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Overexpression of cotton *GhMKK4* enhances disease susceptibility and affects abscisic acid, gibberellin and hydrogen peroxide signalling in transgenic *Nicotiana benthamiana*

YUZHEN LI†, LIANG ZHANG†, WENJING LU, XIULING WANG, CHANG-AI WU AND XINGQI GUO*

State Key Laboratory of Crop Biology, Shandong Key Laboratory of Crop Biology, Shandong Agricultural University, Taian, Shandong 271018, China

SUMMARY

Mitogen-activated protein kinase (MAPK) cascades are involved in plant development, stress responses and hormonal signal transduction. MAPK kinases (MAPKKs), as the key nodes in these cascades, link MAPKs and MAPKK kinases (MAPKKKs). In this study, *GhMKK4*, a novel group C MAPKK gene from cotton (*Gossypium hirsutum*), was isolated and identified. Its expression can be induced by various stresses and signalling molecules. The overexpression of *GhMKK4* in *Nicotiana benthamiana* enhanced its susceptibility to bacterial and fungal pathogens, but had no significant effects on salt or drought tolerance. Notably, the overexpressing plants showed increased sensitivity to abscisic acid (ABA) and gibberellin A3 (GA3), and ABA and gibberellin (GA) signalling were affected on infection with *Ralstonia solanacearum* bacteria. Furthermore, the overexpressing plants showed more reactive oxygen species (ROS) accumulation and stronger inhibition of catalase (CAT), a ROS-scavenging enzyme, than control plants after salicylic acid (SA) treatment. Interestingly, two genes encoding ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (SAMDC), the key enzymes in polyamine synthesis, exhibited reduced *R. solanacearum*-induced expression in overexpressing plants. These findings broaden our knowledge about the functions of MAPKKs in diverse signalling pathways and the negative regulation of disease resistance in the cotton crop.

INTRODUCTION

The adaptive responses of plants to environmental changes are primarily conducted through the perception of external signals. These perceived signals are amplified through multiple signal transduction pathways (Shinozaki *et al*., 2003). Mitogen-activated protein kinase (MAPK) cascades have emerged as common signal transduction pathways for the translation of external stimuli into cellular responses (MAPK Group, 2002).

A typical MAPK cascade consists of a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK. Activation of MAPKKKs by upstream signals results in the sequential phosphorylation of their downstream MAPKKs and MAPKs (Kong *et al*., 2012; Rodriguez *et al*., 2010). MAPK cascades are highly conserved and have been regarded as the integrative points of multiple pathways (Jonak *et al*., 2002). In *Arabidopsis*, there are approximately 60 MAPKKKs, 10 MAPKKs and 20 MAPKs (Rodriguez *et al*., 2010). There are fewer MAPKKs than MAPKKKs and MAPKs, which suggests that individual MAPKKs may function as bifurcation points and are likely to be involved in multiple MAPK cascades to perform diverse biological functions (Andreasson and Ellis, 2010; Heinrich *et al*., 2011; Xu *et al*., 2008). Therefore, the functions of plant MAPKKs have been a major focus of ongoing research. At present, several MAPKKs have been identified in different plants, including *Arabidopsis* AtMKK1-10; alfalfa MsSIMKK and MsPRKK; tomato LeMEK1; tobacco NtMEK1-2 and NtSIPKK; rice OsMKK1, 3, 4, 5, 6 and 10; maize ZmMEK1 and ZmMAPKK1; and cotton GhMKK5 (Cardinale *et al*., 2002; Doczi *et al*., 2012; Kong *et al*., 2011; Zhang *et al*., 2012). MAPKKs are divided into four groups (A, B, C and D) based on the phylogenetic analyses of their amino acid sequences and phosphorylation motifs (Hamel *et al*., 2006; MAPK Group, 2002). *Arabidopsis* AtMKK4 and AtMKK5 are two widely investigated group C MAPKKs that can activate the MAPKs AtMPK3 and AtMPK6, which participate in development, abiotic stress, flagellin perception and innate immunity, as well as in the regulation of the biosynthesis of camalexin (Asai *et al*., 2002; Ren *et al*., 2008). These reports suggest that group C MAPKKs have pivotal roles in plants. Although several group C MAPKKs in other plant species have been isolated recently (Kishi-Kaboshi *et al*., 2010; Kong *et al*., 2011; Zhang *et al*., 2012), the functions of group C MAPKKs have not been investigated fully.

Emerging evidence has revealed the importance of MAPKKs in plant abiotic and biotic stress responses and development. In *Arabidopsis*, constitutively activated AtMKK9 can increase the sensitivity of transgenic plants to salt tolerance (Xu *et al*., 2008). However, AtMKK4 mediates osmotic stress tolerance (Kim *et al*., 2011). The overexpression of *AtMKK3* enhances the resistance to *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (Doczi *et al*., 2007). Similarly, the ectopic expression of *AtMKK7* induces systemic resistance to *Pseudomonas syringae* pv. *maculicola* (*Psm*) ES4326 (Zhang *et al*., 2007). However, some MAPKKs have

**Correspondence*: Email: xqguo@sdau.edu.cn
†These authors contributed equally to this work.

negative effects on pathogen resistance. *Arabidopsis* AtMKK1/MKK2 can activate AtMPK4 to negatively regulate immunity (Kong *et al*., 2012). *AtMKK2*-overexpressing transgenic plants exhibit a markedly enhanced susceptibility to the fungal necrotroph *Alternaria brassicicola*, even though these plants show some resistance to *Pst* DC3000 and *Erwinia carotovora* ssp. *carotovora* (Brader *et al*., 2007). Similarly, our previous study has indicated that the overexpression of *GhMKK5* in *N. benthamiana* enhances the resistance of the plants to the bacterial pathogen *Ralstonia solanacearum*, but increases their susceptibility to the oomycete pathogen *Phytophthora parasitica* var. *nicotianae* Tucker (Zhang *et al*., 2012). During development, *Arabidopsis* AtMKK7 negatively regulates polar auxin transport (Dai *et al*., 2006), and the YODA–MKK4/MKK5–MPK3/MPK6 cascade is involved in stomatal development (Wang *et al*., 2007). In tobacco, NtMEK2 can regulate osmotic stress signals and pollen germination (Mikolajczyk *et al*., 2000). Although these results have broadened our knowledge about plant MAPKKs, the functions of MAPKKs are diverse, and further study of their roles is required.

As the nodal point in signal transduction, MAPKKs are involved in the modulation of the levels of many plant hormones. In apple, the MAPK signalling cascade MdMKK1–MdMPK1 functions in abscisic acid (ABA) signalling by regulating ABI5 or ABI5-like transcription factors (Wang *et al*., 2010). The *Arabidopsis* AtMKK9–MPK3/MPK6 cascade can promote ethylene (ET)-insensitive 3 (EIN3)-mediated transcription in ET signalling, and the *mkk9* mutant exhibits a broad spectrum of moderate ET-insensitive phenotypes (Yoo *et al*., 2008). Furthermore, AtMPK3/MPK6 can also be activated by AtMKK5, resulting in an increase in ET level (Liu *et al*., 2008). The constitutively active form of AtMKK3 has been reported to participate in jasmonic acid (JA) signalling by regulating the expression of JA-related genes (Takahashi *et al*., 2007a). On infection, *AtMKK2*-overexpressing plants show a weaker increase in the production of JA and salicylic acid (SA), suggesting that *AtMKK2* functions in the modulation of hormone levels in response to pathogen infection (Brader *et al*., 2007). Previous reports have focused mainly on the identification of the stressors and signals to which the MAPK cascades respond, especially in model plants. Data describing the functions of MAPKKs in the regulation of hormone levels and the cross-talk among diverse signals during stress responses are limited.

