Identification and functional analysis of mitogen-activated protein kinase kinase kinase (MAPKKK) genes in canola (Brassica napus L.)

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Received 23 September 2013; Revised 9 February 2014; Accepted 10 February 2014

Abstract

Mitogen-activated protein kinase (MAPK) signalling cascades, consisting of three types of reversibly phosphorylated kinases (MAPKKK, MAPKK, and MAPK), are involved in important processes including plant immunity and hormone responses. The MAPKKKs comprise the largest family in the MAPK cascades, yet only a few of these genes have been associated with physiological functions, even in the model plant Arabidopsis thaliana. Canola (Brassica napus L.) is one of the most important oilseed crops in China and worldwide. To explore MAPKKK functions in biotic and abiotic stress responses in canola, 66 MAPKKK genes were identified and 28 of them were cloned. Phylogenetic analysis of these canola MAPKKKs with homologous genes from representative species classified them into three groups (A–C), comprising four MAPKKKs, seven ZIKs, and 17 Raf genes. A further 15 interaction pairs between these MAPKKKs and the downstream BnaMKKs were identified through a yeast two-hybrid assay. The interactions were further validated through bimolecular fluorescence complementation (BiFC) analysis. In addition, by quantitative real-time reverse transcription–PCR, it was further observed that some of these BnaMAPKK genes were regulated by different hormone stimuli, abiotic stresses, or fungal pathogen treatments. Interestingly, two novel BnaMAPKK genes, BnaMAPKK18 and BnaMAPKK19, which could elicit hypersensitive response (HR)-like cell death when transiently expressed in Nicotiana benthamiana leaves, were successfully identified. Moreover, it was found that BnaMAPKK19 probably mediated cell death through BnaMKK9. Overall, the present work has laid the foundation for further characterization of this important MAPKK gene family in canola.

Key words: Abiotic stress, Brassica napus, cell death, MAPKKK, MKK, Sclerotinia sclerotiorum.

Introduction

To survive harsh conditions, plants have developed sophisticated mechanisms to sense environmental cues and transmit these signals to regulate plant development and defence. Plant mitogen-activated protein kinase (MAPK) cascades are vital to these functions (Asai et al., 2002; Ichimura et al., 2002; Rodriguez et al., 2010; Meng and Zhang, 2013). MAPK cascades are conserved in eukaryotes through evolution and are composed of MAPKK kinases (MAPKKKs, MAP3K, or...
MEKK), MAPK kinases (MAPKKs, MAP2Ks, MKks, or MEKs), and MAPKs (MPKs). Basically, extracellular stimuli sensed by receptors are sequentially transmitted through phosphorylation by MAPKKKs to MKks, and then to MPKs to regulate intracellular responses including the transcriptional and metabolic responses (Hamel et al., 2006). The activated MAPKKKs phosphorylate either the serine (S) or threonine (T) residues of MKks and the activated MKks phosphorylate both T and tyrosine (Y) residues of the MPKs (Asai et al., 2002).

Several MKK and MPK genes have been well characterized, including a description of their downstream components and the physiological processes they mediate (Frye et al., 2001; Asai et al., 2002; Jin et al., 2002; del Pozo et al., 2004; Liu et al., 2004; Nakagami et al., 2004; Colcombet and Hirt, 2008; Melech-Bonfil and Sessa, 2010; Ning et al., 2010; Oh et al., 2010). However, only a limited number of MAPKK genes have been functionally characterized (Rodríguez et al., 2010; Meng and Zhang, 2013). A possible reason underlying this is that MAPKKs form a large gene family, making functional redundancy inevitable. In Arabidopsis, MAPKKK is the largest group of the MAPK cascade components, with 80 members classified into three subfamilies, MEKK, Raf, and ZIK, harboring 21, 11, and 48 genes, respectively (Jonak et al., 2002). The MEKK subfamily is the best characterized and includes tobacco NPK1 (Jin et al., 2002; Liu et al., 2004), Arabidopsis MEKK1 (Asai et al., 2002), alfalfa OMTK1 (oxidative stress activated MAPK triple-kinase 1, MEKK1) (Nakagami et al., 2004), tobacco NbMAPKKKa, NbMAPKKKγ, and NbMAPKKKe, and tomato SiMAPKα and SiMAPKKKε (del Pozo et al., 2004; Melech-Bonfil and Sessa, 2010; Oh et al., 2010). Characterized genes of the second subfamily of MAPKKKs include Arabidopsis CTR1/Raf1 (Kieber et al., 1993; Clark et al., 1998), EDR1/Raf2 (Frye et al., 2001), and rice (Oryza sativa) DSM1 (Ning et al., 2010). The ZIK subfamily, also called WNK (With No lysine Kinase), is reported to regulate flowering time and circadian rhythms in rice and Arabidopsis (Wang et al., 2008; Kumar et al., 2011).

Once signals are transmitted from MAPKKKs to terminal MPKs through sequential phosphorylation, activated MPKs then phosphorylate a wide range of substrates, such as WRKY transcription factors or enzymes (Rodríguez et al., 2010; Liang et al., 2013). The first identified complete signalling module is the FLS2–MEKK1–MKK4/5–MPK3/6–WRKY22/29 pathway in Arabidopsis, which is effective against different pathogens including bacteria and the fungal pathogen Botrytis cinerea (Asai et al., 2002; Galletti et al., 2011). Another module, MEKK1–MKK1/2–MPK4, of Arabidopsis was shown to regulate defence responses against biotrophic pathogens negatively while positively regulating defences against necrotrophic fungi (Petersen et al., 2000; Ichimura et al., 2006; Qiu et al., 2008). The induction of salicylic acid (SA) as well as systemic acquired resistance (SAR) in mpk4, mekk1, or mkk1mkk2 double mutants is a result of release and activation of WRKY25 and -33 in nuclei by MAPK substrate 1 (MKS1) in Arabidopsis (Andreason et al., 2005; Petersen et al., 2010). Further study found that AtMPK4 could induce camelexin biosynthesis upon challenge by a bacterial pathogen (Andreason et al., 2005; Ichimura et al., 2006; Qiu et al., 2008) but not by a fungal pathogen (Mao et al., 2011). Recently, it was found that AtMEKK1 and AtMKK1/MKK2 negatively regulate MEKK2-mediated immune responses as well as programmed cell death (PCD) (Kong et al., 2012). In tobacco, NPK1–MEK1–NtN6 mediates resistance to Tobacco mosaic virus (TMV) triggered by the R protein N (Jin et al., 2002; Liu et al., 2004). Moreover, AtEDR1, a Raf-like MAPKKK, could function at the top of a MAPK cascade to regulate SA-inducible defence responses negatively (Frye et al., 2001). However, the functions of most other MAPKKK genes in Arabidopsis are as yet unknown.

Though investigations of the MAPKKK gene family in Arabidopsis, rice, and maize have been reported in recent years (Jouannic et al., 1999; Singh et al., 2012; Kong et al., 2013), no similar study has yet been conducted in canola. Canola is a very important oil crop worldwide and its yield is frequently limited by environmental factors including drought, salinity, cold, and biotic factors, such as stem rot caused by Sclerotinia sclerotiorum. Sclerotinia sclerotiorum is a necrotrophic pathogen and no efficient way has been identified to control this disease. An oxidative burst, or accumulation of reactive oxygen species (ROS), has been associated with many abiotic stresses and pathogen infections (Jaspers and Kangasjarvi, 2010; Heller and Tuzdysynski, 2011), especially S. sclerotiorum (Rietz et al., 2012). Research with MAPK cascades in Arabidopsis has shown that they are involved in signalling multiple defence responses, including the biosynthesis and signalling of plant stress/defence hormones, ROS production, stomatal closure, defence gene activation, phytoalexin biosynthesis, cell wall strengthening, and hypersensitive response (HR) cell death (Meng and Zhang, 2013). However, the identity and role of MAPKKKs in canola responses to abiotic and biotic stresses are unknown. It is therefore necessary to characterize the MAPKKK gene family in canola before stress/disease-tolerant canola species can be developed. In previous transcriptional profiling studies in canola, a few components of the MAPK module were identified, including several MAPKKK genes elicited by S. sclerotiorum (Yang et al., 2007). A few novel BnaMKK (Bna for Brassica napus) and BnaMPK genes and modules were further characterized (Liang et al., 2013). The functions of some of the MKK, MPK, and WRKY genes are under investigation through loss-of-function and gain-of-function strategies. However, the upstream components of MKK–MAPK modules, which are BnaMAPKKKs, have not yet been characterized. Hence, to explore the role of MAPKKK genes in immune and abiotic stress responses in canola, the publicly available expressed sequence tags (ESTs) were mined to identify MAPKKK genes in canola. The cDNA sequences of 28 BnaMAPKKK genes were then cloned, followed by a yeast two-hybrid (Y2H)-based analysis of interactions between canola MAPKKKs and MKKs, and part of the interactions were confirmed in planta. The responses of selected genes under a range of abiotic and biotic stress conditions were also examined. Interestingly, two novel BnaMAPKK genes that could elicit cell death when transiently expressed in tobacco leaves were
successfully identified. These may mediate cell death by regulating specific downstream MKKs. To the authors’ knowledge, this is the first report of canola MAPKKK genes, and the data presented here will lay the foundation for further characterization of this important MAPKKK gene family in canola responses to abiotic and biotic stresses.

