RESEARCH PAPER

Salt-induced subcellular kinase relocation and seedling susceptibility caused by overexpression of Medicago SIMKK in Arabidopsis

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Abstract

Dual-specificity mitogen-activated protein kinases kinases (MAPKKs) are the immediate upstream activators of MAPKs. They simultaneously phosphorylate the TXY motif within the activation loop of MAPKs, allowing them to interact with and regulate multiple substrates. Often, the activation of MAPKs triggers their nuclear translocation. However, the spatiotemporal dynamics and the physiological consequences of the activation of MAPKs, particularly in plants, are still poorly understood. Here, we studied the activation and localization of the Medicago sativa stress-induced MAPKK (SIMKK)–SIMK module after salt stress. In the inactive state, SIMKK and SIMK co-localized in the cytoplasm and in the nucleus. Upon salt stress, however, a substantial part of the nuclear pool of both SIMKK and SIMK relocated to cytoplasmic compartments. The course of nucleocytoplasmic shuttling of SIMK correlated temporally with the dual phosphorylation of the pTEpY motif. SIMKK function was further studied in Arabidopsis plants overexpressing SIMKK–yellow fluorescent protein (YFP) fusions. SIMKK–YFP plants showed enhanced activation of Arabidopsis MPK3 and MPK6 kinases upon salt treatment and exhibited high sensitivity against salt stress at the seedling stage, although they were salt insensitive during seed germination. Proteomic analysis of SIMKK–YFP overexpressors indicated the differential regulation of proteins directly or indirectly involved in salt stress responses. These proteins included catalase, peroxiredoxin, glutathione S-transferase, nucleoside diphosphate kinase 1, endoplasmic reticulum luminal-binding protein 2, and finally plasma membrane aquaporins. In conclusion, Arabidopsis seedlings overexpressing SIMKK–YFP exhibited higher salt sensitivity consistent with their proteome composition and with the presumptive MPK3/MPK6 hijacking of the salt response pathway.

Key words: Arabidopsis, MAPK, Medicago, proteomics, salt stress, SIMK, SIMKK, subcellular relocation.
Introduction

Mitogen-activated protein kinases (MAPKs) perceive and transduce various signals affecting plant life. MAPK pathways are organized into three-tiered modules composed of a MAPK kinase kinase (MAPKKK) activating a dual-specificity Ser/Thr and Tyr MAPK kinase (MAPKK), resulting in the dual phosphorylation and activation of a Ser/Thr MAPK (Jonak et al., 2002; Rodriguez et al., 2010). MAPKKs activate MAPKs by simultaneous phosphorylation of TEY or TDY signature motifs, within the kinase activity loop (Kiegerl et al., 2000; Calderini et al., 2001; Keshet and Seger, 2010). In Arabidopsis thaliana L., 20 different MAPK pathways have been identified in the complete annotated genome (MAPK Group, 2002; Colcombet and Hirt, 2008; Dóczi et al., 2012; Janitz et al., 2012).

Plant MAPKs can be activated by multiple abiotic stimuli such as wounding, drought, cold, and salinity (Rodriguez et al., 2010; Šiná et al., 2011; Šamajová et al., 2013a) and biotic stresses such as diverse pathogens, pathogen-derived toxins, and microbe-associated molecular patterns or MAMPs (Pitzschke et al., 2009; Rasmussen et al., 2012). In response to environmental and developmental cues, plant MAPKs mediate hormonal responses, cell-cycle regulation, and development (Komis et al., 2011; Sasabe and Machida, 2012; Šamajová et al., 2013a). In this respect, MAPKs phosphorylate, and thereby regulate, diverse intracellular targets including other protein kinases, nuclear transcription factors, cytoskeletal components, and proteins involved in vesicular trafficking in eukaryotic cells (Cargnello and Roux, 2011; Komis et al., 2011; Šamajová et al., 2013b). Therefore, MAPKs represent versatile transducers of plant signalling.

In model systems such as yeast and mammalian cells, MAPK modules undergo dynamic compartmentalization. The prototypical extracellular signal-regulated kinase 1 (ERK1) exhibits conditional nucleocyttoplasmic shuttling in response to mitogen stimulation (Volmat et al., 2001). Cellular localization of plant MAPK cascades and their molecular interactions are much less understood. Previous reports in dividing plant suspension cells showed relocation of MAPKs during biotic stress (Ligterink et al., 1997; Lee et al., 2004). The differential subcellular localization of MAPKs was particularly described during developmental processes including cytokinesis and root hair formation (Calderini et al., 1998; Bögre et al., 1999; Coronado et al., 2002; Šamaj et al., 2002; Beck et al., 2010, 2011; Kosetsu et al., 2010; Müller et al., 2010). Co-localization of MAPKs with their upstream MAPKKs and their joint translocation to certain cellular compartments has been documented only rarely, including the ethylene-induced simultaneous nuclear translocation of MKK9–MPK3/6 (Yoo et al., 2008). However, insight into the regulation and function of the subcellular localization of MAPK pathways is lacking in plants.

In Medicago sativa, SIMK (stress-induced MAPK) was identified as a salt stress- and elicitor-induced MAPK (Munnik et al., 1999; Cardinale et al., 2000). Yeast two-hybrid screening and in vitro and in vivo activation studies identified SIMK kinase (SIMKK) as the upstream activator of SIMK. SIMKK was shown to activate SIMK in response to salt stress (Kiegerl et al., 2000; Cardinale et al., 2002). This study aims to provide insight into the subcellular localization of SIMKK and SIMK, revealing that a significant part of the nuclear pool of the two kinases becomes relocated to the cytoplasm upon activation by salt stress. Interestingly, upon salt activation, SIMKK and SIMK are concentrated in unknown subcellular cytoplasmic punctate structures that require further investigation. Finally, we have presented proteomic, biochemical, and phenotypic characterization of Arabidopsis plants overexpressing SIMKK. These plants contained altered levels of proteins involved in salt and oxidative stress, higher activity levels of MPK6 and MPK3 after short salt treatment, and they were more susceptible to long-term salt stress.