At present, the investigation of plant MAPKKs has focused mainly on model plants and food crops. Cotton (*Gossypium hirsutum*) is one of the oldest and most important fibre and oil crops. Research on the cotton MAPKKs has far-reaching significance in improving the growth and yield under various stress conditions. In this study, a novel group C MAPKK gene from cotton, *GhMKK4*, was isolated and identified. The expression of *GhMKK4* could be induced by biotic and abiotic stresses and various signalling molecules. The ectopic expression of *GhMKK4* in *N. benthamiana* enhanced its susceptibility to bacterial and fungal pathogens, but did not affect significantly its salt or drought tolerance. The *GhMKK4*-overexpressing plants showed elevated ABA levels, but not GA levels, under normal conditions, and increased reactive oxygen species (ROS) accumulation after SA treatment. These results suggest that *GhMKK4* plays a pivotal role in pathogen responses and multiple signal transduction pathways.

RESULTS

Sequence analysis of *GhMKK4*

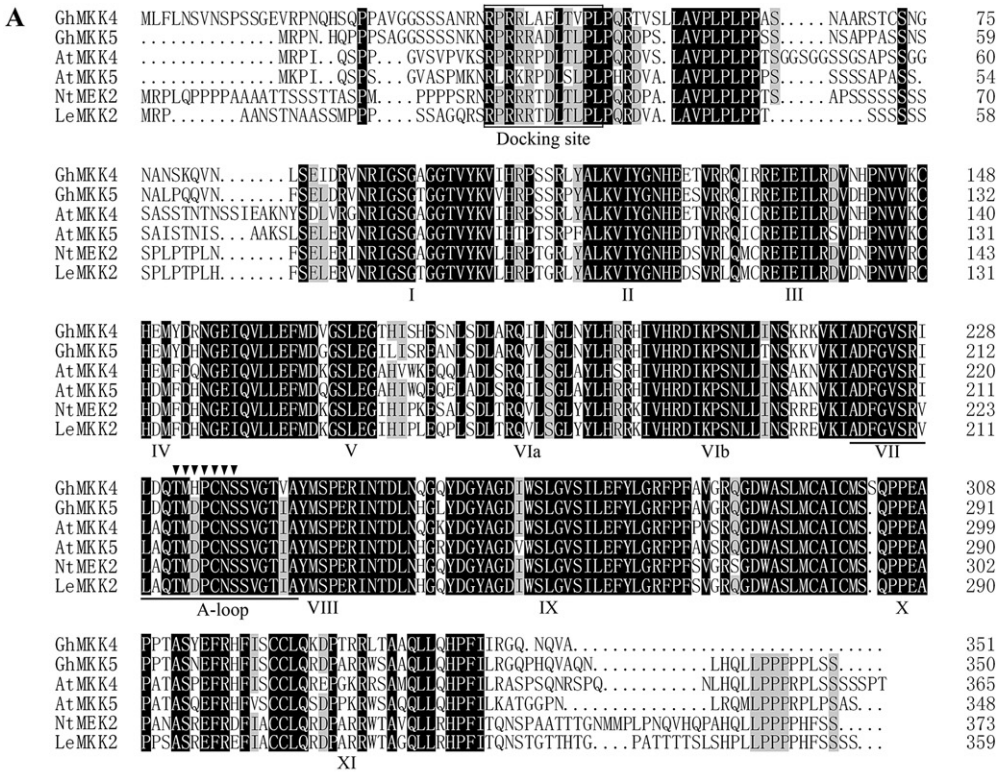
The full-length cDNA of *GhMKK4* (FJ966886) consisted of 1338 nucleotides, containing a 1056-bp open reading frame (ORF), a 26-bp 5'-untranslated region (5'-UTR) and a 256-bp 3'-UTR. *GhMKK4* was predicted to encode a protein of 351 amino acid residues with a putative molecular weight of 39.07 kDa and an isoelectric point of 9.55. The GhMKK4 protein exhibited the same family signature as other plant MAPKKs, including 11 conserved subdomains, a conserved S/TXXXXXS/T motif, an activation loop and a docking site, as demonstrated by multiple sequence alignments. Furthermore, GhMKK4 showed 66.93% homology to AtMKK4 and 65.58% to AtMKK5 from *Arabidopsis thaliana*, 64.38% to NtMEK2 from *Nicotiana tabacum*, 64.19% to LeMKK2 from *Lycopersicon esculentum* and 77.11% to GhMKK5 from *Gossypium hirsutum* (Fig. 1A). As shown in Fig. 1B, GhMKK4 was highly similar to group C MAPKKs, such as AtMKK4, AtMKK5, NtMEK2 and GhMKK5. These results suggest that GhMKK4 is a group C MAPKK.

The genome sequence of *GhMKK4* was also analysed. Sequence comparisons revealed that *GhMKK4* had no intron structure, similar to other group C and D MAPKKs (Fig. 2). This result further indicates that GhMKK4 is a member of group C MAPKKs.

Expression patterns of *GhMKK4* mRNA

Reverse transcription-polymerase chain reaction (RT-PCR) was conducted to investigate the expression patterns of *GhMKK4* in 7-day-old cotton seedlings. The relative expression of *GhMKK4* is shown in Fig. 3. *GhMKK4* was expressed much more strongly in the leaves and roots than in the stems (Fig. 3A). This tissue-specific expression suggests that *GhMKK4* may serve specific functions in different tissues.

The environmental stress response assays showed that the expression of *GhMKK4* was induced significantly by NaCl and mannitol, and that NaCl had a relatively long-lasting effect (Fig. 3B,C). Treatment with *Rhizoctonia solani* significantly induced the expression of *GhMKK4*, peaking at day 5 or 6 during the indicated time (Fig. 3D). To explore the mechanism through which *GhMKK4* is involved in signal transduction, the responsiveness of *GhMKK4* to diverse signalling molecules was also examined. As