Materials and methods

Database search and identification of MAPKKK ESTs in canola

The identification of canola ESTs representing MAPKKK genes was performed as described previously (Liang et al., 2013). In brief, ESTs representing MAPKKKs were retrieved through BLAST search in the NCBI dbEST (http://www.ncbi.nlm.nih.gov/dbEST/index.html, release 01012012) and DFCI (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=oilseed_rape,release5.0) using the 80 public AitMAPKKK cDNA sequences downloaded from TAIR9.0 (www.arabidopsis.org) as the queries with an e-value cut-off <10-4. After manual curation, these ESTs were clustered and assembled by DNASTAR (DNASTAR Inc., USA). Subsequently, contigs and singletons were run in a reciprocal BLAST search against the Arabidopsis database to assign a putative orthologue based on the best hit (Supplementary Table S1 available at JXB online).

Plant growth and gene cloning

Canola (double haploid DH12075) plants were grown as described previously (Liang et al., 2013). RNA was isolated using the Plant RNA kit (Omega bio-tek, USA). First-strand cDNA synthesis and high-fidelity PCR amplification using PrimeSTAR HS DNA polymerase (TaKaRa, Japan) were performed as previously described (Liang et al., 2013) with the primers listed in Supplementary Table S2 available at JXB online. PCR products were purified and cloned into the pJET1.2 vector supplied in the CloneJET PCR cloning kit (Fermentas, USA), sequenced, and analysed by DNASTAR. The cDNA sequences of genes cloned in this study were deposited into the GenBank database under the accession numbers KC190095–KC190110 and KF129395–KFI29406.

Phylogenetic tree reconstruction, multiple alignment analysis, and conserved signature detection

The MAPKK genes of rice were downloaded from the rice genome annotation project (http://rice.plantbiology.msu.edu), while those of other species were either identified from Phytozome v9.1 (http://www.phytozome.net/) or retrieved from the NCBI by a keyword search (Supplementary Table S3 available at JXB online). To investigate the evolutionary relationship among MAPKKK genes, the predicted amino acid sequences of the products of the MAPKKK genes of canola and other species were aligned and a phylogenetic tree was reconstructed as described previously (Liang et al., 2013). Motif analysis of BnaMAPKKKs was determined by using the Prosite program (http://prosite.expasy.org/prosite.html), and a schematic diagram of amino acid motifs of each BnaMAPKKK was drawn accordingly. The respective domains of MAPKKK proteins were aligned using ClustaX1.83 and analysed in MEME 4.9.0 (release date 3 October 3, 11:07:26 EST 2012) or illustrated by Boxshade (http://www.ch.embnet.org/software/BOX_form.html). The percentage identity and similarity of sequences was calculated using the program MatGAT v2.02 (Campanella et al., 2003).

Subcellular localization and confocal microscopy

To examine the localization of selected BnaMAPKKKs in planta, the coding regions were amplified using Pfu polymerase (Bioer, China) with the primers listed in Supplementary Table S2 available at JXB online. These were digested by the corresponding restriction enzymes and fused upstream of the green fluorescent protein gene (GFP) in the pYJGFP vector. These constructs and p19 protein of Tomato bushy stunt virus were transformed into Agrobacterium tumefaciens GV3101 individually, and overnight cell cultures were resuspended in infiltration media before being infiltrated into 5-week-old leaves of Nicotiana benthamiana (Liang et al., 2013). Two days later, leaf discs were plasmolyzed with 500mM mannitol or not treated, and observation of GFP was conducted under an AIR confocal microscope (Nikon, Japan).

Quantitative reverse transcription–PCR (qRT–PCR) assay

Eighteen-day-old canola grown in a greenhouse with a photoperiod of 16h light/8h dark was treated with S. sclerotiorum inoculation, 5mM oxalic acid (OA; Sigma-Aldrich) inoculation, and agar inoculation as a control for S. sclerotiorum and OA treatment. Different chemical treatments include 200mM NaCl, 100 μM jasmonic acid (JA; Sigma-Aldrich, USA), 2mM SA (Sigma-Aldrich), 50 μΜ abscisic acid (ABA; Invitrogen, USA), 25 μM 1-aminoacyclopropane-1-carboxylic acid (ACC; Sigma-Aldrich), 10 μM methyl viologen (MV; Sigma-Aldrich); alternatively, plants were treated at 4 °C (cold) and 37 °C (heat), and mock-treated plants were used as the control (Liang et al., 2013). Leaves were collected at 1h (except S. sclerotiorum and OA, which were collected after 3h) and 24h post-treatments, flash frozen in liquid nitrogen, and stored at –80 °C. Total RNA samples were isolated and the first-strand cDNAs were synthesized from 2.5 μg of total RNA as described previously (Liang et al., 2013). Three independent biological replicates of each sample were prepared at different times. qRT–PCR was performed using 10-fold diluted cDNA and a SYBR Green I kit (CWBio, China) on a CFX96 real-time PCR machine (Bio-Rad, USA). Primers used for qRT–PCR were designed using the PrimerSelect program (DNASTAR Inc.), which was targeted mainly at the 3’-untranslated region (UTR) with an amplicon size of 75–250bp (Supplementary Table S2 available at JXB online). The specificity and amplification efficiency of each pair of primers were examined through both a BLASTn search in the NCBI database and by running standard curves with melting curves. Three independent biological replicates and two technical replicates for each biological replicate were run and the significance was determined through t-test of SPSS statistical software (P<0.05).

Yeast two-hybrid assay

The coding regions of canola MAPKK and M KK genes were subcloned into pGBK T7 (BD) and pGAD T7 (AD) vectors, respectively, using the primers listed in Supplementary Table S2 available at JXB online. Then recombinant plasmids were transformed sequentially into yeast AH109 competent cells according to the Yeast Protocols Handbook (Clontech, USA). The interactions between BnaMAPKKKs and BnaM KKs were tested by streaking both on non-selective SD-LW (synthetic dropout without leucine and tryptophan), and on selective SD-LW–His (+5 mM 3’AT) and SD-LWHA (–SD-Leu-Trp-His-Ade) media. Plates were incubated at 30 °C for up to 7 d before being photographed. The titration and colony-lift filter assays were conducted as described previously (Liang et al., 2013).