Materials and methods

Plant material and treatments

Seeds of M. sativa L. cv. Europe were placed on moist filter paper in Petri dishes and germinated in culture chambers in darkness at 25 °C. Three-day-old seedlings were selected for salt treatments, immunoblotting, and immunolocalization experiments. Seeds of wild-type A. thaliana L., cv. Columbia and stably transformed lines were germinated and grown on agar or Phytagel plates containing half-strength Murashige and Skoog medium under standard culture conditions. Protoplasts were isolated from Arabidopsis suspension cultures as described previously (Kiegerl et al., 2000; Cardinale et al., 2002). Transiently transformed protoplasts, M. sativa roots, and seedlings of stably transformed Arabidopsis lines were treated with 250 mM NaCl diluted in the culture medium. Stably transformed Arabidopsis plants with fluorescently tagged SIMKK constructs were also used for MAPK salt activation (treatment with 250 mM NaCl for 10 and 30 min) and for long-term salt treatments with 100 mM NaCl. Images of the Petri dishes were taken 14 d after the transfer of 5-d-old plants to salt-containing medium. For germination tests, seeds of control and stably transformed Arabidopsis lines were sown on control medium or medium containing 100 mM NaCl, kept at 4 °C for 48 h, and transferred to a growing chamber under standard culture conditions. Germination rate was evaluated under a stereomicroscope on day 1, 2, and 3 after transfer to the chamber. Each experiment was repeated in five biological repeats.

Vector constructs

Both SIMKK and SIMK were tagged on their C terminus with reporter genes encoding cyan fluorescent protein (CFP), yellow fluorescent protein (YFP) or the haemagglutinin (HA) epitope. All tags were subcloned as NovI/NovI fragments. Reading frames of the SIMKK and SIMK genes tagged with CFP, YFP, or HA were cloned into pSH9 or PRT100 vectors using HindIII and PstI under the control of the cauliflower mosaic virus (CaMV) 35S promoter. For stable expression in Arabidopsis plants, expression cassettes with SIMKK–CFP/YFP constructs under the control of the CaMV 35S promoter were cloned into the binary vector P-Green II.

Plant transformation

Arabidopsis plants were stably transformed with SIMKK–CFP/YFP constructs using the standard floral-dip method (Clough and Bent, 1998). Protoplasts were transformed with SIMKK–YFP/CFP and SIMK–YFP constructs using a polyethylene glycol
method as described previously (Kiegerl et al., 2000; Cardinale et al., 2002). *M. sativa* root cells were transiently transformed with SIMKK–YFP using the gene gun method according to the manufacturer’s instructions (Helios gene gun system; Bio-Rad) and the fluorescence of individually transformed cells was observed the next day.

**Antibodies and immunoblotting**

Both protein A- and immunofinity-purified polyclonal antibodies N103 (recognizing the CTDFMpTEpYVVTRWC peptide of SIMK) and M23 (recognizing the C-terminal heptapeptide FNPEYQQ of N103 (recognizing the CTDFMpTEpYVVTRWC peptide of SIMK) were tested on root and protoplast extracts as described by Kiegerl et al. (2000) and Šamaj et al. (2002). For protein extraction, roots were homogenized in ice-cold extraction buffer [50 mM Tris/HCl, pH 8, 150 mM NaCl, 1% (v/v) NP-40, and 0.1% (w/v) SDS] and the protein content was measured using a Bradford assay. Protein extracts were separated by SDS-PAGE (MINI-Protein II cell system; Bio-Rad) and blotted onto nitrocellulose membrane. The membrane was blocked with 3% (w/v) bovine serum albumin and 3% (w/v) non-fat dried milk powder in Tris-buffered saline (TBS. 100 mM Tris/HCl, pH 7.4, 1.5 mM NaCl) for 1 h, and subsequently incubated with a primary anti-green fluorescent protein (GFP) antibody (Sigma), diluted 1:1000 in TBS-T [TBS plus 0.1% (v/v) Tween 20] containing 1% (w/v) bovine serum albumin at room temperature for 1.5 h.

For MAPK activation study, proteins were extracted from liquid nitrogen powders of roots or aerial parts of Arabidopsis plants before and after salt treatment (250 mM for 30 min) in 2 vols of RIPA buffer [50 mM Tris/HCl, pH 7.4, 150 mM KCl, 5 mM EGTA, 0.5% (w/v) sodium deoxycholate, 0.1% (v/v) Triton X-100, 0.1% (w/v) SDS, phosphatase and protease inhibitors (PhosStop™ and EDTA-free Complete™, both from Roche)]. Extracts were then cleared (13 000g, 10 min, 4 °C) and the supernatant was used as a source of total protein after quantitative determination by a Bradford assay. If the quantity of starting material was low, then aliquots of the supernatant were precipitated with 4 vols of anhydrous acetone (overnight at −20 °C), pelleted (13 000g, 10 min, 4 °C), resuspended in 1/10 vol. of rehydration buffer [8 M urea, 2 M thiourea, 2% (w/v) CHAPS], mixed proportionally with 4× Laemmli sample buffer and separated by 7.5% SDS-PAGE without heating. If the quantity of starting material was sufficient, then it was mixed proportionally with 4× Laemmli sample buffer and heat denatured before separating by 7.5% SDS-PAGE.

After transfer to polyvinyldene difluoride membranes and Ponceau S visualization of the bands, the corresponding lanes were cut into strips and probed with anti-pTEpY (20 min) followed by successive methanolic ammonium acetate and acetone washes. The strips were manually aligned under a Leica TCS4D system using a 514 nm Argon laser line for photobleaching of YFP. FRAP analysis of YFP-tagged SIMKK was performed employing a Leica TCS4D system using a 514 nm Argon laser line for photobleaching of YFP. For quantitative analysis of FRAP experiments, the recovery of fluorescence was determined using Leica TCS4D software. Briefly, nuclei were selected as regions of interest with the zoom function and these areas were repeatedly bleached at full laser power so that the post-bleach fluorescence value represented maximally 10% of the original pre-bleach fluorescence value. The pre-bleach/post-bleach fluorescence and fluorescence recovery rates were recorded every 10 seconds using a time-lapse function. Fluorescence intensities were expressed in arbitrary units and normalized to absolute fluorescence intensities in the nuclei before bleaching. Corresponding half-time values of signal recovery and the portion of immobile protein fractions were calculated from FRAP data. Data from 12 regions of interest were exported to Microsoft Excel software, averaged and plotted as means±standard deviation.