A
GhMKK4 MLFLNSVNSPSSGEVRPNQHSQPAVGGSSSANRNRPRRLAELTVPLPQRTVSLLAVPLPLPPASNAARSTCSNG 75
GhMKK5 MRPN.HQPPPSAGGSSSSNKNRPRRRADLTLPLPQRDPSLAVPLPLPPSSNSAPPASSNS 59
AtMKK4 MRPI..QSPP...GVSVPVKSRPRRRPDLTLPLPQRDVSLAVPLPLPPTSGGSGGSSGSAPSSGG 60
AtMKK5 MKPI..QSPS...GVASPMKNRLRKRPDLSLPLPHRDVALAVPLPLPPPSSSSAPASS. 54
NtMEK2 MRPLQPPPPAAAATTSSSTTASPM....PPPPSRNRPRRRTDLTLPLPQRDPALAVPLPLPPTSAPSSSSSSSS 70
LeMKK2 MRP.....AANSTNAASSMPPP....SSAGQRSRPRRRTDLTLPLPQRDVALAVPLPLPPT.....SSSSSS 58
Docking site
GhMKK4 NANSKQVN.....LSEIDRVNRIGSGAGGTVYKVIHRPSSRLYALKVIYGNHEETVRRQIRREIEILRDVNHPNVVKC 148
GhMKK5 NALPQQVN.....FSELDRVNRIGSGTGGTVYKVVHRPSSRPYALKVIYGNHEESVRRQIRREIEILRDVDHPNVVKC 132
AtMKK4 SASSTNTNSSIEAKNYSDLVRGNRIGSGAGGTVYKVIHRPSSRLYALKVIYGNHEETVRRQICREIEILRDVNHPNVVKC 140
AtMKK5 SAISTNIS...AAKSLSELDRVNRIGSGAGGTVYKVIHTPTSRPFALKVIYGNHEDTVRRQICREIEILRSVDHPNVVKC 131
NtMEK2 SPLPTPLN.....FSELDRINRIGSGAGGTVYKVLHRPTGRLYALKVIYGNHEDSVRLQMCREIEILRDVDNPNVVRC 143
LeMKK2 SPLPTPLH.....FSELDRVNRIGSGTGGTVYKVLHRPTGRLYALKVIYGNHEDSVRLQMCREIEILRDVDNPNVVRC 131
I II III
GhMKK4 HEVYDRNGEIQVLLEFMDVGSLEGTHISHFSNLSDLARQILNGLNYLHRRHIVHRDIKPSNLLINSKRKVKIADFGVSRI 228
GhMKK5 HEVYDHNGEIQVLLEFMDGGSLEGILISREANLSDLARQVLSGLNYLHRRHIVHRDIKPSNLLTNSKKVVKIADFGVSRI 212
AtMKK4 HEVFDQNGEIQVLLEFMDKGSLEGAHVWKEQQLADLSRQILSGLAYLHSRHIVHRDIKPSNLLINSAKNVKIADFGVSRI 220
AtMKK5 HDVFDHNGEIQVLLEFMDQGSLEGAHIWQEQELADLSRQILSGLAYLHRRHIVHRDIKPSNLLINSAKNVKIADFGVSRI 211
NtMEK2 HDVFDHNGEIQVLLEFMDKGSLEGIHIPKESALDLTRQVLSGLYYLHRRKIVHRDIKPSNLLINSRREVKIADFGVSRV 223
LeMKK2 HDVFDHNGEIQVLLEFMDKGSLEGIHIPLEQPLSDLTRQVLSGLYYLHRRKIVHRDIKPSNLLINSRREVKIADFGVSRV 211
IV V VIa VIb VII
GhMKK4 LDQTMHPCNSSVGTVAYMSPERINTDLNQGQYDGYAGDIWSLGVSILEFYLGRFPFAVGRQGDWASLMCAICMSSQPPEA 308
GhMKK5 LDQTMDPCNSSVGTIAYMSPERINTDLNHGLYDGYAGDIWSLGVSILEFYLGRFPFAVGRQGDWASLMCAICMS.QPPEA 291
AtMKK4 LAQTMDPCNSSVGTIAYMSPERINTDLNQGKYDGYAGDIWSLGVSILEFYLGRFPFPVSRQGDWASLMCAICMS.QPPEA 299
AtMKK5 LAQTMDPCNSSVGTIAYMSPERINTDLNHGRYDGYAGDVWSLGVSILEFYLGRFPFAVSRQGDWASLMCAICMS.QPPEA 290
NtMEK2 LAQTMDPCNSSVGTIAYMSPERINTDLNHGQYDGYAGDIWSLGVSILEFYLGRFPFSVGRSGDWASLMCAICMS.QPPEA 302
LeMKK2 LAQTMDPCNSSVGTIAYMSPERINTDLNHGQYDGYAGDIWSLGVSILEFYLGRFPFSVGRQGDWASLMCAICMS.QPPEA 290
A-loop VIII IX X
GhMKK4 PPTASVEFRHFISCCLQKDETRRLTAAQLLQHPFIIRGQ.NQVA. 351
GhMKK5 PPTASVEFRHFISCCLQRDEARRWSAAQLLQHPFILRGQPHQVAQN.....LHQLLPPPPPLSS..... 350
AtMKK4 PATASPEFRHFISCCLQREEGKRRSAMQLLQHPFILRASPSQNRSPQ.....NLHQLLPPPRPLSSSSSPT 365
AtMKK5 PATASQEFRHFVSCCLQSDEPKRWSAQQLLQHPFILKATGGPN.....LRQMLPPPRPLPSAS... 348
NtMEK2 PANASREFRDFIACCLQRDEARRWTAVQLLRHPFITQNSPAATTTGNMMPLPNQVHQPAHQLLPPPPHFSS..... 373
LeMKK2 PPSASREFREFIACCLQRDEARRWTAGQLLRHPFITQNSTGTTHTG....PATTTTSLSHPLLPPPPHFSSSS... 359
XI

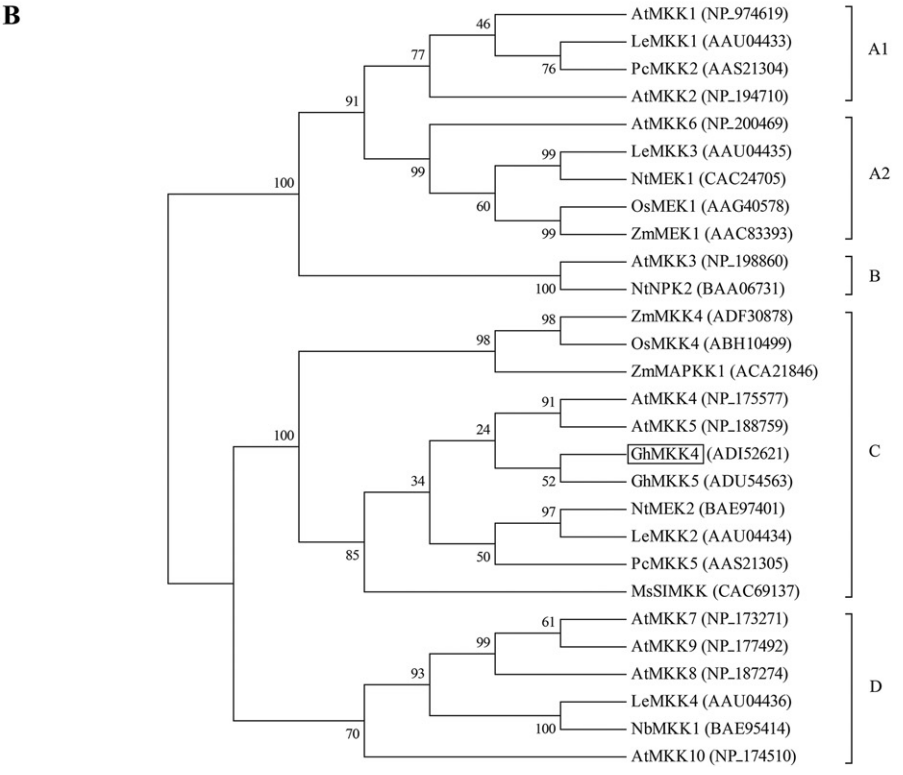

B
AtMKK1 (NP_974619)
LeMKK1 (AAU04433)
PcMKK2 (AAS21304)
AtMKK2 (NP_194710)
AtMKK6 (NP_200469)
LeMKK3 (AAU04435)
NtMEK1 (CAC24705)
OsMEK1 (AAG40578)
ZmMEK1 (AAC83393)
AtMKK3 (NP_198860)
NtNPK2 (BAA06731)
ZmMKK4 (ADF30878)
OsMKK4 (ABH10499)
ZmMAPKK1 (ACA21846)
AtMKK4 (NP_175577)
AtMKK5 (NP_188759)
GhMKK4 (ADI52621)
GhMKK5 (ADU54563)
NtMEK2 (BAE97401)
LeMKK2 (AAU04434)
PcMKK5 (AAS21305)
MsSIMKK (CAC69137)
AtMKK7 (NP_173271)
AtMKK9 (NP_177492)
AtMKK8 (NP_187274)
LeMKK4 (AAU04436)
NbMKK1 (BAE95414)
AtMKK10 (NP_174510)
A1
A2
B
C
D