Bimolecular fluorescence complementation (BiFC)

To verify interaction partners in planta, yellow fluorescent protein (YFP)-based BiFC analysis was performed. The coding region of BnaMAPKKK and BnaM KK genes were subcloned into pSPYNE(R)173 and pSPYCE(M) vectors, respectively (Waad et al., 2008). Primers used are listed in Supplementary Table S2 available at JXB online. The recombinant plasmids were transformed into Agrobacterium GV3101 competent cells before being used to infiltrate the leaves of 5-week-old N. benthamiana plants as described previously (Liang et al., 2013). Three to four days later, YFP signals in at least three slides were examined under an AIR confocal microscope (Nikon, Japan).
Site-directed mutagenesis

The coding regions of relevant BnaMAPKKK and BnaM KK genes were PCR amplified using Pfu polymerase and Gateway-compatible gene-specific primers (Supplementary Table S2 available at JXB online) before being introduced into a Gateway entry vector pDONR/Zeo (Invitrogen, USA). For substitution of one amino acid residue with another, two overlapping primers harbouring mutated nucleotides in the middle were used to run PrimeSTAR-mediated PCR (Li and Wilkinson, 1997). The BnaM3K18-K32G-F/BnaM3K18-K32G-R and BnaM3K19-K37G-F/BnaM3K19-K37G-R primers were used to change the lysine (K) at residues 32 and 37 to glycine (G), respectively (Melech-Bonill and Sessa, 2010; Hashimoto et al., 2012). Primers BnaMKK9-K74R-F and BnaMKK9-K74R-R were designed to mutate lysine (K) at residue 74 to arginine (R), while primers of BnaMKK9-S193D/S199E-F and BnaMKK9-S193D/S199E-R were designed to mutate serine (S) on residues 193 and 199 to aspartic acid (D) and glutamic acid (E) individually. BnaMKK9K74R and BnaMKK9S193D/S199E are constitutively inactive and active forms of BnaMKK9, respectively (Popescu et al., 2009). PCR products were purified, followed by DpnI (Fermentas, USA) digestion overnight. After purification, the restricted PCR product was transformed into Escherichia coli DH5α competent cells and was selected on low-salt LB medium supplemented with 50 μg ml⁻¹ zeocin, with the plasmid isolated and sequenced to confirm that the mutated regions were correct.

Transient expression and physiological assay

The coding regions of the respective genes and their mutated derivatives were isolated by restriction digestion of the aforementioned pJET or pDONR/Zeo recombinant plasmids, which was sometimes preceded by PCR amplification using high-fidelity Pfu polymerase and primers containing corresponding restriction sites, as listed in Supplementary Table S2 available at JXB online. After digestion, the products were inserted downstream of a double Cauliflower mosaic virus (CaMV) 35S promoter in the binary vector pYBHA or pYB-Myc, which was modified from the pYGFP vector (Li et al., 2013). Recombinant plasmids were transformed into A. tumefaciens GV3101 and overnight cell cultures were resuspended in infiltration media containing 10 mM MES-KOH (pH 5.6), 10 mM MgCl₂ and 0.15 mM acetosyringone, adjusted to an OD₆₀₀ of 0.5 before equal volumes of each cell culture were infiltrated into the lower epidermal side of 4-week-old leaves of N. benthamiana plants. For each construct, 21 independent leaves of seven independent plants (three leaves per plant) were used for each time point tested. After that, infiltrated plants were kept under normal growth conditions with the phenotype observed and recorded daily beginning 2 d after infiltration and continuing until day 7. To quantify the degree of PCD, electrolyte leakage was measured according to Oh and Martin (2011) with modifications. In brief, five leaf discs (10 mm in diameter) were taken from each agro-infiltrated area and kept in deionized water under vacuum for 10 min, followed by incubation for 30 min at 25 °C. Ion conductivity was measured using a DDS-307 ion conductivity meter (Leici, China). After boiling for 5 min and cooling to room temperature, the ion conductivity was measured again. Distribution of hydrogen peroxide (H₂O₂) was detected by 3,3'-diaminobenzidine (DAB) staining according to Daudi et al. (2012).

Results and Discussion

Identification and cloning of MAPKKK genes from canola

Although the functions of a few MAPKKK genes in Arabidopsis and a few other plant species have been reported, little is known about this gene family in the important oilseed crop, canola (B. napus). A previous transcriptomic study identified several pathogen- or defence hormone-responsive MAPKKK genes, including BnaMAPKKK17 and BnaMAPKKK18 (Yang et al., 2007), suggesting that MAPKKKs may play a role in canola defence against fungal pathogens. More recently, as a follow-up work, MKK and MAPK genes, as well as the downstream WRKY transcription factor genes in canola were systemically studied and part of them were characterized (Yang et al., 2009; Liang et al., 2013). Ongoing work with selected components of MKK–MPK–WRKY cascades in canola demonstrated interesting phenotypes of gain- and loss-of-function plants (unpublished data). However, the upper components of MAPK cascades, namely MAPKKKs, have not yet been described in canola. All of these prompted the authors to clone and study them in the context of abiotic and biotic stress conditions.

To this end, public EST databases were mined, since whole-genome sequencing of B. napus is not yet complete. Eighty cDNA sequences of AtMAPKKK genes were used to search for ESTs of canola that showed high similarity to AtMAPKKK genes. Altogether 839 unique ESTs were obtained including 80 singletons and 145 contigs representing putative MAPKKK genes in canola (Supplementary Table S1 available at JXB online). To facilitate comparisons between species, the established nomenclature of AtMAPKKK genes was followed when naming the BnaMAPKKK (Brassica napus MAPKKK) genes. As a result, 66 MAPKK genes from canola were identified, which are composed of 18 MAPKK genes, nine ZIK genes, and 39 Raf genes. It was noted that among all the BnaMAPKK genes identified, BnaRaf28 has the largest number (73) of ESTs, followed by BnRaf22 and BnRaf21 with a total of 63 and 51 ESTs, respectively, while BnaMAPKKK2, BnaMAPKKK19, ZIK3, ZIK7, Raf2, and Raf7 have only one EST each (Table 1; Supplementary Table S1 available at JXB online). To facilitate the following work, primers were designed based on the identified ESTs to obtain full-length cDNA sequences, at least for the coding regions, employing RT–PCR together with rapid amplification of cDNA ends (RACE). As a result, the cloning of cDNA sequences of 28 BnaMAPKK genes was achieved (Table 1). Conceptual translation of these cDNA sequences and reciprocal BLAST searches against the Arabidopsis genome indicated that they bore domains and motifs that were typical of MAPKKK proteins. The number of amino acids in each BnaMAPKKK protein ranged from 337 to 1062, with a pI of 4.62–9.42. A previous report identified three MAPKKK genes (MAP3Kα1, MAP3Kβ1, and MAP3Kε1) from B. napus (Jouannic et al., 1999), which corresponds to BnaMAPKKK3, -6, and -8, respectively (Supplementary Table S1 available at JXB online), and they were added to the analysis. Further comparison of these 31 BnaMAPKKK genes with their 31 respective orthologous AtMAPKKK genes demonstrated that they show an identity of 14.2–91%, with a similarity of 14.2–91.5% at the nucleotide level. At the protein level, the maximum identity is 95.2% with a minimum of 6.6%, whereas the similarity ranges from 10.5% to 97.7% (Supplementary Table S4 available at JXB online). However, a comparison of BnaMAPKKKs with all the 75 OsMAPKKs showed identity of 6.6–71.7%, with a similarity of 6.6–72.9% at the nucleotide level. At the protein level, the maximum identity is 74.6% with the minimum
Table 1. MAPKK genes identified and cloned from canola

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<th>pI value</th>
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3.8%, whereas the similarity ranges from 5.6% to 87.8% (Supplementary Table S4 available at JXB online). At this step, it is possible to identify putative orthologues of these BnaMAPKKK genes in both Arabidopsis and rice using the program InParanoid (Table 1).

Brassica napus is an amphidiploid species with an AACC genome (2n=38), which is presumably derived from interspecific hybridization of Brassica rapa (2n=20, AA) and Brassica oleracea (2n=18, CC) (Rana et al., 2004). Although partial genome sequencing and comparative chromosome painting suggest that ancestral segmental chromosomal duplications led to effective triplication in Brassica diploids (Lysak et al., 2005, 2007), various mechanisms of genome evolution have contributed to many situations where fewer than three paralogous genes, corresponding to single orthologues in Arabidopsis, are present in the Brassica A or C genome (Osborn, 2004; Parkin et al., 2005). MAPKKK sequences from the sequenced genome of B. rapa were therefore analyzed. As a result, 118 MAPKKK genes were identified from B. rapa (Supplementary Table S3 available at JXB online), which supports that there are not necessarily three paralogous genes existing in the AA genome of B. rapa compared with that in Arabidopsis. Since the CC genome of B. oleracea is not yet known, there is no way to determine the exact number of MAPKKK genes in B. napus. However, since a double haploid canola variety was used, it should greatly facilitate the identification and functional characterization of MAPKKK genes.