**Fluorescence recovery after photobleaching (FRAP)**

**Immunolocalization of inactive and active SIMKK and microscopy**

For double immunolocalizations, the N103 and M23 antibodies were directly coupled to Alexa Fluor 488 (green fluorescence) and 568 (red fluorescence), with a commercial protein labelling kit (Invitrogen, Leiden, The Netherlands). Immunolocalization was performed on sections from Steedman’s wax embedded material as described previously (Šamaj et al., 2002). Images of CFP/YFP/GFP-tagged SIMKK and SIMK in living cells as well as immunolabelled SIMKK in fixed cells were acquired with an Axioplan 2 (Zeiss, Oberkochen, Germany) laser-scanning confocal microscope (Zeiss LSM710; Carl Zeiss, Jena, Germany) and Leica TCS4D system (Leica, Mannheim, Germany), or a high-speed confocal microscope UltraVIEW life cell imager (PerkinElmer Life Sciences, Boston, USA) equipped with a spinning disc. Images were processed using Adobe Photoshop. Microfluorimetric image analysis of the immunofluorescence intensities of labelling in root cells was determined from Steedman’s wax sections according to Šamaj et al. (2002).

**Protein extraction for proteomics and trypsin digestion**

Roots of 14-d-old transgenic Arabidopsis seedlings (in five independent biological replicates) carrying the SIMKK–YFP construct (line Y11) were used for proteomic analysis. The preparation of trypsin-digested extracts was performed as described by Takáč et al. (2011). Briefly, roots were homogenized in liquid nitrogen using a mortar and pestle and proteins were extracted by phenol extraction followed by successive methanolic ammonium acetate and acetone precipitation (Hurkman and Tanaka, 1986). The precipitates were dissolved in 6 M urea and in total 100 μg of protein was reduced and alkylated with dithiothreitol and iodoacetamide, respectively. Peptides were digested with 20 μg of trypsin (0.01% (w/v) trypsin) overnight. Digestion was stopped by the addition of 4 μl of 1% (v/v) formic acid. The peptides were desalted using SEPAK light C18 columns (Waters, UK) according to manufacturer’s instructions.

**Mass spectrometric analysis and quantification including one-dimensional nanoACQUITY ultraperformance liquid chromatography (UPLC) was performed according to Uváčková et al. (2013) with minor modifications. First, the trypsin-digested extracts were concentrated under vacuum to 10 μl. The final volume was then adjusted to 50 μl by adding 5% (v/v) acetonitrile in 0.1% (v/v) formic acid. Each sample was spiked with pre-digested bovine haemoglobin (prepared by digesting of HBA UNIPROT P01966) internal standard (Waters 186002327, UK) at a level of 1 pmol per 2 μl injection (2 μg of proteins). For sample analysis, 2 μg protein aliquots were analysed using a nanoACQUITY UPLC system (Waters) coupled
to a Premier quadrupole time-of-flight (Q-TOF) mass spectrometer (Waters). The peptide mixture was injected onto a reverse-phase column (nanoACQUITY UPLC column BEH 130 C18, 75 μm×1.5 mm, 1.7 μm particle size) and an acetonitrile gradient (10–50% acetonitrile containing 0.1% formic acid for 60 min) at a flow rate of 350 nl min⁻¹ was employed to elute the peptides into the Q-TOF. The column was connected to PicoTip emitters (New Objective, USA) mounted into the nanospray source of the Q-TOF Premier. A nano-electrospray voltage of 3.5 kV was applied, with the source temperature set to 70 °C. The spectral acquisition scan rate was 1 s with a 0.05 s interscan delay. A novel multiplex MS² approach, in which MS data were collected in an alternating low-energy (MS) and elevated energy (MS³) mode, was used for protein identification (Plumb et al., 2006; Li et al., 2009). For MS² quantification, the average MS signal response from bovine haemoglobin was used to determine the universal signal response factor (counts per mol of protein). This information was then used to determine the concentrations for each of the target proteins by dividing the MS by the universal signal response factor. In the low-energy MS mode, data were collected at a constant collision energy of 2 eV. In the elevated energy MS³ mode, the collision energy was ramped from 25 to 38 eV during each integration. During data acquisition, the quadrupole analyser was not mass selective but operated in the radiofrequency-only mode. Thus, all ions were passed to the TOF analyser. Glu-1-fibrinopeptide B at a concentration of 1 pmol ml⁻¹ was infused via the source at a flow rate of 500 nl min⁻¹ and sampled every 30 s as the external mass calibrant.

Data processing and database search

The MS² data were processed using the ProteinLynx Global Server v.2.4 (PLGS 2.4; Waters), which provides background subtraction, smoothing, centroiding, and deisotoping. All data were then lock-spray calibrated against Glu-1-fibrinopeptide B using data collected from the reference line during acquisition and charge-state reduced to produce precursor monoisotopic mass lists as well as associated product ion mass lists. Each processed file was searched against the non-redundant Arabidopsis UniProt database downloaded on 2 April 2013 (31 821 entries) with the addition of internal standard HBA_BOVIN haemoglobin subunit α sequence (Water: 186002327; NCBI P01966) using the search algorithm within the PLGS 2.4. The initial correlation of a precursor (MS) and possible fragment ions (MS/MS) was achieved by means of time alignment. The elution profile of precursor masses from the LC component was used to deconvolute the mass of fragments from many precursor ions. Default search parameters were applied including the ‘automatic’ setting for mass accuracy (10 ppm for precursor ions and 15 ppm for product ions), a minimum of one peptide match per protein, a minimum of three consecutive product ion matches per protein, and a minimum of seven total product ion matches per protein. The maximum false-positive rate against the randomized forward database was set at 4%, and the protein quantification functionality was enabled using the internal standard (1 pmol of HBA_BOVIN haemoglobin subunit α). Only one missed tryptic cleavage site was allowed during the search. A fixed carbamidomethyl-Cys modification was used, in addition to the following variable modifications: deamidation of Asn and Gln, oxidation of Met, and dehydration of Ser and Thr. In order to determine protein quantities, the combined intensity of the multiply charged ions for the three most abundant tryptic peptides of a quantitatively added internal standard was compared with the observed response for any identified protein in a complex mixture. One-way analysis of variance statistical analysis was carried out to identify statistically significant (P<0.05) differences in protein amount.