Fig. 1 Comparison of the deduced amino acid sequences of GhMKK4 and closely related plant mitogen-activated protein kinase (MAPK) kinases (MAPKKs). (A) The amino acid sequence alignment of GhMKK4 (ADI52621), GhMKK5 (ADU54563), AtMKK4 (NP_175577), AtMKK5 (NP_188759), NtMEK2 (BAE97401) and LeMKK2 (AAU04434). Identical amino acids are shaded in black. The protein kinase subdomains are shown with Roman numerals (I–XI) at the bottom of the sequences, and the activation loop (A-loop) is underlined. The serine (Ser) and/or threonine (Thr) residues in the conserved S/TXXXXXS/T consensus motif between MAPKK subdomains VII and VIII are marked with arrowheads (▼). The docking site is boxed. (B) The phylogenetic relationships between GhMKK4 and other plant MAPKK proteins. The neighbour-joining phylogenetic tree was created with CLUSTALW in MEGA 4.1. The numbers above and below the branches indicate the bootstrap values (>50%) from 500 replicates. The gene name is followed by the protein ID. The species of origin of the MAPKKs is indicated by the abbreviation before the gene names: At, *Arabidopsis thaliana*; Gh, *Gossypium hirsutum*; Le, *Lycopersicon esculentum*; Ms, *Medicago sativa*; Nb, *Nicotiana benthamiana*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Pc, *Petroselinum crispum*; Zm, *Zea mays*.

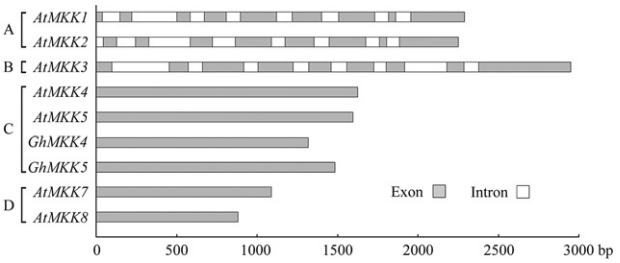


Fig. 2 Comparison of the genomic DNA sequences of *GhMKK4* and several mitogen-activated protein kinase (MAPK) kinase (MAPKK) genes of *Arabidopsis* available in GenBank. The white boxes indicate the introns, and the grey boxes represent exons. The scale indicates the length of the sequence. A, B, C and D indicate the MAPKK groups.

shown in Fig. 3E, H_2O_2 markedly induced the accumulation of *GhMKK4* at 2 h, with *GhMKK4* expression then decreasing gradually to the level of the control. Notably, *GhMKK4* expression was rapidly and strongly induced by ABA, SA and methyl jasmonate (MeJA) (Fig. 3F–H). Another signalling molecule, ET, which is released from ethephon, also induced *GhMKK4* accumulation (Fig. 3I). However, 10 μM GA3 had a negligible effect on *GhMKK4* expression (Fig. 3J). These results indicate that *GhMKK4* is responsive to environmental stresses and may be involved in multiple signal transduction pathways in stress responses.

Enhanced susceptibility of *GhMKK4*-overexpressing plants to bacterial pathogens

Transgenic *N. benthamiana* plants overexpressing *GhMKK4* were produced to further evaluate the role of *GhMKK4*. The *GhMKK4* expression levels in 12 randomly selected overexpressing (OE) lines of T1 progeny plants were detected using RT-PCR (Fig. 4A). Three representative lines (1#, 2# and 4#) were selected, and their T3 progeny were used for functional analyses. Interestingly, the OE lines, especially lines 2# and 4#, grew relatively more slowly than the vector control (Vec) plants at the early vegetative stage (Fig. 4B). However, the differences in plant size diminished gradually by the late vegetative stage (data not shown).

When the detached leaves were injected with *R. solanacearum* over 4 days, the leaves of the OE plants showed notable signs of chlorosis and relatively larger lesion diameters than those of the Vec plants (Fig. 4C,D). The trickle irrigation method was also used to examine the response to the pathogen. The OE plants displayed marked wilting, whereas the Vec plants showed a relatively higher survival rate than the OE plants (Fig. 4E,F). These results indicate that the overexpression of *GhMKK4* enhances the susceptibility to the bacterial pathogen in the transgenic plants.

To elucidate the reasons underlying the enhanced susceptibility, the relative expression levels of pathogenesis-related protein (*PR*) genes were examined using quantitative real-time PCR (qPCR). The *PR* genes, including *PR1a*, *PR1c*, *PR2*, *PR4* and *PR5*, were expressed at much lower levels in the OE plants than in the Vec plants (Fig. 4G). This reduced expression of *PR* genes may be the reason for the enhanced susceptibility to the bacterial pathogen in the transgenic plants.

Enhanced susceptibility of *GhMKK4*-overexpressing plants to a fungal pathogen

To test the response to a fungal pathogen, the detached leaves from 2-month-old T3 generation transgenic plants were incubated with *R. solani*. After incubation for 6 days, marked lesions were observed in the leaves of both Vec and OE plants. However, the lesions on the OE leaves were much larger than those on the Vec leaves, and many more hyphae and spores were present in the OE leaves than in the Vec leaves (Fig. 5A,B). When the plants were infected by the trickle irrigation method for approximately 10 days, the OE plants displayed significant wilting, with more serious caudex rot and a much lower survival rate than the Vec plants (Fig. 5C,D). These results indicate that the transgenic plants overexpressing *GhMKK4* show an enhanced susceptibility to the fungal pathogen.

GhMKK4-overexpressing plants do not show significant salt or drought stress tolerance

To test the function of *GhMKK4* in response to abiotic stresses, transgenic plants were subjected to NaCl and drought stress. There was no significant difference in the growth and germination rates of the transgenic seeds during germination on water-moistened filter paper. Under the 50 mM NaCl condition, the germination of both OE and Vec seeds was inhibited slightly. Although a larger