Phylogenetic tree reconstruction and domain analysis of BnaMAPKKK proteins

To examine the evolutionary relationships of canola MAPKKKs to other representative crops and models, a rooted phylogenetic tree was produced by alignment of full-length amino acid sequences using a maximum parsimony (MP) algorithm. The species used represented the major land plant lineages including several mono- and eudicotyledonous
angiosperms, namely: eudicots A. thaliana (At), B. rapa (Br), N. benthamiana (Nb), and Solanum lycopersicum (Sl); monocot O. sativa (Os); brephyte Physcomitrella patens (Pp); lycophyte Selaginella moellendorfii (Sm), as well as the pico-eukaryotic prasinophyte green alga Ostreococcus lucimarinus, which has one of the highest gene densities known in eukaryotes (Lanier et al., 2008). Furthermore, an MAPKKK from a marine green alga Ostreococcus tauri (Ot), the world’s smallest free-living eukaryote, has also been identified and was used to root the tree (Fig. 1; Supplementary Fig. S1, Supplementary Table S3 available at JXB online). It can be seen that most of the characterized MAPKKKs from other species including NbMAPKKK, NbMAPKα, LeMAPKα, NbMAPKκ, SIMAPKκ, OsNK1, and OsCDR1 were clustered within the MEKK subfamily and the others were clustered into the Raf subfamily (Supplementary Fig. S1 available at JXB online).

Moreover, the presence of a smaller set of MAPKKK members in the green alga O. lucimarinus and primitive land plants including P. patens and S. moellendorfii compared with eudicots and monocots indicated that recent genome duplications may have caused expansion of the MAPKKK gene family during the evolution of the angiosperms, which is especially evident within the Raf subfamily (Rao et al., 2010). This is further supported by the fact that the lower plants S. moellendorfii and P. patens have only 38 and 61 MAPKKK genes, respectively (Supplementary Table S3 available at JXB online), which is much fewer than found in higher plants such as Arabidopsis or rice, which have 80 and 75 members, respectively (Rao et al., 2010).

On the basis of the above phylogenetic analysis, the 31 BnaMAPKKKs could be divided into three major groups, namely groups A(1–4), B(1–4), and C(1–7), which are each supported by significant bootstrap values (Fig. 1; Supplementary Fig. S1 available at JXB online). This is in agreement with the conclusion reached with 60 Arabidopsis MAPKKKs (Ichimura et al., 2002; Jonak et al., 2002). In total, there were four BnaMAPKKKs in the MEKK subfamily, while there were seven in the ZIK subfamily and 17 in the Raf subfamily among the 28 genes cloned (Fig. 1; Supplementary Fig. S1 available at JXB online). It was found that BnaMAPKKK17, -18, -19, and -20 and BnaZIK2, -3, -4, and -8 are in group A, whereas the 17 cloned BnaRaf genes are distributed in the B and C groups. For example, BnaRaf35 and BnaCTR1 are in group B, while BnaRaf17, -21, -22, -23, -27, -28, -29, -30, -33, -34, -36, -37, -39, -41, and -46 belong to group C (Fig. 1). It was also observed that in group A, only A1–A4 have been assigned to AtMAPKKKs, as only 60 AtMAPKKK genes were used to infer the phylogenetic relationship. The remaining 20 novel AtMAPKK genes, including 11 AtZIK genes (from AtZIK1 to AtZIK11) and nine more AtMAPKKK genes (from AtMAPKKK13 to AtMAPKKK21) were not included in the previous phylogenetic analysis, nor was any specific subgroup assigned. Based on the analysis presented here, it is proposed to assign AtMAPKKK13–AtMAPKKK21 to subgroup A4, since MAPKKKs within each subgroup were clustered together and were supported by high bootstrapping values (Fig. 1; Supplementary Fig. S1 available at JXB online). It is noted that orthologous pairs of MAPKKKs between Arabidopsis and canola, and between canola and B. rapa were clustered in the same subclades of the phylogenetic tree, indicating a higher identity/similarity between them (Fig. 1; Supplementary Fig. S1 available at JXB online). Reciprocal Blast analysis and sequence comparison also indicates that the 28 BnaMAPKKK genes cloned are paralogous and are not homeologous genes.

Conserved domains and motifs within BnaMAPKKK proteins were further examined. As reported with Arabidopsis MAPKKK proteins, the characteristic of the MEKK subfamily in canola includes a conserved kinase domain of G(T/S)Px(W/Y/F)MAPEV. The BnaZIK subfamily has GTPEFMAPE(L/V)Y while the BnaRaf-like subfamily has GTxxx(W/Y)MAPE (Supplementary Figs S2, S4 available at JXB online) (Jonak et al., 2002). Analysis of the domain architectures of BnaMAPKKKs revealed that most of the 17 BnaRafs have a kinase domain in the C-terminus and a long regulatory domain in the N-terminus. In contrast, eight BnaZIKs have a kinase domain at the N-terminus. As for the four BnaMAPKKKs reported here, the kinase domain is located in the N-terminus (Fig. 1), which is consistent with observations in Arabidopsis and rice (Jouannic et al., 1999; Ichimura et al., 2002; Rao et al., 2010). It is proposed that the long regulatory domain in the N-terminus of the Raf subfamily may function to interact with other proteins and hence to regulate or specify their kinase activity (Jouannic et al., 1999).

A careful examination of orthologous MAPKKK17, -18, -19, and -20 genes between Arabidopsis and canola showed that they had a GxGxxS/AxV motif instead of the typical sequence GxGxxGxV in subdomain I, which was also the ATP active site (Supplementary Fig. S2A, B available at JXB online). These could be assigned to the A4 subgroups as they existed as an independent subclade within the group A (Supplementary Fig. S1 available at JXB online). In addition, the ATP-binding region signature within the kinase domain of BnaMAPKKKs in subgroup A4 was totally different from that of other members in MEKK, Raf, or even the ZIK subfamilies (Supplementary Fig. S2 available at JXB online).

The ZIK or WNK subfamily genes, with 10 and nine members in Arabidopsis and rice, respectively, are reported to regulate flowering time and circadian rhythms in rice and Arabidopsis (Wang et al., 2008; Kumar et al., 2011). However, AtZIK8 was not considered to belong to any of the groups in WNK. The protein kinase ATP-binding region signature of the ZIK subgroup was therefore inspected; this is (L/I)GXG(A/S) (F/V/S)XXXX(G/A)X(5–18)AVK of Motif 1. The second lysine (K) in domain II was replaced by an asparagine (N) residue, while it is serine (S) in BnaZIK2 (Supplementary Fig. S3A available at JXB online), which was consistent with previous findings in rice and Arabidopsis (Wang et al., 2008; Kumar et al., 2011). In OsWNK2, -3, -7, and -8, R is present in place of K, while in OsWNK4 and -9, S is present in place of K, and G replaces K in OsWNK6 (Kumar et al., 2011). The missing K residue in domain II in the ZIK subgroup, originally responsible for the coordination of ATP in the active centre, was replaced by a K residue in subdomain I (Xu et al., 2000). It was also noted that there is a 60 amino acid region at the C-terminus of BnaZIKs, which is likely to be the auto-inhibitory domain (Supplementary Fig. S3A available at JXB online). This is similar to the Arabidopsis orthologues (Wang et al., 2008).
Furthermore, from the phylogenetic study of ZIKs, it was noted that they could be clustered into four clades or subgroups, with each containing ZIKs from both monocots and eudicots (Supplementary Fig. S3C available at JXB online), suggesting that about four ZIK ancestral genes existed before the split of monocots and eudicots. As multiple ZIK genes exist in higher plants such as *Arabidopsis*, rice, and canola, this suggests a relatively recent duplication of ZIK genes (Wang et al., 2008). In addition, it was obvious that AtZIKs and BnaZIKs were always clustered together, suggesting a higher similarity of orthologous pairs between *Arabidopsis* and canola, the two representative species of the Brassicaceae family. A previous study identified that plant and animal WNKs form completely different groups, indicating divergence from a common ancestor (Wang et al., 2008). ZIK/WNK genes do not exist in either yeast or bacterial genomes but exist in some other unicellular eukaryotic genomes such as oomycetes and diplomonads, indicating the origin of early eukaryotes (Wang et al., 2008). Moreover, the monophyletic clade for eudicots indicates the same ancestor before splitting of the monocots and eudicots, while a polyphyletic group may suggest that more than one gene existed before the splitting of the monocots and eudicots (Wang et al., 2008).