Results

In order to study the subcellular localization of SIMKK and its downstream target SIMK, we tagged these proteins with spectral variants of GFP (CFP and YFP) and expressed the gene constructs under the control of a CaMV 35S promoter. Individual or tandem expression was studied transiently in protoplasts isolated from A. thaliana suspension cells or in M. sativa roots. Expression after stable transformation was studied in Arabidopsis plants.

Salt stress triggers nuclear export of YFP-tagged SIMKK and its association with cytoplasmic compartments in vivo

In non-stressed transiently transformed Arabidopsis protoplasts, YFP-tagged SIMKK was localized predominantly in the nucleus and in the cytoplasm (Fig. 1A). Salt treatment (10–30 min) reduced the nuclear content of SIMKK–YFP, whilst it relocated, at least partially, to cytoplasmic punctate compartments (Fig. 1B). No association of YFP alone (used as a control) with cytoplasmic compartments was observed under the same conditions of salt stress. In both control and salt-treated protoplasts, YFP alone remained predominantly in the nucleus (Fig. 1C, D). Thus, in vivo localization of YFP-tagged SIMKK in Arabidopsis protoplasts revealed salt stress-induced relocation of the kinase and its specific association with punctate compartments in the cytoplasm.

Localization of YFP-tagged SIMKK into stress-induced cytoplasmic compartments and their dynamic mobility was also studied in non-dividing elongated epidermal root cells of M. sativa following transient transformation. These cytoplasmic structures representing vesicular organelles were highly motile and moved with different velocities independently of cytoplasmic streaming, whilst they also accumulated in larger patches around the nucleus (Fig. 1E). Salt treatment of these cells significantly enhanced localization of SIMKK–YFP to cytoplasmic compartments occasionally fusing to each other (Fig. 1E; Supplementary Video S1 available at JXB online).

Persistent salt-induced activation of SIMKK, a downstream target of the SIMKK, in intact roots of M. sativa

The concentration and time-dependent salt-induced activation of SIMKK, which is a direct target of SIMKK (Kiegerl et al., 2000), was characterized in intact M. sativa roots, which are the organs typically affected by soil salinity. Within 2 min, salt induced the activation of SIMK which persisted for at least 1 h (Fig. 2A, upper panel) without affecting SIMK protein levels (Fig. 2A, lower panel). Thus, salt-induced SIMK activation occurred by a post-translational mechanism. These data showed that salt stress rapidly activates SIMK in a dose-dependent manner by phosphorylation of the TEY motif.

Salt-induced activation of SIMKK correlates with its relocation to cytoplasmic compartments in intact M. sativa roots

Next, the effects of salt on the subcellular localization of SIMK in M. sativa roots were addressed. The persistent salt-induced activation of SIMK in intact roots of M. sativa
Fig. 1. In vivo localization of YFP-tagged SIMKK in transiently transformed *Arabidopsis* proplasts and in *M. sativa* roots. (A) SIMKK–YFP was located predominantly in the nucleus, but lower levels could also be detected in the cytoplasm of control protoplasts. (B) SIMKK–YFP relocates to cytoplasmic punctate compartments (indicated by arrows) in protoplasts treated with 250 mM salt for 30 min. (C, D) No relocation could be observed in salt-treated protoplasts transformed with control non-fused YFP. (E) In elongating epidermal root cell of *M. sativa*, SIMKK–YFP was located predominantly in the nucleus and partly in cytoplasmic motile compartments. Additional salt stress (250 mM NaCl for 30 min) caused accumulation of SIMKK–YFP in patches localized around nucleus. Arrows indicate some fast-moving and sometimes fusing cytoplasmic vesicular organelles with associated SIMKK–YFP. Figures represent individual frames (with indicated time points) from Supplementary Video S1 available at JXB online. Stars indicate nuclei. Bar, 7 μm (A–D); 10 μm (E).
coincided with the depletion of SIMK from nuclei and its increased association with punctate cytoplasmic structures (Fig. 2B). As revealed by immunofluorescence microscopy using the SIMK protein-specific M23 antibody, these compartments frequently coalesced into patchy structures around nuclei (Fig. 2B; see also Fig. 4D). Partial depletion of the strong nuclear SIMK signal upon salt treatment of root cells (Fig. 2B) suggested that SIMK was partially relocated from this compartment.

Next, it was examined whether salt-induced activation of SIMK was correlated with its relocation. Therefore, the SIMK activation state within the nucleus and the cytoplasmic compartments were studied by using the phospho-specific antibody N103 (raised against the SIMK phosphopeptide CTDFMpTEpYVVTRWC; see also Šamaj et al., 2002). Specificity tests revealed that the N103 antibody recognized activated SIMK in Arabidopsis protoplasts upon co-expression of HA-tagged SIMK and its upstream activator SIMKK (Fig. 3A). Immunoblots using crude M. sativa root extracts and N103 antibody showed little active SIMK in control roots (Fig. 3B, 0 min). Within 10 min of salt treatment, however, N103 antibody clearly recognized a 46 kDa band, which corresponded to SIMK (Fig. 3B). These data showed that salt stress in M. sativa roots activates SIMK through dual phosphorylation of the TEY motif.

In order to localize the pool of active SIMK, M23 (protein-specific) and N103 (activity-specific) SIMK antibodies were covalently coupled to two different fluorochromes (Alexa Fluor 568 and 488, respectively). In control M. sativa root cells, the co-localization experiments revealed a predominant nuclear localization of SIMK (Fig. 4A) and very low levels of dually phosphorylated SIMK (Fig. 4B, C). In contrast, after salt treatment, a significant amount of dual-phosphorylated SIMK was found in the cytoplasm and mostly in patchy