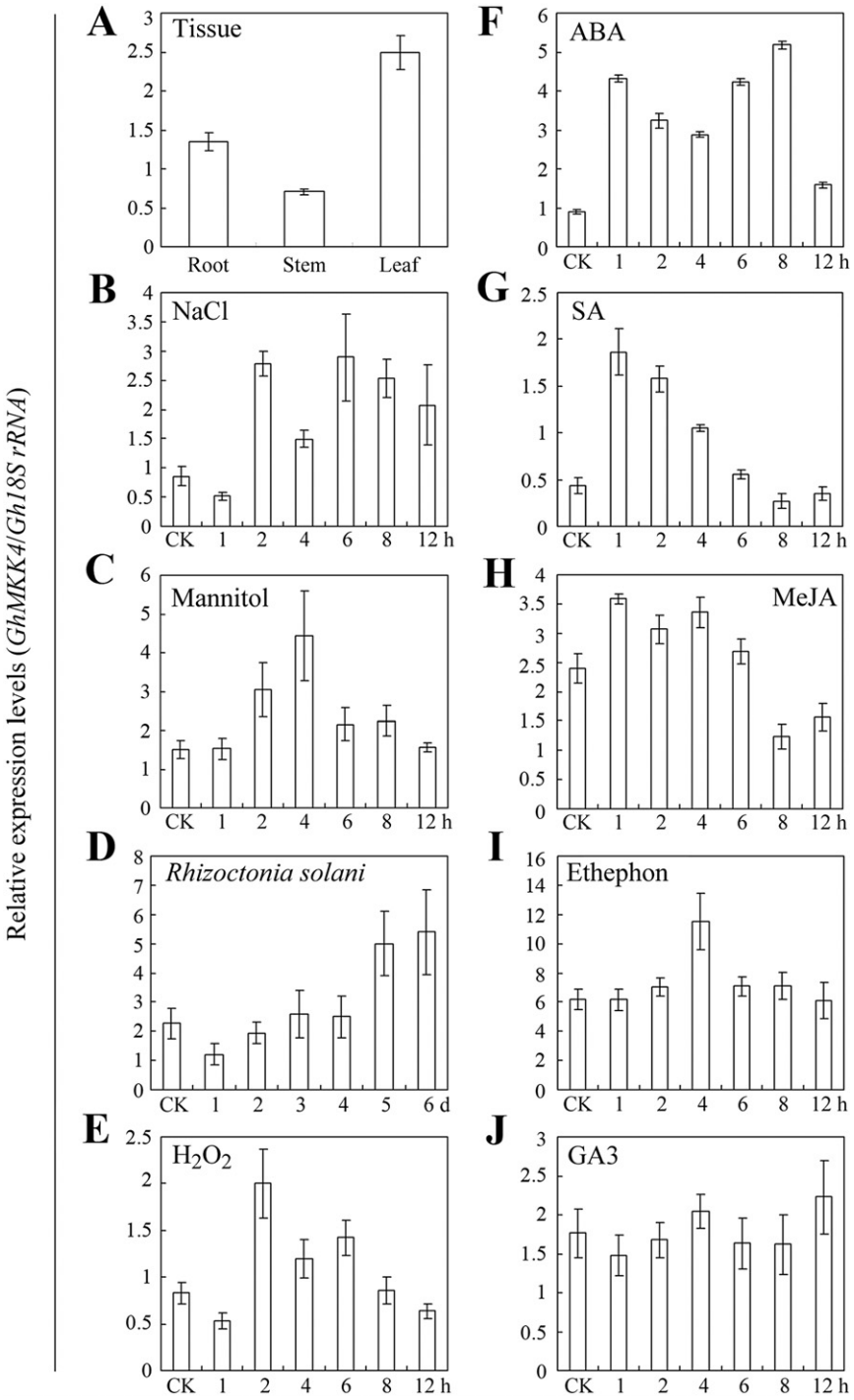


Fig. 3 Relative expression of *GhMKK4* in different tissues and in response to different stress factors. These results are the quantification of the *GhMKK4* band intensity over the expression of *Gh18S rRNA* genes by Quantity One software. For the tissue-specific expression of *GhMKK4*, the roots, stems and cotyledon leaves of 7-day-old cotton seedlings were used. For different treatments, the seedlings were treated with 200 mM NaCl (B), 200 mM mannitol (C), *Rhizoctonia solani* (D), 100 μM H_2O_2 (E), 100 μM abscisic acid (ABA) (F), 10 mM salicylic acid (SA) (G), 100 μM methyl jasmonate (MeJA) (H), 5 mM ethylene (ET) released from ethephon (I) or 10 μM gibberellins A3 (GA3) (J).

number of OE seeds germinated slightly earlier at the very beginning of the experimental period, there was no significant difference between the overall germination rates of the transgenic seeds during germination (Fig. 6A,B). Similar results were observed under the 200 mM NaCl condition (data not shown). When germinated on filter paper moistened with 100 mM mannitol, OE seeds showed a relatively higher germination rate than Vec seeds, but this difference was not significant. A similar result was observed under the 200 mM mannitol condition (data not shown). Furthermore, the withholding of water for 12 days during the vegetable growth stage (drought stress) caused both OE and Vec plants to wilt. No noticeable differences were found

