species bearing one or ≥4 charges were excluded from fragmentation; dynamic exclusion of already fragmented precursor ions was applied for 90 s, with a repeat count of 1, a repeat duration of 30 s and an exclusion mass width of +/- 5 ppm. The lock mass option using as reference the ion at m/z 445.120 was activated to ensure more accurate mass measurements in FTMS mode. The automatic gain control (AGC) allowed accumulating up to $10^6$ ions for FTMS scans and $10^4$ ions for ITMS⁰ scans. Maximum injection time was set to 500 ms for FTMS scans and 100 ms for ITMS⁰ scans.

Data analysis and interpretation of MS data

RAW data files acquired on the LTQ-Orbitrap instrument were converted to Mascot-compatible ASKs le.MGF files using the software Proteome Discoverer 1.2. Database searches were performed using the Mascot server v2.2.07 with the following parameters: database TAIR8; enzymatic specificity: tryptic or semi-tryptic with two allowed missed cleavages; fixed modification of cysteine residues (methylthio(C)); possible phosphorylation of S, T and Y residues; 5 ppm tolerance on precursor masses and 0.6 Da tolerance on fragment ions; fragment types taken into account were those specified in the configuration ‘ESI-trap’. The positioning of phosphate groups in the identified phosphorylated sequences was systematically assessed by checking the Mascot score difference between the first and second peptide matches which was required to be >5 to validate the phosphosite(s). Phosphopeptides of GST-MKK4 identified both in samples of interest (gel bands of GST-MKK4+GST-BIN2, whose tryptic digests were either analysed directly or further enriched for phosphopeptides by IMAC) and in control samples (GST-MKK4 alone) were considered not to be specific of GST-BIN2 kinase activity. The presence of MKK4 phosphopeptides exclusively identified in the samples of interest was further searched for in the control samples. The chromatographic peaks of these species were manually extracted from the total MS signals detected during LC-MS/MS analysis of the different samples. If no clear chromatographic peak was obtained in the control samples, the peptide species was considered to be specifically present in the samples of interest, and thus to be due to the presence of GST-BIN2.
SUPPLEMENTARY FIGURE LEGENDS

SUPPL. FIG. 1. Expression analysis of different ASKs in transiently transformed arabidopsis protoplast. YFP reporter fusion constructs of the indicated genes were transiently transformed into arabidopsis protoplasts by PEG mediated transformation. The protoplasts were then analyzed with an Olympus fluorescent microscope applying an equal exposure time of 639.10 ms.

SUPPL. FIG. 2. Mass Spectra of phosphosite identification in BIN2 phosphorylated MKK4. A. Spectrum leading to the identification of the sequence that may contain phosphorylated S230 or S231. The experimental spectrum was interpreted by the program Mascot. The asterisk indicates the position of the phosphorylation. B. Table of fragments corresponding to the above spectrum. C. Spectrum leading to the identification of the sequence that may contain phosphorylated T234. The experimental spectrum was interpreted by the program Mascot. The asterisk indicates the position of the phosphorylation. D. Table of fragments corresponding to the above spectrum. E. A second spectrum leading to the identification of the sequence that may contain phosphorylated T234. The experimental spectrum was interpreted by the program Mascot. The asterisk indicates the position of the phosphorylation. F. Table of fragments corresponding to the above spectrum.

SUPPL. FIG. 3. BIN2 phosphorylation of MKK4 is not altered in a S6A mutant. In vitro kinase assays using recombinant GST-BIN2, GST-tagged wild-type MKK4 and a S6A mutant of MKK4.

SUPPL. FIG. 4. Mutations at T234 renders MKK4 inactive. In vitro kinase assays using recombinant HIS-MPK6, GST-tagged wild-type and mutant variants of MKK4. Proteins were incubated in kinase buffer in the presence of \([\gamma^{32}\text{P}]\)-ATP as a co-substrate. Subsequently samples were separated by SDS-PAGE and subjected to autoradiography (AR). Stainings with coomassie brilliant blue (CBB) are shown as loading controls in the lower panel.

SUPPL. FIG. 5. Localization of MKK4p:MKK4-YFP in arabidopsis protoplasts. Arabidopsis protoplasts expressing pMKK4:MKK4-YFP treated with epi-BL (1\(\mu\)M) or bikinin (30 \(\mu\)M) for 2 hrs as compared to an untreated control. Representative protoplasts are shown.

SUPPL. FIG. 6. MKK5 over-expression suppresses stomata clustering induced by bikinin treatment. A. Western blot analysis of wild-type and homozygous lines expressing either 35S:MKK2-MYC or 35S:MKK5-YFP with anti-MYC or anti-YFP antibody respectively. B. Phenotype of seedlings grown on
plates with or without 1\(\mu\)M Brz. C. Frequency of stomata clustering in wild-type, 35S:MKK2-MYC and 35S:MKK5-YFP plants in the presence or absence of 1 \(\mu\)M Brz as determined by light microscopy. The results are shown in \%. P-values derived of a two-tailed \(\chi^2\)-test are given.
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Precursor: m/z = 739.322 (2+)

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pMKK4:MKK4-YFP
+ 1 μM epi-BL

pMKK4:MKK4-YFP
+ 30 μM bikinin

bright field  filter  overlay
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C

![Bar chart showing clustered stomata in %](chart.png)