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**Fig. 2.** Salt-dependent activation and localization of SIMK in *M. sativa* roots. (A) Immunokinase analysis of time-dependent, persistent salt-induced activation of SIMK in intact *M. sativa* roots. Upper panel: *M. sativa* roots treated with 250 mM NaCl for the indicated times. Root cell extracts were immunoprecipitated with protein G-purified SIMK-specific antibody M23. Kinase reactions were performed using MBP as a substrate. Lower panel: the same crude extracts from salt-treated roots were subjected to immunoblotting with the M23 antibody to check SIMK protein levels. (B) Immunolocalization of SIMK with M23 antibody in control and salt-treated (250 mM NaCl) non-dividing cells of intact *M. sativa* roots. SIMK was abundant in the nuclei of control cells, whilst it relocated to punctate cytoplasmic compartments in salt-treated root cells. With increasing time (60 min) of salt treatment, these cytoplasmic structures coalesced into larger patches that accumulated around the nuclei. Note the change in shape of the nuclei due to NaCl-induced plasmolysis. Images in the middle panel show nuclear DAPI staining. DIC, differential interference contrast. Bar: 5 μm (B).
Salt-induced relocation and function of SIMKK

To obtain semi-quantitative data for this process, microfluorimetric measurements were performed on the nucleocytoplasmic partitioning of phosphorylated versus non-phosphorylated SIMK in control and salt-stressed *M. sativa* root cells. The pools of inactive and active SIMK were quantified by measuring the mean fluorescence intensity of total SIMK immunolocalized with M23 antibody in comparison with dual-phosphorylated SIMK labelled with N103 antibody within the nuclear and cytoplasmic fractions of control and salt-treated cells (Fig. 4G, H). These measurements clearly demonstrated that a pool of non-phosphorylated SIMK was concentrated within the nuclei of control cells (Fig. 4G), whereas phosphorylated SIMK accumulated in the cytoplasm of cells treated with salt (Fig. 4H).

Altogether, both single and double immunolocalization studies together with quantitative and immunobiochemical analyses strongly suggested that salt-induced phosphorylation and activation of SIMK is correlated with its recruitment to cytoplasmic compartments in intact roots of *M. sativa*.

Salt stress can trigger the association of both SIMKK and SIMK with cytoplasmic compartments

Both SIMKK and SIMK were tagged with different fluorescent proteins (CFP and YFP) and co-expressed in *Arabidopsis* protoplasts in order to investigate their co-localization. Under control conditions, both CFP-tagged SIMKK and YFP-tagged SIMK preferentially localized to the nuclei, but they were also dispersed throughout the cytoplasm (Fig. 5A–C). In contrast, both SIMKK–CFP and SIMK–YFP relocated to cytoplasmic punctate compartments where they co-localized after 30 min of salt treatment (Fig. 5D–F).

Immunoprecipitation with GFP- and SIMK-specific antibodies and subsequent immunokinase assays revealed that SIMKK–CFP was functional, being able to phosphorylate SIMK–YFP (Fig. 5G, H). As SIMK protein levels remained constant in these experiments, SIMK–YFP appeared to be post-translationally activated by SIMKK–CFP (Fig. 5G). These data suggested that salt treatment triggered SIMKK–CFP mediated activation of SIMK–YFP, which was associated with concomitant enrichment and co-localization of both kinases in cytoplasmic compartments. Relocation and co-localization of both SIMK and SIMKK to cytoplasmic compartments suggested that both kinases were actively targeted to these structures in a coordinated manner.

Constitutive import of SIMKK into the nucleus under control and salt-stress conditions

To investigate the mechanism of nuclear import of SIMKK in living cells, FRAP was used in *Arabidopsis* protoplasts transformed with SIMKK–YFP. As the YFP-tagged SIMKK fusion protein is much larger (>70kDa) than the exclusion limit of nuclear pores (maximum 45–50kDa), it requires an active nuclear import mechanism. During laser bleaching of the nuclear compartment, the whole nuclear area was first depleted and subsequently replenished quickly, indicating that YFP-tagged SIMKK was mobile. During the recovery, we always observed structures around the nuclei (Fig. 4D–F). Although some inactive SIMK remained in the nuclei of salt-treated roots (Fig. 4D), these data suggested that salt stress-induced dual phosphorylation and activation of SIMK induced its relocation to the cytoplasm in punctate compartments.
a simultaneous overall decrease in cytoplasmic fluorescence with increased time (Supplementary Fig. S1A available at JXB online). These data suggested that cytoplasmic SIMKK–YFP steadily enters the nuclei. The FRAP rate of SIMKK–YFP in bleached nuclei was similar between control and salt-stressed cells, although the slope of the curves reflected some differences (Supplementary Fig. S1B, C). After photobleaching of nuclei, nuclear SIMKK–YFP fluorescence recovered at similar rates in control and salt-stressed Arabidopsis protoplasts, with half-life times between 31.5 and 32.5 s (Supplementary Fig. S1D). The immobile protein fractions, however, showed a significant reduction after salt stress (Supplementary Fig. S1E). These results suggested that, although the mobility of SIMKK–YFP protein is not considerably affected by salt, the mobile pool of SIMKK–YFP in the nucleus is altered, probably as a consequence of salt-induced SIMKK–YFP activation.

Salt-induced accumulation of YFP-tagged SIMKK into cytoplasmic compartments in Arabidopsis plants

In order to extend our results with transiently transformed protoplasts and M. sativa root cells, we generated 19 independent
Arabidopsis lines stably expressing CFP- or YFP-tagged SIMKK under the 35S CaMV promoter. Fluorescently (CFP or YFP) tagged SIMKK was predominantly nuclear under control conditions in epidermal cells of Arabidopsis root tip (Fig. 6A), but it was enriched in cytoplasmic punctate compartments following salt treatment (Fig. 6D). On the other hand, the distribution of fluorescence was identical in control plants transformed with GFP, irrespectively of salt treatment, showing GFP accumulation within the nuclei but no association with cytoplasmic compartments after salt treatment (Fig. 6B, E). As another control, nuclear protein histone 2B (H2B) tagged with YFP was used to confirm the integrity of nuclei under the plasmolytic conditions caused by 250mM NaCl. Similarly to GFP alone, H2B–YFP was also not associated with cytoplasmic compartments upon salt stress but rather showed exclusive and constitutive nuclear localization (Fig. 6C, F).

The expression level of the SIMKK–YFP in independently transformed Arabidopsis lines fluctuated according to the roughly estimated fluorescence intensity. Therefore, we characterized the level of SIMKK–YFP expression in two selected lines (Y4 and Y11) by immunoblotting using anti-GFP antibody recognizing the YFP tag of SIMKK. The expression level of SIMKK–YFP in line Y4 was 2.5-fold higher than in line Y11 (Fig. 6G, H). As the levels of constitutively overexpressed SIMKK may considerably influence properties of transformed plants, these two lines with altered levels of SIMKK–YFP overexpression were further studied by phenotypic and biochemical analyses for their sensitivity to salt stress.