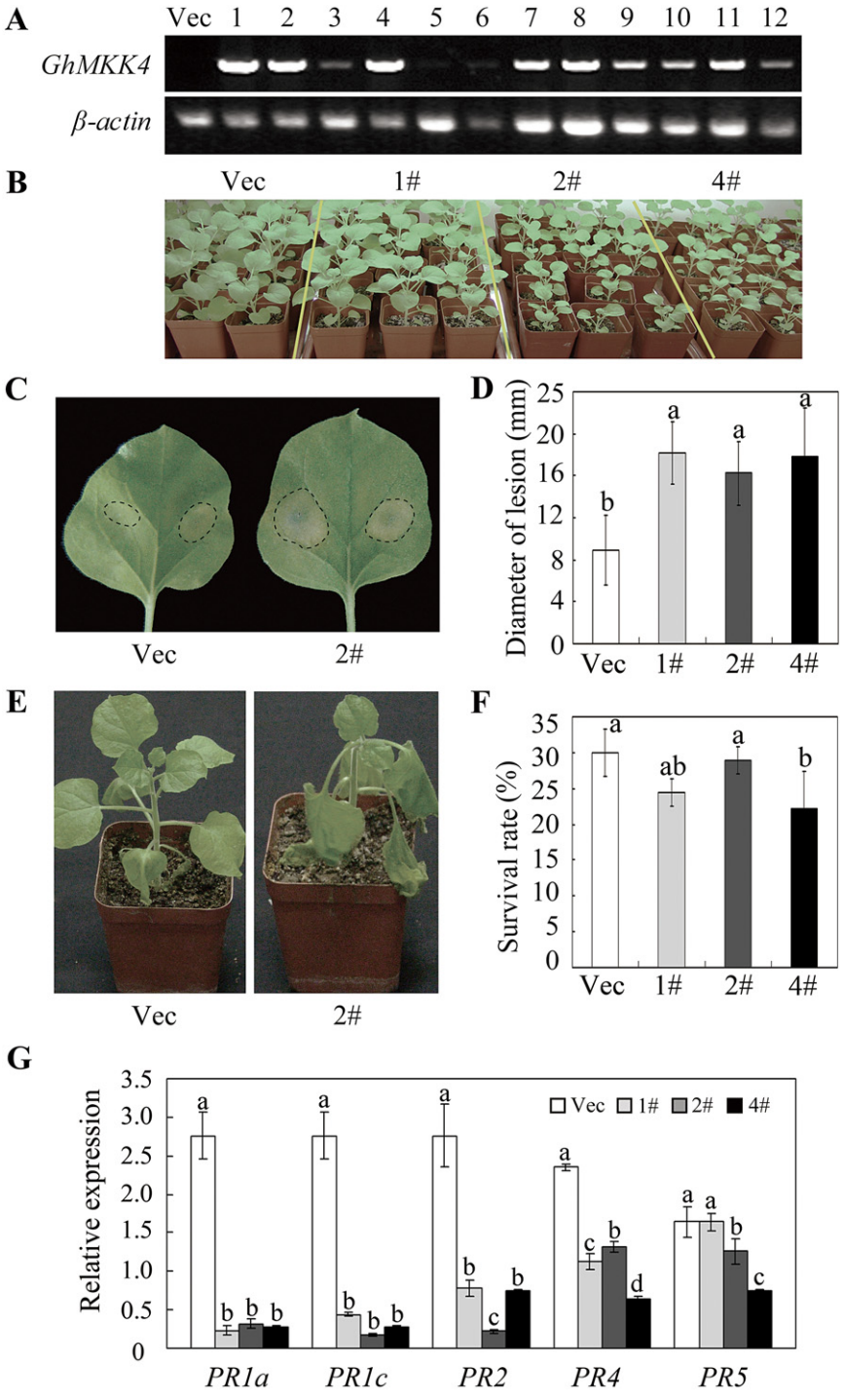


Fig. 4 Identification of transgenic plants and subsequent bacterial pathogen resistance analysis. (A) The evaluation of *GhMKK4* expression in the T1 progeny of transgenic plants. (B) The developmental phenotype of the transgenic plants. The photograph was taken approximately 6 weeks after the seedlings had been transplanted into the tubs. (C) The symptoms of detached leaves from 2-month-old T3 generation transgenic plants at 4 days after *Ralstonia solanacearum* injection. The lesion diameters are indicated in (D). Different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. (E) The phenotypes of the transgenic plants after inoculation with *R. solanacearum* using the trickle irrigation method. The survival rates of these plants are shown in (F). (G) The expression of the *pathogenesis-related* (*PR*) genes in transgenic plants as analysed by quantitative real-time polymerase chain reaction (qPCR). Different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. Each experiment was repeated at least three times. Vec, vector control; 1#, 2#, 4#, overexpressing lines.

between these plants during the drought or recovery periods, and the plant survival rate was similar in both groups (Fig. 6C,D). In addition, there was no significant difference in the rate of water loss during the drought treatment between OE and Vec plants (Fig. 6E). These results indicate that *GhMKK4*-overexpressing plants do not show significant abiotic stress tolerance.

Overexpression of *GhMKK4* affects ABA and GA signalling in transgenic plants

To identify the signal transduction mechanisms in which *GhMKK4* might be involved, we evaluated the germination of transgenic seeds treated with signalling molecules. When germinated on filter

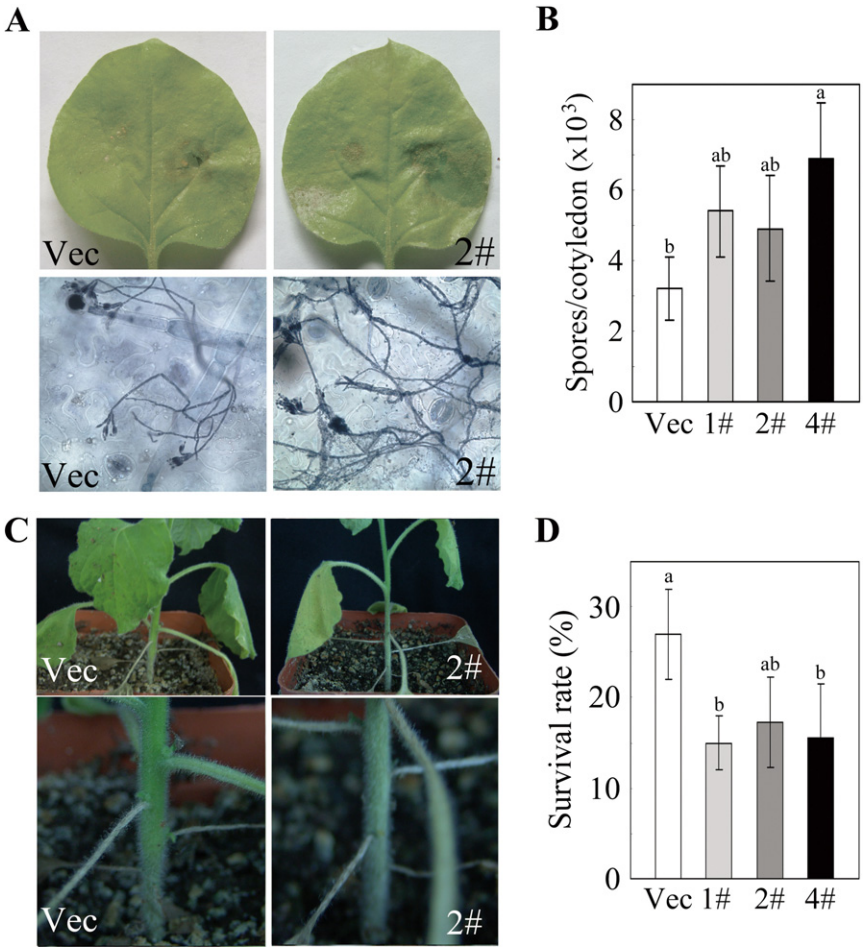


Fig. 5 *GhMKK4*-overexpressing plants exhibit enhanced susceptibility to *Rhizoctonia solani*. (A) Signs of disease on the detached leaves of transgenic plants 6 days after inoculation. The hyphae were visualized by trypan blue staining. (B) The number of spores per cotyledon at 6 days after *R. solani* infection. The data shown represent the means ± standard errors of three independent experiments. Different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. (C) The symptoms of the transgenic plants inoculated with *R. solani* using the trickle irrigation method. (D) The survival rates of the transgenic plants during *R. solani* infection using the trickle irrigation method. The data shown indicate the means ± standard errors of three independent experiments ($n \geq 30$). Different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. Vec, vector control; 1#, 2#, 4#, overexpressing lines.

paper moistened with 5 μM ABA, the green cotyledon rate of OE seeds was more than 10% lower than that of Vec seeds (Fig. 7A). To confirm this change in response to ABA, the post-germination growth of seedlings was measured on filter paper moistened with 0.1 or 0.3 μM ABA. There were no significant differences in the root length or fresh weight at 0.1 μM ABA between OE and Vec seedlings. However, when treated with 0.3 μM ABA, OE seedlings showed a noticeably shorter root length and lower fresh weight than Vec seedlings (Fig. 7B). On GA3 treatment, although there was no noticeable difference in the seed germination rate (data not shown), the OE seedlings exhibited a shorter root length and lower fresh weight post-germination (Fig. 7C).

Previous experiments have demonstrated that OE plants show an enhanced susceptibility to *R. solanacearum*. Here, we measured the ABA and GA levels in 2-month-old transgenic plants to better understand the basis of the increased susceptibility. Notably, the ABA levels in OE plants were higher than those in Vec plants (Fig. 7D). In contrast, the GA levels in OE plants were lower than those in Vec plants (except for line 1#), which was consistent with the developmental phenotype described previously (Fig. 4B). When infected with *R. solanacearum*, although the difference was not significant, the ABA levels in the OE plants were depressed slightly, and the ABA levels in the Vec plants were barely affected. Interestingly, the GA levels were significantly depressed in all lines except for line 4# after *R. solanacearum* treatment. However, there was no significant difference in the GA levels between the OE and Vec plants after treatment. Together, these results indicate that overexpression of *GhMKK4* affects ABA and GA signalling in transgenic plants.