From a multiple alignment of the Raf subfamily of canola MAPKKKs, it was inferred that BnaRaf5s contain a conserved [RK][IV][GXS][SF][FY][G][TE][V][KRH][GA][X][WF][HFN] G sequence, which is the signature sequence of subdomain I and also discriminates them from the other MAPKKKs (Supplementary Fig. S4 available at JXB online) (Jouannic et al., 1999). It was noted that an ACT domain is present...
only in BnaRaf21, -29, and -30, and this domain is reported to be involved in amino acid metabolism or protein–protein interaction (dimerization) (Feller et al., 2006); however, the significance of it in Raf protein function awaits experimental study. The presence of an ACT domain in some Raf kinases was also observed in rice (Rao et al., 2010). It was also identified that BnaRaf23, -27, and -46 have three ankyrin (ANK) repeats in the N-terminus while BnaRaf17 has only one (Supplementary Fig. S4 available at JXB online). The ANK repeat is one of the most common protein–protein interaction motifs and has been found in proteins with diverse functions, such as transcriptional initiators, cell cycle regulators, the cytoskeleton, ion transporters, and signal transducers (Sedgewick and Smerdon, 1999). In Arabidopsis, there are 105 predicted ANK repeat-containing proteins, and most of them are transmembrane proteins (Becerra et al., 2004). Since no interactions were detected between BnaRaf23, -27, -46, and eight BnaMKKs (see below), they may interact with some of the unidentified BnaMKKs or other unknown proteins. Another feature of the Raf proteins in plants is the existence of subdomain VIII, which is GTXX(W/Y)MAPE or [LIM]X[SD]X[ST]X[AK]GTP[EQ]W (Juannic et al., 1999) (Supplementary Fig. S4 available at JXB online); however, the conservation of amino acid residues within this subdomain is limited, except at a few sites, as shown by the MEME analysis (Supplementary Fig. S4B available at JXB online).

Through the aforementioned phylogenetic analysis, multiple alignments and domain analysis of BnaMAPKKKs in canola, it was concluded that MAPKKK genes are ancestral and conserved from lower to higher plants. The classification and function of members of this important gene family may be rather conserved between monocots and dicots. Compared with other species, such as O. lucimarins, P. patens, and S. moellendorfii, the increasing number of MAPKKK genes is probably due to evolutionary events such as genome duplication or expansion. Taken together, during the long history of evolution, the MAPK signalling cascades were relatively conserved, though subfunctionalization or neofunctionalization may have resulted in differences in gene function in specific species.

Subcellular localization of canola MAPKKK proteins

To investigate further the function of the 28 BnaMAPKKK genes cloned, the localization of the encoded proteins was first predicted by using PSORT (http://psort.hgc.jp/), CELLO v2.5 (http://cello.life.nctu.edu.tw), and ESLPred (http://www.imtech.res.in/raghava/eslpred/index.html). It was found that most of the BnaMAPKKKs were predicted to be localized to the nucleus, cytoplasm, or plasma membrane, with the exception of BnaRaf17, -28, -29, and BnaZIK2, which were also present in the cytoskeleton and chloroplast, respectively (Supplementary Table S5 available at JXB online). For instance, BnaRaf17 was predicted to be localized to chloroplast stroma, microbody (peroxisome), chloroplast thylakoid membrane, and chloroplast thylakoid space, while BnaRaf28 was localized to the endoplasmic reticulum (membrane), plasma membrane, chloroplast thylakoid membrane, and mitochondrial inner membrane. BnaRaf29 was predicted to sit in the cytoplasm, microbody (peroxisome), chloroplast, stroma and chloroplast thylakoid membrane. Transmembrane helices (TMH) of these BnaMAPKKKs were also predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/); however, no TMH was identified for any of the 28 BnaMAPKKK proteins (data not shown), suggesting that none of these proteins is a transmembrane protein.

To investigate further the subcellular localization of canola MAPKKK proteins, selected genes representing the three subfamilies of BnaMAPKKKs, namely BnaMAPKKK18, -19, -20, BnaZIK2, and -4, as well as BnaRaf17, -28, -29, -30, -34, and -36 were expressed as fusion proteins with GFP in N. benthamiana. The recombinant plasmids were transformed into agrobacteria and then infiltrated into the lower epidermal leaves of N. benthamiana, with the GFP signals observed 2 d later. It was found that in leaf cells harbouring the fusion proteins of BnaMAPKKK18, -19, and -20, BnaZIK2 and -4, and BnaRaf17, -28, -29, -30, -34, and -36, the GFP signals were present in the cytoplasm and nucleus (Fig. 2). For BnaZIK2, the signals were observed in the chloroplast as well as in the cytoplasm and nucleus, while for BnaRaf30, the GFP signals were only present in nuclei. As a control, the subcellular localization of the GFP protein was also examined in tobacco leaf cells, and green signals were obviously present in both the cytosol and nuclei (data not shown). Leaf discs from the agroinfiltrated plants were further treated with a high osmotic solution (500 mM mannitol) for 1 h, and the GFP signals were observed again under the same confocal settings. As shown, most of the BnaMAPKKKs tested were still localized in both the cytoplasm and nuclei, except BnaRaf30 (Fig. 2). The in planta demonstration of the subcellular localizations of BnaMAPKKKs is expected to reflect more accurately the natural subcellular localization of these proteins, as compared with the in silico predictions.

Identification and validation of BnaMAPKKK and BnaMKK interactions

To explore the function of MAPKKK genes in canola, it is essential to identify their direct MKK targets. Therefore, a Y2H assay was performed to identify the BnaMKKs interacting with each of the 28 BnaMAPKKKs. To this end, the coding regions of 28 BnaMAPKK and eight BnaMKP genes were first cloned into the GAL4-BD bait and GAL4-AD prey vectors, respectively. The autoactivation activity of each of the 28 BnaMAPKKK proteins in yeast was then tested, and it was found that BnaMAPKKK18, BnaZIK3, BnaZIK4, and BnaRaf35 showed evident autoactivation activity (data not shown). To solve this issue, the coding regions of BnaMAPKKK18, BnaZIK3, BnaZIK4, and BnaRaf35 were fused in-frame with the GAL4-AD domain whereas those of the eight BnaMKK genes were fused with the GAL4-BD domain. Thirdly, individual BnaMAPKKK–BnaMKK pairs were co-transformed into yeast cells, with colonies tested on both selective media, followed by titration and β-galactosidase activity assay to examine the strength and genuineness of interactions.
As a result, 22 pairs of interactions were identified, and six of the BnaMKK proteins were found to interact with at least one BnaMAPKKK protein in the Y2H assay, while BnaMKK1 and BnaMKK4 did not interact with any of the 28 BnaMAPKKK proteins assayed (Table 2; Supplementary Fig. S5 available at JXB online), suggesting

Fig. 2. Subcellular localization of BnaMAPKKK proteins in N. benthamiana cells using green fluorescent protein (GFP). The six panels represented BnaMAPKKK18, -19, -20, and BnaRaf17, -28, and -29, respectively, under normal and mannitol treatment (500 mM for 1 h). The red arrows indicate the nuclei or dots of cytosol in the focused cells. In each panel, the extreme left is GFP fluorescence, the middle bright field, and the right an overlay of the two images. Scale bar=50 μm.
that these two MKK proteins may be transducer signals from other upstream MAPKKK proteins. There were six BnaMAPKKKs, BnaMAPKKK17, -18, and -20, BnaZIK3 and -4, and BnaRaf35, that interacted with BnaMKK3; six BnaMAPKKKs, BnaMAPKKK17, -19, -20, BnaZIK2, -9, and BnaRaf28, with BnaMKK9; three BnaMAPKKKs, BnaMAPKKK17, -18, and -20, BnaZIK2, -9, and BnaRaf28, with BnaMKK5; and five BnaMAPKKKs, BnaMAPKKK17, -19, -20, BnaZIK2, -9, and BnaRaf28, with BnaMKK8. As regards BnaMKK2 and -6, only BnaRaf28 interacted with both of them (Table 2).