Overexpression of SIMKK in Arabidopsis causes enhanced MPK6 and MPK3 activation and altered sensitivity to salt stress

Stably transformed Arabidopsis lines overexpressing SIMKK–YFP and non-transformed Col-0 seedlings, untreated or
Ovečka et al. treated with 250 mM NaCl for 30 min, were analysed and compared for MPK6 and MPK3 expression and activation. Protein extracts separated by SDS-PAGE were probed with affinity-purified antibodies against the dual-phosphorylated pTEpY motif of mammalian ERK1/2 (phospho-p44/42), and with affinity-purified antibodies against A. thaliana MPK6 and MPK3. The protein separation setup allowed unambiguous discrimination between MPK6 and MPK3 in identical SDS-PAGE-separated samples using commercially available, affinity-purified MPK6 and MPK3 antibodies (Fig. 7). The activation of MPK6 and MPK3 was studied using the pTEpY-specific phospho-p44/42 antibody. Its application identified a band persisting at ~46 kDa in Col-0 plants in control conditions, which, by size comparison, clearly corresponded to MPK6 (Fig. 7). This band was evident in all non-induced samples, including the stably transformed lines Y11 and Y4 (Fig. 7). However, the second band at ~43 kDa, corresponding to MPK3, was weakly detectable in Y4 but absent in Col-0 and Y11 plants (Fig. 7). A 30 min salt treatment induced phosphorylation of both MPK6 and MPK3 in Col-0 and Y11 plants but considerably stronger activation in the Y4 line (Fig. 7). These data suggested that SIMKK–YFP overexpression in Arabidopsis causes the upregulation of both MPK6 and MPK3 kinase activities (Fig. 7) in a quantitative manner that correlates with the level of overexpression of the MAPKK (Fig. 6G, H).

Importantly, the SIMKK–YFP overexpressor lines Y4 and Y11 were clearly more sensitive to salt when 5-d-old plants were transferred for long-term growth on 100 mM NaCl for 14 d (Fig. 8A, B). Comparison of Y4 and Y11 lines revealed that line Y4 was more sensitive to salt because more plants of this line exhibited dwarfism or completely inhibited growth (Fig. 8B). These data suggested that constitutive overexpression of SIMKK–YFP in growing Arabidopsis plants compromises their ability to withstand moderate salt stress, and it was, at least partially, related to the level of SIMKK–YFP overexpression in the Y4 and Y11 lines. These Arabidopsis lines, however, were salt insensitive during germination (Fig. 8C, D; Supplementary Figs S2 and S3). The two SIMKK–YFP overexpressing lines Y4 and Y11, as well as wild-type Col-0 and a GFP overexpressing line germinated effectively within 24 h in control medium (Fig. 8C; Supplementary Fig. S3A available at

![Fig. 6. Localization of SIMKK in non-dividing epidermal cells in the elongating zone of the root tip of the stable transformed Arabidopsis line Y11. (A) YFP-tagged SIMKK in root epidermal cells under control conditions. (B) Localization of GFP in line G5 expressing GFP alone under control conditions. (C) Localization of nuclear marker H2B–YFP under control conditions. (D) YFP-tagged SIMKK in root epidermal cells upon salt stress (250 mM NaCl for 30 min). (E) Localization of GFP in line G5 expressing GFP alone upon salt stress (250 mM NaCl for 30 min). (F) Localization of nuclear marker H2B–YFP upon salt stress (250 mM NaCl for 30 min). (G) Immunoblot analysis of the recombinant SIMKK–YFP protein expression in roots of two Arabidopsis lines (Y4 and Y11) overexpressing SIMKK–YFP and wild type (Col-0) using anti-GFP antibody. A band corresponding to the recombinant protein with molecular weight of 70 kDa is shown. (H) Graph showing the relative optical density of bands in (G) corresponding to SIMKK–YFP. Bar, 10 μm (A–F).](https://academic.oup.com/jxb/article-abstract/65/9/2335/522564)
Fig. 7. Salt stress-induced MPK6 and MPK3 activation in stably transformed Arabidopsis lines Y4 and Y11 overexpressing SIMKK–YFP. Seedlings (16 d old) of line Y4, line Y11, and Col-0 (control) grown on solidified culture medium were treated with 250 mM NaCl for 30 min. (A) Root protein extracts separated by SDS-PAGE were probed with affinity-purified antibodies against mammalian ERK1/2 (phospho-p44/42, pERK), Arabidopsis MPK6, and Arabidopsis MPK3. Molecular mass is indicated. Staining with anti-actin antibody was used as a loading control. (B, C) Graphs showing quantitative analysis of the relative optical densities of pERK bands corresponding to activated MPK6 (B) and activated MPK3 (C) in control and salt-treated samples.

Fig. 8. Salt sensitivity test of Arabidopsis plants stably transformed with YFP-tagged SIMKK. (A, B) Plates containing control plants of wild-type Col-0, control plants stably transformed with GFP alone (line G5), and transformed plants expressing YFP-tagged SIMKK (lines Y4 and Y11). Five-day-old seedlings germinating on control medium were transferred to control medium (A), and to medium containing 100 mM NaCl (B). Plants were photographed 14 d after the transfer. (C, D) Evaluation of the germination rate of wild-type Col-0, line G5, and YFP-tagged SIMKK lines Y4 and Y11 in control medium (C) and medium containing 100 mM NaCl (D).
Proteomic analysis of SIMKK–YFP overexpressor plants reveals a lower abundance of stress-related proteins

Proteomic analysis was performed to better characterize SIMKK–YFP overexpressor plants at the molecular level. Protein identification details from each replicate, as well as quantification of significantly differentially regulated proteins, are shown in Supplementary Tables S1 and S2, respectively, available at JXB online. On average, this one-dimensional LC/MS\(^6\) proteomic analysis resulted in the identification of 237 proteins in SIMKK–YFP transgenic plants and 256 proteins in the wild-type plants (Supplementary Table S1). Of these, 22 proteins were detected as significantly differentially abundant between SIMKK–YFP transgenic plants and wild-type Col-0 plants (Table 1). Ten of these proteins are involved in stress responses, representing the most abundant functional class of differentially regulated proteins (Table 1). Notably, proteins involved in salt-induced oxidative stress (e.g. in hydrogen peroxide detoxification) such as catalase, peroxiredoxin, and glutathione S-transferase, as well as nucleoside diphosphate kinase 1, a signalling protein involved in reactive oxygen species signalling and interacting with catalase (Fukamatsu et al., 2003), were all less abundant in transgenic SIMKK–YFP plants.