Overexpression of *GhMKK4* increases SA-induced ROS accumulation in transgenic plants

SA is essential for the establishment of systemic acquired resistance (SAR) (Loake and Grant, 2007). In this study, we observed that SA affected H_2O_2 accumulation in transgenic plants, consistent with previous reports. Without any treatment, there was no significant difference in H_2O_2 accumulation in the plants (Fig. 8A). After SA treatment, both the Vec and OE plants exhibited a marked increase in H_2O_2 levels. However, H_2O_2 accumulation in OE plants was much higher than that in Vec plants. Moreover, the accumulation of superoxide anion (O_2^-), another primary component of

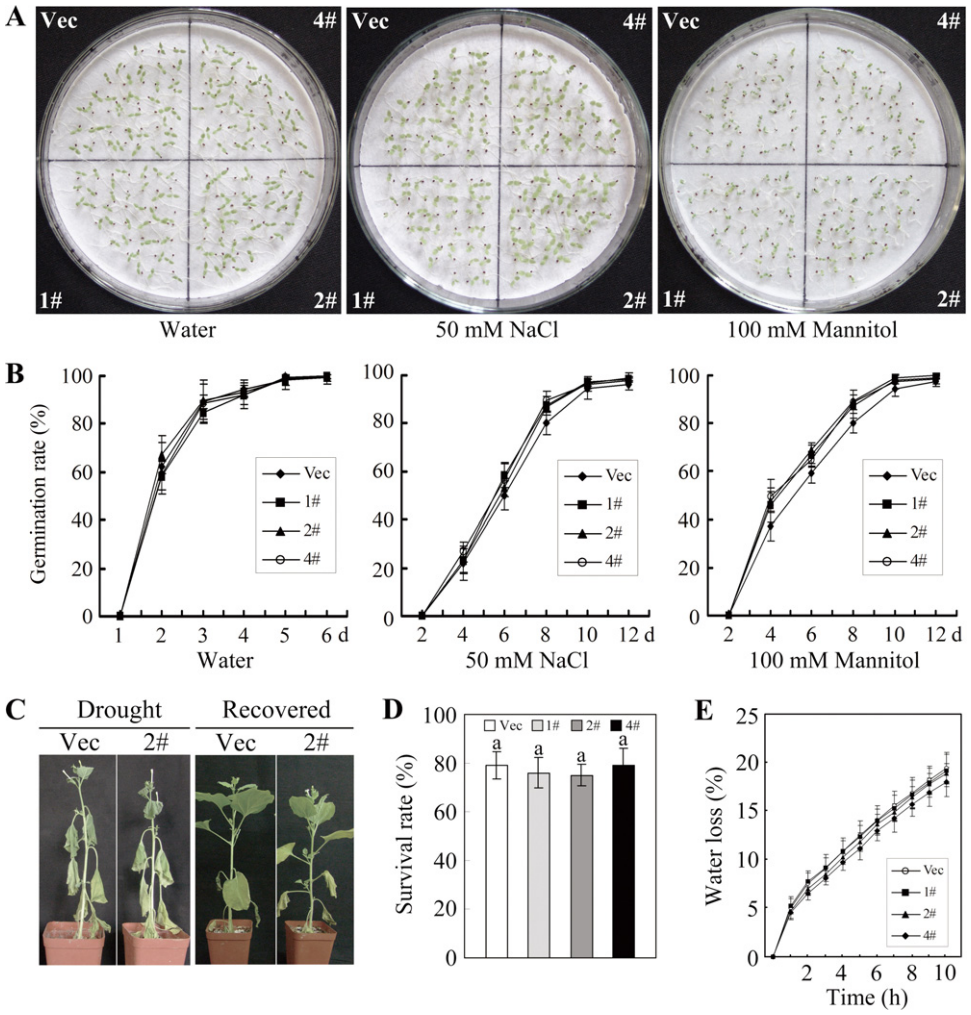


Fig. 6 Tolerance analysis of transgenic plants to salt and drought stresses. (A) The seed germination phenotype. The seeds were germinated on filter paper soaked with 50 mM NaCl or 100 mM mannitol. The photographs shown were taken approximately 14 days after the seeds had been sown. (B) The germination rates of the seeds under normal and stress conditions. Germination was scored daily, and the results of germination on filter paper soaked with 50 mM NaCl or 100 mM mannitol are presented. (C) The phenotypes of plants subjected to drought stress at the late vegetable stage. Water was withheld from transgenic plants for 12 days, and the plants were then watered for 2 days to allow recovery. (D) The survival rates of the transgenic plants under drought conditions. The data shown represent the means ± standard errors of three independent experiments ($n \geq 30$). (E) The water loss of detached leaves from the OE and Vec plants. The rates of water loss were calculated by the loss in fresh weight of the samples. The data shown represent the means ± standard errors of three independent experiments ($n = 6$). OE, overexpressing; Vec, vector control; 1#, 2#, 4#, overexpressing lines.

ROS, showed a similar pattern to that of H_2O_2 (Fig. S1, see Supporting information). Too much ROS accumulation can result in cell death. After SA treatment, the OE plants showed a more serious cell death phenotype than Vec plants, as visualized by trypan blue staining (Fig. 8B). To investigate the possible mechanisms underlying the increase in SA-induced ROS accumulation in OE plants, we evaluated the relative expression of genes that encode ROS-scavenging enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and the antioxidative enzyme glutathione-*S*-transferase (GST), as well as the ROS-producing enzymes, respiratory burst oxidase homologue (RbohA and RbohB), using qPCR (Fig. S2, see Supporting information). In the absence of SA treatment, the expression of *SOD*, *CAT*, *APX* and *GST* showed different levels of decrease in OE plants relative to Vec plants. After SA treatment, the expression of *SOD* in Vec plants and of *CAT* in both Vec and OE plants was decreased, but the difference in the expression of *SOD* and *CAT* between OE and Vec plants was not significant. The expression of *APX*, especially for lines 1# and 4#, showed a significantly low level in OE plants. By contrast, the expression of *GST*, *RbohA* and *RbohB* was relatively higher in OE plants than in Vec plants. To confirm these results, the activities of SOD, peroxidase (POD) and CAT were measured. Prior to SA treatment, there was no marked difference in the activities of these enzymes between OE and Vec plants. After SA treatment,