Parts of the protein–protein interactions identified in the Y2H assays were confirmed using the BiFC procedure in plant cells. The presence of yellow fluorescence signals showed that BnaMAPKKK17, -19, -20, BnaZIK2, -9, and BnaRaf28 interacted with BnaMKK9; BnaRaf28 with BnaMKK6; BnaZIK2 and BnaRaf28 with BnaMKK5; BnaMAPKKK17, -20, and BnaZIK4 with BnaMKK3; and BnaRaf28 with BnaMKK2 in the epidermal cells of N. benthamiana (Supplementary Fig. S6 available at JXB online). The respective negative controls (target proteins fused to half of YFP co-expressed with the other half of the YFP) did not yield detectable YFP signals (Supplementary Fig. S6 available at JXB online). Taken together, the identification of BnaMAPKKK–BnaMKK pairs provided useful information to dissect their function in canola response to abiotic and biotic stresses.

Since MAPKKKs usually exert their function through phosphorylation of downstream MKK proteins, co-localization of interacting MAPKK and MKK proteins is normally expected for signal transduction. Therefore, the in vivo localizations of pairs of interacting BnaMAPKKKs and BnaMKKs was compared, including Raf28–MKK2, MAPKKK18–MKK3, MAPKKK20–MKK3, and ZIK4–MKK3. The subcellular localization of MKK2 and MKK3 was previously shown to be the cytoplasm and nucleus (Liang et al., 2013). The co-localization of MKK2 and MKK3 with BnaMAPKKKs probably facilitates their interactions in the cytoplasm as well as the nuclei (Fig. 2; Supplementary Fig. S6 available at JXB online). Hence, the co-localization of BnaMAPKKK–BnaMKK interaction pairs facilitates their interaction and phosphorylation to mediate timely responses to external and internal stimuli. However, it should be noted that even if two proteins are identified to interact in a Y2H assay or after co-expression in plants, they may not necessarily do so under natural conditions, as a result of differences in spatiotemporal expression patterns.

### Table 2. Summary of yeast two-hybrid assay of interactions between BnaMAPKKK and BnaMKK proteins

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The interaction strength was scored visually, from no interaction (–) to strong interaction (++). BiFC indicates interactions confirmed through bimolecular fluorescence complementation in planta.
Expression analysis of BnaMAPKK genes in response to stress treatments

To elucidate of the functions of BnaMAPKK genes in the context of abiotic and biotic stresses, the expression patterns of 16 selected BnaMAPKK genes, including four MAPKK genes, four ZIK genes, and eight Raf genes, were studied using qRT-PCR. Among the stress treatments applied, ABA is a well-known abiotic stress hormone, and JA, ethylene, and SA are well-known defence hormones, while MV can trigger ROS burst in plant cells. OA, on the other hand, is a pathogenicity factor produced by S. sclerotiorum, and can elicit PCD and suppress the oxidative burst of host plants (Cessna et al., 2000; Guimaraes and Stotz, 2004; Kim et al., 2008). Canola seedlings were subjected to moderate stress treatments at two time points to better monitor the transcript changes of the investigated BnaMAPKK genes. Data of three independent biological replicates were subjected to statistical analysis to identify BnaMAPKK genes responsive to one or a combination of stress conditions (Fig. 3; Supplementary Fig. S7A available at JXB online). It was found that JA modulated expression of five genes, among which BnaRaf30 was induced while BnaZIK3, BnaZIK4, BnaRaf36, and BnaRaf39 were repressed at one or both time points tested. Five genes, BnaMAPKKK18, BnaZIK3, BnaZIK8, BnaRaf29, and BnaRaf36, were responsive to ACC, among which BnaZIK8 and BnaRaf29 were up-regulated while BnaMAPKKK18, BnaZIK3, and BnaRaf36 were down-regulated. SA significantly induced BnaMAPKKK18 and BnaRaf28 while it repressed BnaZIK2, BnaRaf34, and BnaRaf36 at specific time points. Expression of BnaMAPKKK17, BnaRaf34, and BnaRaf36 was down-regulated by MV treatment at one time point tested. ABA seemed only to repress BnaZIK3 expression and exerted no effect on the transcription of the other genes. On the other hand, salinity induced BnaMAPKKK20 and BnaRaf36 at the 1 h and 24 h time points, respectively, whereas it down-regulated BnaMAPKKK20 (24 h), BnaZIK3, BnaZIK8, and BnaRaf17 (1 h and 24 h), as well as BnaRaf29 and BnaRaf34 (1 h). Seven genes were affected by heat treatment, among which the transcript levels of BnaMAPKKK18, -19, and BnaRaf35 were increased at the 1 h time point, while those of BnaZIK2, -3, -4, and BnaRaf17 were decreased at 24 h or at both time points by heat stress. In addition, moderate cold stress increased the transcript abundance of BnaMAPKKK20 and BnaRaf29 at the 1 h time point, but decreased that of BnaMAPKKK17, ZIK2, -3, -4, and BnaRaf39 mostly at the 24 h time point. Upon challenge by the fungal pathogen S. sclerotiorum, BnaMAPKKK19, ZIK4, Raf34, -35, and -39 were significantly induced at the 3 h time point, while BnaRaf28, -29, -30, and -36 were repressed at the 24 h time point. Lastly, only BnaMAPKKK20 was induced by OA treatment (Fig. 3; Supplementary Fig. S7A available at JXB online), although to a rather small extent. Taken together, these data indicate that on one hand, some BnaMAPKKK genes participate in transcription of multiple stresses; and, on the other hand, a specific stress activates transcription of more than one BnaMAPKKK gene, providing evidence that BnaMAPKKK plays a role in the cross-talk of multiple stresses, including both abiotic and biotic stresses.

Since public data on Arabidopsis gene expression are available and Arabidopsis is a close relative of canola, comparative analysis of orthologous MAPKKK genes between these two species was performed to examine to what extent the behaviours of AtMAPKKK and BnaMAPKKK genes in response to stress are correlated. To this end, expression profiles of AtMAPKKK genes in response to different hormone and stress treatments were investigated using ATH1 GeneChip data. It should be noted that the stress conditions applied to canola seedlings for the qRT-PCR assay are very similar to those for the Arabidopsis data set. As shown in Supplementary Fig. S7B available at JXB online, AtMAPKKK17 was induced in seedlings 3 h after treatment with 10 μM ABA and 6 h after heat (38 °C) treatment. AtMAPKKK18 was up-regulated by 10 μM ABA at 1 h and 3 h after treatment. AtMAPKKK19 was induced by B. cinerea, 10 μM MeJA at 30 min, 1 h, and 3 h, and 10 μM SA, while it was repressed after treatment with 150 mM NaCl. Botrytis cinerea is a grey mould fungus and it is taxonomically closely related to the white mould fungus S. sclerotiorum (Amselem et al., 2011). Both pathogens are necrotrophic by producing similar virulence factors such as OA, cell wall-degrading enzymes, etc. (Gentile, 1954; Choquer et al., 2007; Kim et al., 2008; Amselem et al., 2011). AtMAPKKK20 was up-regulated by 10 μM MeJA after 1 h treatment. It was also identified that both AtMAPKKK17 and -18 are also induced in the root samples after treatment with 150 mM NaCl for 30 min to 24 h. MAPKKK19 orthologues in both canola and Arabidopsis are induced by necrotrophic pathogens (Fig. 3; Supplementary Fig. S7 available at JXB online). However, differences in the expression profiles of BnaMAPKKK and AtMAPKKK after some treatments are also evident (Supplementary Fig. S7 available at JXB online). For instance, AtZIK4 was repressed by SA and heat, whereas it was induced by the biotrophic bacterial pathogen P. syringae. BnaZIK4 was also repressed by JA, heat, cold, and ACC, but was induced by S. sclerotiorum (Fig. 3; Supplementary Fig. S7A available at JXB online). AtZIK8 was induced by B. cinerea, SA, and drought (Supplementary Fig. S7B available at JXB online), whereas BnaZIK8 was induced by ACC only (Fig. 3). AtRaf17 was induced by SA treatment, whereas BnaRaf17 was repressed by NaCl and heat. AtRaf18 was induced by SA while it was repressed by salt treatment (Supplementary Fig. S7 available at JXB online). AtRaf29 was induced by ABA; AtRaf30 was induced by MeJA, SA, and cold, while it was repressed by salt. AtRaf34 was repressed by cold and heat, and AtRaf35 was induced by ABA, heat, and salt treatments (Supplementary Fig. S7B available at JXB online).