Two peroxidases, namely peroxidase 69 and peroxidase 23, belonging to the class III of peroxidase superfamily, were slightly upregulated in the transgenic plants (Table 1). Aquaporin PIP2, which is involved in water transport across the plasma membrane, was more abundant in transgenic SIMKK–YFP plants (Table 1), indicating the accelerated water conductance followed by increased loss of water (Katsuhara et al., 2003). Germin-like protein subfamily 2 member 1, upregulated 2.6-fold in SIMKK–YFP plants (Table 1), was shown to be a plasmodesmata-localized protein, which facilitates the plasmodesmata permeability when overexpressed in Arabidopsis (Ham et al., 2012). Furthermore, the actin-binding protein profilin 1, which promotes actin polymerization (Staiger et al., 2010) was also downregulated in these transgenic plants (Table 1). Upregulation of luminal-binding protein 2 suggested altered protein folding in the SIMKK–YFP plants.

Discussion

MAPK signalling cascades regulate cellular processes at different subcellular compartments. A proper understanding of the spatiotemporal organization of individual MAPK modules requires studying the dynamic localization and mode of activation/inactivation of MAPKs during plant development and stress responses (Šamajová et al., 2013b). The data presented in this work provide compelling evidence of a coordinated and activity-dependent relocation of SIMKK and SIMK during salinity stress in plant cells. In a complementary transgenic approach in Arabidopsis, SIMKK–YFP overexpression resulted in enhanced MPK3 and MPK6 kinase activation and conferred altered sensitivity to salt stress. Considering that SIMKK-overexpressing plants have changed amounts of some proteins functioning in salt and oxidative stress responses, these data suggest that SIMKK may play a role as possible negative regulator of salt stress tolerance.

Phospho-specific anti-pTEpY antibodies allow the identification of the activated MAPKs at the cellular level. With such an antibody, it was shown previously that activated SIMK is recruited to tips of growing root hairs (Šamaj et al., 2002). Here, we aimed to study the activation and localization of SIMK upon salt stress in intact roots, representing a plant organ that typically has to cope with increased soil salinity in nature. We showed that SIMK is persistently activated in intact Medicago roots and enriched in cytoplasmic compartments following salt treatment. Our data are supported by several independent lines of evidence: (i) immunokinase assays and immunoblotting with phospho-specific antibodies, both showing persistent activation of SIMK in intact roots upon salt stress; (ii) immunolocalization with protein- and phospho-specific antibodies, both showing relocation of SIMK to cytoplasmic compartments in intact roots; (iii) in vivo relocation of SIMK to cytoplasmic compartments in non-dividing protoplasts; and (iv) similar relocations of SIMKK (involved in salt stress activation of SIMK) into cytoplasmic compartments in root cells and non-dividing protoplasts.

The immunolocalization results with phospho-specific SIMK antibody demonstrated the requirement of SIMK activation for its relocation and helped to explain the coordinated accumulation of SIMKK and SIMK into cytoplasmic compartments during salt stress. It is possible that SIMKK...
functions as a scaffold protein for SIMK or, alternatively, other unknown scaffold proteins that bind both activated SIMKK and SIMK could be involved in their coordinated relocation to cytoplasmic compartments upon salt stress. It is interesting to note that in this context activated kinase modules have been localized to clathrin-coated vesicles and signaling endosomes in animals (Howe et al., 2001; Sorkin and von Zastrow, 2002), but so far nothing is known about similar processes in plants. Our recent data showed the co-localization of Arabidopsis MPK6 and clathrin at the plasma membrane and at TGN vesicles (Müller et al., 2010). These findings are consistent with previous reports suggesting cytoplasmic functions for plant MAPKs and MAPKKs in specific situations such as cell division (Calderini et al., 1998; Bögre et al., 1999; Nishihama et al., 2001) and root hair development (Šamaj et al., 2002), both representing processes associated with vigorous vesicular trafficking.

The dynamic relocalization of the MAPK and MAPKK components from the nucleus to the cytoplasm seems to challenge the traditional view of MAPK translocation from the cytoplasm to the nucleus upon its activation. SIMK and SIMKK relocation into the cytoplasmic compartments upon salt treatment could represent an intermediate stage within the activation-dependent relocation to certain membrane targets that are supposed to be regulated upon salt stress. Supporting evidence that the process of activated MAPK relocation to membrane targets may be related to plant adaptation strategies to salt stress comes from studies of the salt overly sensitive (SOS) pathway. Within this pathway, a complex of SOS2, a Ser/Thr protein kinase (Liu et al., 2000), with SOS3, a RhoGAP-like protein, phosphorylates and activates the transport activity of SOS1, a plasma membrane Na⁺/H⁺ antiporter (Shi et al., 2000; Qiu et al., 2002). MPK6 phosphorylates and activates SOS1,
whilst the MAPK module encompassing MPK6 is located at the plasma membrane (Yu et al., 2010; Kim et al., 2012).

Salt stress triggers different MAPK signalling pathways in Arabidopsis resulting in positive or negative outcomes in terms of plant tolerance. It is well documented that both MPK6 and MPK3 are activated by NaCl stress, and their activation seems to be required for salt tolerance in A. thaliana (Ichimura et al., 2000; Droillard et al., 2002; Teige et al., 2004; Yu et al., 2010). High concentrations of salt activate MKKK20 in Arabidopsis, which is upstream of MPK6 (Kim et al., 2012), but in the MEKK1-dependent signalling pathway involving MPK4, mkk1 mutants exhibited improved growth under high salinity (Su et al., 2007). These results suggest that, in contrast to MKKK20, MEKK1 may negatively regulate the salt response in Arabidopsis. MKK2 is another major activator of MPK4 and MPK6 in cold and salinity conditions, as mkk2 null mutants show salt hypersensitivity (Teige et al., 2004). On the other hand, loss of MKK9 activity as an activator of MPK6 reduced salt sensitivity, indicating its negative impact in salinity tolerance (Xu et al., 2008). Similar results have been shown in mkk1 knockout plants, which were able to tolerate elevated salt concentrations in both germination and post-germination seedling development, indicating that MKK1 is probably a negative regulator of salt stress (Conroy et al., 2013).

Generation of transgenic Arabidopsis lines overexpressing orthologous members of MAPK signalling pathways from other species facilitates their functional characterization (Ren et al., 2002; Han et al., 2010). In this study, constitutive overexpression of YFP-tagged SIMKK in Arabidopsis resulted in lower abundance of some salt-stress-related proteins in roots and enhanced salt stress sensitivity at the stage of seedling growth. It is well known that salt stress induces reactive oxygen species production and antioxidant defence in plants (Munnis and Tester, 2008; Miller et al., 2010), whilst increased antioxidant defence is linked to higher tolerance against salinity (Badawi et al., 2004; Ashraf, 2009). Arabidopsis plants overexpressing SIMKK–YFP exhibited constitutively decreased abundance of proteins such as catalase, glutathione S-transferase and peroxidoredoxin, all involved in antioxidant defence. Other enzymes putatively involved in hydrogen peroxide decomposition, class III peroxidases (peroxidase 23 and 69), were slightly upregulated in the transgenic plants. These enzymes have, except for hydrogen peroxide-decomposing activity, multiple functions in cells including cell-wall metabolism (Passardi et al., 2004), and they can generate highly reactive oxygen species (Liszkay et al., 2003). This suggests that the contribution of peroxidases to hydrogen peroxide removal in SIMKK–YFP plants is not unambiguous and they may exert functions that are not connected to salt-induced oxidative burst.

In addition, nucleoside diphosphate kinase 1 was downregulated in these transgenic plants. Interestingly, nucleoside diphosphate kinases interact with catalase and are involved in salt-stress tolerance (Fukamatsu et al., 2003; Verslues et al., 2007). Thus, SIMKK overexpression might negatively affect nucleoside diphosphate kinase 1-mediated signalling towards catalase, leading to increased sensitivity to salt in SIMKK–YFP plants. Other molecular mechanisms determining the higher susceptibility of SIMKK–YFP overexpressing plants to salt stress may be suggested by the increased constitutive abundance of the aquaporin PIP2, as shown by the proteomic analysis. The accelerated water conductance driven by the higher abundance of aquaporins (Javot et al., 2003) together with restricted capacity of plants for water uptake under salt stress (Romero-Aranda et al., 2001) may result in the limited growth and development of SIMKK–YFP transgenic plants. This mechanism was also suggested for rice plants overexpressing barley HvPiP2.1, which showed higher sensitivity to salt stress (Katsuhara et al., 2003). Reduction of the hydraulic conductivity in Arabidopsis root cells upon NaCl treatment was caused not only by an overall decrease in the abundance of aquaporins but also by their relocation from the plasma membrane (Boursiac et al., 2005). In addition to this stimulus-dependent relocation of aquaporins, stress-induced changes in their phosphorylation status have been described. Phosphorylation of aquaporins is required for regulation of their gating (Tornroth-Horsefeld et al., 2006). However, in response to salinity stress, specific changes in the C-terminal phosphorylation of AtPiP2;1 were characterized, and these were related to relocation of AtPiP2;1 from the plasma membrane to intracellular compartments (Prak et al., 2008). Abundance of aquaporins, their phosphorylation status, and their subcellular localization in particular developmental stages may act as one of the key determinants of altered salt susceptibility of SIMKK–YFP-overexpressing plants. Obviously, the relevance of aquaporins as potential substrates for MAPK-mediated phosphorylation should be addressed in future studies, as it might be an important aspect of the complex reactions of plants to abiotic stresses. Germin-like protein subfamily 2 member 1 was defined as a protein localized to the plasmodesmata. This protein contributes to the increased plasmodesmata conductance and possibly enhances the symplastic intercellular water transport. The overexpression of SIMKK–YFP in Arabidopsis also led to substantial upregulation of molecular chaperone luminal-binding protein 2, perhaps indicating the demand for accelerated protein folding in these plants. The biological relevance of this phenomenon remains to be substantiated experimentally.

In conclusion, this study has revealed dynamic aspects of MAPK signalling components of intact plants challenged by salt stress and has provided compelling evidence that coordinated and activity-dependent relocations of SIMKK and SIMK are tightly linked with salt stress in plant cells. Overexpression of SIMKK in Arabidopsis plants leads to higher activation of endogenous MPK6 and MPK3 following short-term salt treatment, whilst it results in enhanced sensitivity of these plants to long-term salinity stress. Overall, our study revealed that MAPK signalling is a highly coordinated process in both space and time that is still poorly understood and needs to be investigated in more depth. In addition, our work also indicates that a predictive biological engineering of signalling pathways will require a much better understanding of the full set of protein partners that make up the signalling pathways and interact with each other in a given process.
Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Fluorescence recovery after photobleaching (FRAP) analysis of YFP-tagged SIMKK in transiently transformed *Arabidopsis* protoplasts.

Supplementary Fig. S2. Overview of germination test of *Arabidopsis* plants stably transformed with YFP-tagged SIMKK under salt stress.

Supplementary Fig. S3. Details of germination test of *Arabidopsis* plants stably transformed with YFP-tagged SIMKK under salt stress.

Supplementary Video S1. SIMKK–YFP in *Medicago sativa*, salt stress. This movie shows transiently transformed *M. sativa* root epidermal cell. The SIMKK–YFP is localized to the nucleus and cytoplasmic vesicles exhibiting fast movements. Salt treatment is enhancing relocation of SIMKK–YFP to these cytoplasmic vesicles. The time lapse covers a period of 15 s (accelerated 2.4 times; avi; 26.1 MB).

Supplementary Table S1. Details of protein identification and quantification for biological replicates pertinent for proteomic analysis of roots of SIMKK–YFP transgenic plants and wild-type plants.

Supplementary Table S2. The quantification of significantly differentially abundant root proteins between SIMKK–YFP transgenic plants and wild-type plants.

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