In summary, comparison of transcript expression profiles of some presumed orthologous MAPKK genes in Arabidopsis and canola show similarities in response to abiotic stress, hormone, and pathogen treatments. For instance, Raf28 was induced by SA, Raf30 was induced by JA, and MAPKKK19 was induced by necrotrophic fungi. However, significant differences were also observed between these two species and this may be attributed to evolutionary differences in gene expression that have occurred, and also experimental differences, especially sampling time.
Characterization of BnaMAPKKK gene functions in cell death

In the above Y2H and qRT–PCR studies, certain BnaMAPKKK genes were identified whose encoded proteins interacted with specific BnaM KKs, and that also increased in transcript abundance in response to multiple stress conditions. To explore their functions further, it was selected to express multiple genes transiently, including BnaMAPKKK18, -19, and BnaMKK9 as well as their constitutively inactive (CI) mutant forms individually under the CaMV 35S promoter through agroinfiltration into N. benthamiana leaves. The

Fig. 3. Expression analyses of BnaMAPKKK genes in response to various treatments, including 20 μM JA, 1 mM ACC, 2 mM SA, 10 μM paraquat (MV), 50 μM ABA, 200 mM NaCl, heat (37 °C), cold (4 °C), S. sclerotiorum infection, and 5 mM oxalic acid (OA). Data are the mean of three biological replicates ±SE. Asterisks denote significant differences (compared with 1) by Student t-test analysis (*P≤0.05; **P≤0.01).
inactive form of the MAPKKKs or MKKs was achieved by replacing the ATP-binding site (lysine, K) with an arginine (R) or with a methionine (M) (Melech-Bonfil and Sessa, 2010; Hashimoto et al., 2012). Interestingly, ectopic expression of either BnaMAPKKK18 or -19 caused pathogen-independent cell death compared with the CI version of their respective genes or empty vector control, beginning 48 h or 72 h post-infection (hpi), and this lasted till 144 hpi (Fig. 4A). It was also observed that the symptom of a water-soaked area became apparent as early as 24 hpi (Fig. 4A). To explore the role of hydrogen peroxide (H$_2$O$_2$) during cell death, DAB staining was performed and the results showed that there was strong staining in sites expressing only BnaMAPKKK18 or -19 beginning at 48 hpi and continued till 144 hpi, but not in any control sites (Fig. 4A, B). Nitroblue tetrazolium (NBT) staining of superoxide showed similar changes (data not shown). Moreover, the electrolyte leakage of leaf discs taken from leaves expressing BnaMAPKKK18, BnaMAPKKK18K32R, BnaMAPKKK19, BnaMAPKKK19K37G, and the empty vector was examined. The results showed that a significant increase in ion leakage was visible 3 d after agroinfiltration of BnaMAPKKK18 or -19, in contrast to that of leaves expressing BnaMAPKKK18K32R or the control plasmid (Fig. 4C), which further demonstrates that the hypersensitive response (HR)-like cell death associated with hydrogen peroxide production is triggered by high expression of BnaMAPKKK18 and -19. From the phylogenetic analysis, BnaMAPKKK18 and -19 were classified into subgroup A4 (Supplementary Fig. S1 available at JXB online). Interestingly, a literature search and phylogenetic analysis identified that SlMAPKKK1 and NtMAPKKK1 in the A4 group positively regulate cell death signalling in plant immunity (Supplementary Fig. S1 available at JXB online) (Melech-Bonfil and Sessa, 2010; Hashimoto et al., 2012). Epistasis experiments showed that SIMAPKKKe and NbMAPKKKe in the A4 group positively regulate cell death signalling in plant immunity (Supplementary Fig. S1 available at JXB online) (Melech-Bonfil and Sessa, 2010). The results therefore implied that BnaMAPKKK18 and -19 are two novel kinases mediating cell death in plants.

From our Y2H results, BnaMAPKKK18 interacted with BnaMKK3. However, expression of BnaMKK3 in N. benthamiana did not induce significant cell death (data not shown). Though the function of BnaMKK3 has not been identified yet, its orthologue AtMKK3 is known to regulate the JA signal transduction pathway together with AtMPK6 (Takahashi et al., 2007) and also plays an important role in plant immune and stress responses possibly through interacting with group C MPKs, including MPK1, -2, -7, and -14, and the downstream pathogenesis-related 1 (PR1) (Doczi et al., 2007).

In the above Y2H assay, it was found that BnaMAPKKK19 interacted with BnaMKK9. Interestingly, it was also found that BnaMKK9 also induced cell death when transiently expressed in N. benthamiana leaves (Fig. 4D). Interestingly, expression of the constitutively active BnaMKK9 (CA-MKK9) also induced strong cell death, whereas the CI BnaMKK9 (CI-MKK9) or empty plasmid did not (Fig. 4D). DAB and NBT staining demonstrated that there was evident accumulation of ROS at sites expressing BnaMKK9 or its constitutive form, but not in the sites expressing the inactive form or empty vector (Fig. 4D). To quantify further the extent of cell death, electrolyte leakages of leaf discs taken from leaves expressing the wild-type or mutant form of the BnaMKK9 gene and the empty vector were monitored. The results showed that a significant increase in ion leakage was visible 3 d after agroinfiltration of canola MKK9 or CA-MKK9 in contrast to that of leaves expressing CI-MKK9 or the control plasmid (Fig. 4E). A literature search indicated that Arabidopsis MKK9 can similarly accelerate cell death in N. benthamiana through Sgt1, a known regulator of cell death (Popescu et al., 2009), which suggests that orthologous MKK9 between canola and Arabidopsis may have a conserved function.

To understand further the role of the MAPKKK–MKK signaling cascade in cell death and ROS accumulation, co-overexpression analysis of BnaMAPKKK19 and BnaMKK9 in N. benthamiana was performed, considering the fact that BnaMAPKKK19 and BnaMKK9 interacted in both Y2H and BiFC. As shown in Supplementary Fig. S8 available at JXB online, BnaMKK9 alone is already active, and co-expression of MKK9 and MAPKKK19 does not have a significant additive or synergistic effect, as shown from statistical analysis of the cell death index and electrolyte leakage (Supplementary Fig. S8B, C available at JXB online). Meanwhile, cell death induced by expression of BnaMKK9 was not significantly influenced in the absence of upstream BnaMAPKKK19, neither was BnaMAPKKK19 in the absence of downstream BnaMKK9. It would be interesting to identify the substrates of MAPKKK19 and MAPKKK18 in N. benthamiana, and to explore the relationship between these two MAPKKKs and ROS-generating enzymes Rbohs (respiratory burst oxidase homologues) (Torres and Dangl, 2005). Moreover, whether the other cloned BnaMAPKK and BnaMKK genes could regulate cell death and ROS signalling is still under investigation in our lab.

A complete module, which includes BnaMKK9–BnaMPK1/2–BnaWRKY53 was previously identified (Liang et al., 2013), which links the BnaMKK9–MPK1/2 cascade to downstream WRKY transcription factor(s), and they may together regulate cell death and/or leaf senescence, since AtWRKY53 was identified to regulate leaf senescence (Miao et al., 2007). Whether BnaMAPKKK19 mediates cell death through MKK9–BnaMPK1/2–BnaWRKY53 and how the ethylene signalling pathway is integrated by this module need to be further elucidated. Besides BnaMAPKKK19, four more BnaMAPKKKs, namely BnaMAPKKK17, -20, BnaZIK2, and BnaRafl28, were also identified to interact with BnaMKK9 (Table 2; Supplementary Figs S5, S6 available at JXB online); however, none of these BnaMAPKKKs was shown to elicit cell death (data not shown), indicating that they are possibly involved in other biological processes.

The activation of AtMKK5 can lead to HR-like cell death through ethylene signalling perception (Liu et al., 2008). Arabidopsis MKK4 and MKK5 belong to group C MKKs with D sites K/R-K/R-X(1–5)-L/I-X-L/I at the N-termini (Bardwell et al., 2001; Grewal et al., 2006). More recently, it is reported that the D site in SIMKK2 of S. lycopersicum is critical for interacting with SIMPK3 and triggering PCD (Oh et al., 2013). Though Arabidopsis YODA–MKK4/MKK5–MPK3/MPK6 modules regulate
Fig. 4. Overexpression of BnaMAPKKK18, -19, and MKK9 induced pathogen-independent cell death in N. benthamiana leaves. Leaves were infiltrated with agrobacteria carrying wild-type genes or mutated versions. All experiments were performed three times with similar results obtained. (A) Symptoms of N. benthamiana leaf areas expressing BnaMAPKKK18 and BnaMAPKKK19 genes and their mutated forms 24, 48, 72, 96, 120 and 144 h post-infiltration (hpi). The left, middle, and right panels represent the front and back, and DAB staining, respectively. (B) Quantification of cell death in N. benthamiana leaves expressing BnaMAPKKK18 or BnaMAPKKK19 and their mutated derivates by examining the percentages of leaf sites with water-soaked symptoms at various time points. (C) Measurement of electrolyte leakage in leaf discs expressing BnaMAPKKK18 or BnaMAPKKK19 and their mutated derivates at 48, 96, and 144 hpi. (D) Symptoms of N. benthamiana leaf areas expressing BnaMKK9 or its mutated versions at 48 and 60 hpi. (E) Measurement of electrolyte leakage in leaf discs transiently expressing BnaMKK9 and its mutated derivates at 60 hpi. Values represent the means of three independent assays for each time point ±SE. Identical and different letters represent non-significant and significant differences (P≤0.05), respectively.
stomatal development and patterning (Wang et al., 2007), the direct upstream component(s) of AtMKK5 has not been reported yet. In the Y2H assay, two BnaMAPKKKs, namely BnaZIK2 and BnaRaf28, interacted with BnaMKK5 (Table 2; Supplementary Fig. S5 available at JXB online), and the interactions were confirmed through BiFC (Supplementary Fig. S6 available at JXB online). However, no cell death induced by BnaZIK2 and BnaRaf28 overexpression in N. benthamiana was observed (data not shown). Whether there are other canola MAPKKK genes mediating cell death or ROS signalling awaits further investigation. Also, whether and how BnaMAPKKK18 and -19 modulate plant immunity against fungal pathogens such as S. sclerotiorum needs to be experimentally determined.

Conclusion

MAPK signalling pathways are very important in plant development, abiotic stress, and defence responses. So far, the function of only a few MAPKKK genes in Arabidopsis, rice, tomato, and tobacco have been reported (Rodriguez et al., 2010; Meng and Zhang, 2013). In the present study, the identification of the MAPKKK gene family in the important oilseed crop, canola, was described. A total of 28 novel MAPKKK genes in canola were cloned and characterized, and two novel MAPKKK genes, BnaMAPKKK18 and -19, mediating cell death were identified. A few complete MAPK modules were identified through linking MAPKKKs to recently identified downstream components (Liang et al., 2013). For example, BnaZIK2/BnaRaf28–BnaMKK5–BnaMPK3/6–BnaWRKY20/26, BnaRaf28–BnaMKK2/5–BnaMPK3/6–BnaWRKY20/26, and BnaMAPKKK17/19/20/ZIK2/BnaRaf28–BnaMKK9–BnaMPK5/-9/-19/-20 (Fig. 5). Many of these have not been reported even in Arabidopsis, indicating that different combinations of BnaMAPKKK–BnaMKK–BnaMPKs are very likely to be involved in the responses to different external and internal stimuli in canola. In the present and previous studies, it was found that there are differences in expression patterns of orthologous MAPKKK, MKK, and MPK genes in response to different stimuli between Arabidopsis and canola, and it was also identified that even the interaction pairs are not conserved between these two species, which highlights the limitations of applying conclusions from the model species Arabidopsis to canola (Liang et al., 2013).

In a previous study, canola MAPKKK17, MAPKKK18, as well as MPK3, MPK4, MPK6, and MPK17 were identified as being responsive to S. sclerotiorum infection (Yang et al., 2007). Seven MKK and 12 MPK genes from canola were recently identified, and their function was analysed in the context of abiotic and biotic stresses (Liang et al., 2013). Here the identification and characterization of their upstream regulators, MAPKKKs, were described. Although the present 28 BnaMAPKKK genes comprise only 44% of the 66 identified genes for canola, 15 BnaMAPKKK–BnaMKK interaction pairs were identified, which provide clues to dissect the function of individual BnaMAPKKKs in canola. For instance, BnaRaf28 could interact with BnaMKK2, -5, -6, -8, and -9, while six different BnaMAPKKKs could interact with BnaMKK9, and six BnaMAPKKKs interacted with BnaMKK3 (Table 2; Fig. 5). It is proposed that the different combinations of BnaMKK–BnaMKK pairs may fulfil their function in response to diverse abiotic stress signalling pathways and plant immune responses. However, the possibility cannot be excluded that even if two proteins are found to interact in Y2H assay or after co-expression in N. benthamiana,
they may not necessarily do that in the natural situation. Moreover, it was identified that two novel MAPKK genes, BnaMAPKKK18 and -19, could induce cell death when transiently expressed in leaves of N. benthamiana, possibly through accumulation of ROS. Moreover, BnaMKK9 and its constitutively active form also induced strong cell death and H2O2 accumulation. However, a more complete picture of MAPKK genes and the related signalling cascade in canola will not be available before the remaining MAPKK genes are cloned from canola and characterized. Overall, the present study of MAPKK genes in canola lays the foundation for further exploration of their roles in abiotic stress signalling and in plant immunity against S. sclerotiorum. It also provides important information for genetically manipulating the abundance and/or activity of related MAPKKKs and their components in the MAPK pathway to improve stress tolerance of canola.

Supplementary data

Supplementary data are available at JXB online.

- Figure S1. Phylogenetic analysis of MAPKKKs from representative species.
- Figure S2. Multiple alignment of MEKK subfamily MAPKKK proteins in representative species.
- Figure S3. Multiple alignment of ZIK subfamily MAPKKK proteins in representative species.
- Figure S4. Multiple alignment of Raf subfamily MAPKKK proteins in representative species.
- Figure S5. Yeast two-hybrid (Y2H) assay of interactions between MAPKKK and MKK proteins in canola.
- Figure S6. Analysis of BnaMAPKKK and BnaMKK interactions in N. benthamiana through bimolecular fluorescence complementation (BiFC).
- Figure S7. The heat maps of the expression profiles of MAPKK genes of canola and Arabidopsis in responses to abiotic and biotic stresses.
- Figure S8. Co-expression analysis of BnaMAPKKK19 and M KK9 in eliciting pathogen-independent cell death in N. benthamiana leaves.
- Table S1. BnaMAPKK EST summary.
- Table S2. Primers used in this study.
- Table S3. MAPKKK sequences from different species used for phylogenetic analysis.
- Table S4. Similarity and identity analysis of MAPKK genes/proteins between Arabidopsis, rice, and canola.
- Table S5. Computational prediction of subcellular localizations of MAPKKK proteins in canola.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (no. 31301648 to BY and 31270293 to Y-QJ) and the Chinese Ministry of Education Program for New Teachers in Universities (no. 20110204120005 to BY). We would like to acknowledge Professor Lili Huang (Northwest A&F University) for providing the Sclerotinia sclerotiorum strain SN09-904, and Professor Jörg Kudla (Universität Münster, Germany) for the BiFC vectors. We would also thank the anonymous reviewers for constructive advice on improving the manuscript.

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