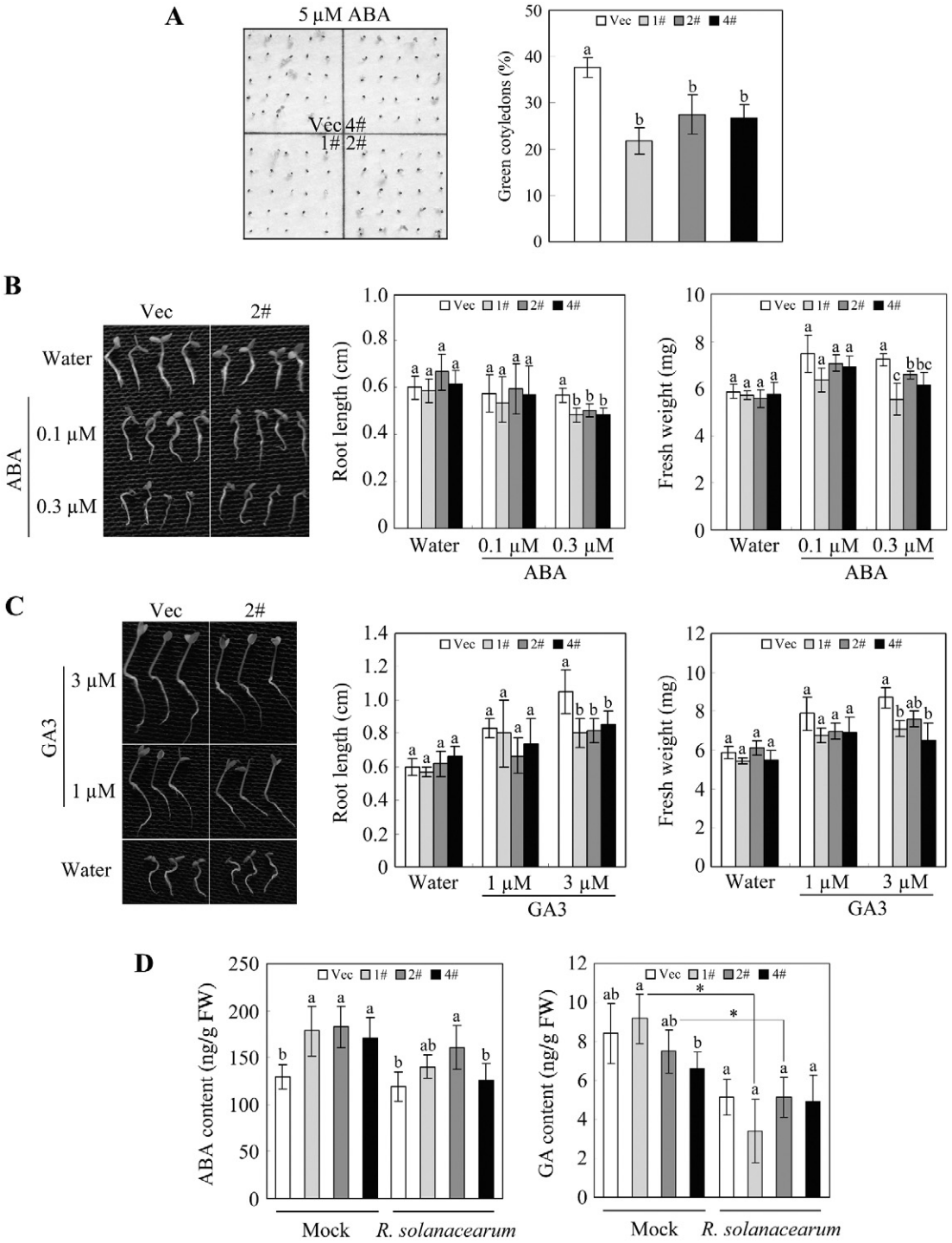


Fig. 7 The influence of *GhMKK4* overexpression on abscisic acid (ABA) and gibberellin (GA) signalling. (A) Seed germination under the ABA condition. The seeds germinated and developed green cotyledons on filter paper soaked with 5 μM ABA. (B) The post-germination growth of seedlings under the 0.1 or 0.3 μM ABA condition. The root length and fresh weight (weight of 10 seedlings) of the seedlings were recorded 10 days after sowing. (C) The post-germination growth of seedlings under the 1 or 3 μM GA3 condition. The root length and fresh weight (weight of 10 seedlings) of seedlings were recorded 7 days after sowing. (D) The ABA and GA levels in the transgenic plants determined by enzyme-linked immunosorbent analysis (ELISA) under normal and *Ralstonia solanacearum*-induced conditions. Leaves of 2-month-old plants were used for hormone measurement. The data shown indicate the means ± standard errors of three independent experiments. Different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. FW, fresh weight; Vec, vector control; 1#, 2#, 4#, overexpressing lines.

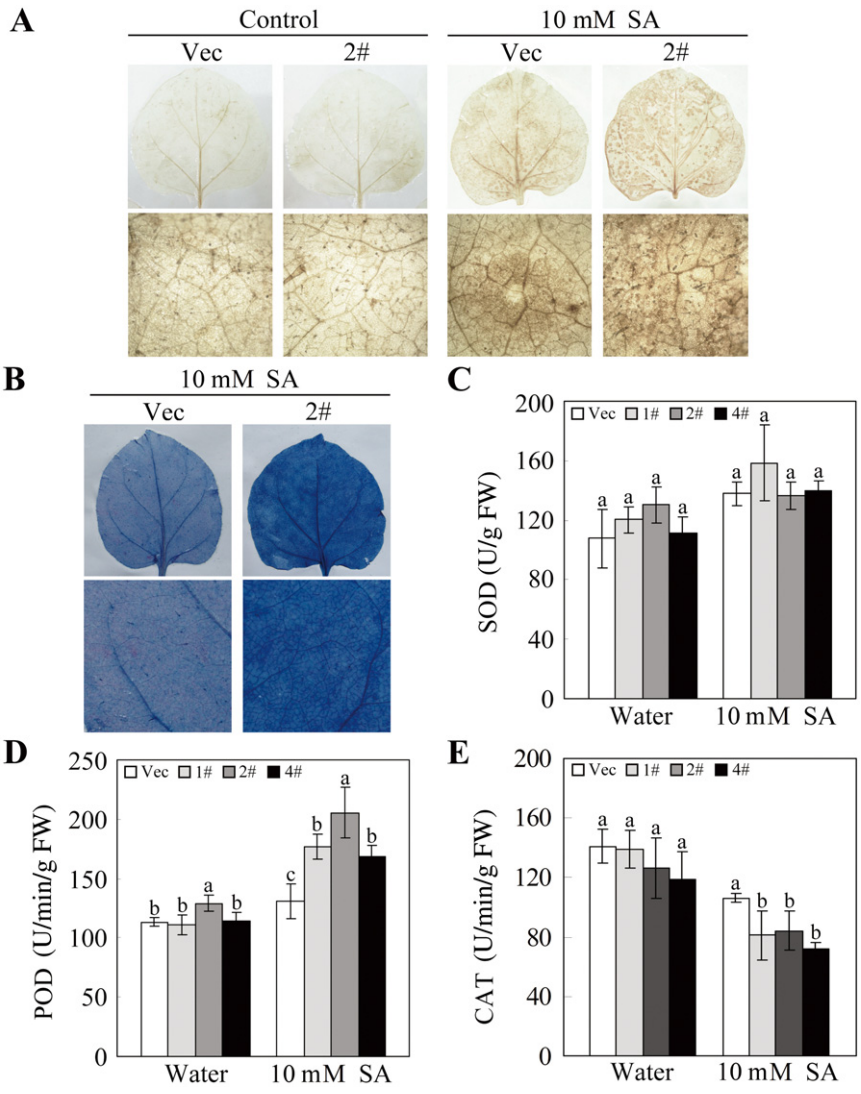


Fig. 8 The increased accumulation of H_2O_2 induced by salicylic acid (SA) in the overexpressing plants. (A) The accumulation of H_2O_2 in transgenic plants, visualized by 3,3-diaminobenzidine (DAB) staining under normal and SA-induced conditions. (B) The trypan blue staining of the leaves after SA treatment. (C–E) The activities of the reactive oxygen species (ROS)-scavenging enzymes under normal and SA-induced conditions. Leaves were treated with 10 mM SA for 2 h, and three leaves at least of each line were used. The data shown indicate the means ± standard errors of three independent experiments. Different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. FW, fresh weight; Vec, vector control; 1#, 2#, 4#, overexpressing lines.

SOD activity increased slightly, and POD activity was significantly induced (Fig. 8C,D). Interestingly, the activity of CAT was more significantly repressed in OE lines after SA treatment (Fig. 8E). These results indicate that *GhMKK4* overexpression confers increased SA-induced ROS accumulation in transgenic plants, probably by the inhibition of CAT activity.

Expression profiling of signalling pathway-related genes in transgenic plants

To test other possible signalling pathways during defence responses that involve *GhMKK4*, certain signalling pathway-related genes were analysed using qPCR after *R. solanacearum* treatment. As shown in Fig. 9, the expression of *1-aminocyclopropane-1-carboxylate oxidase* (*ACO*) (except line 4#) and *nitric oxide-associated 1* (*NOA1*), which are involved in ET and nitric oxide (NO) signalling, respectively, was lower in OE plants

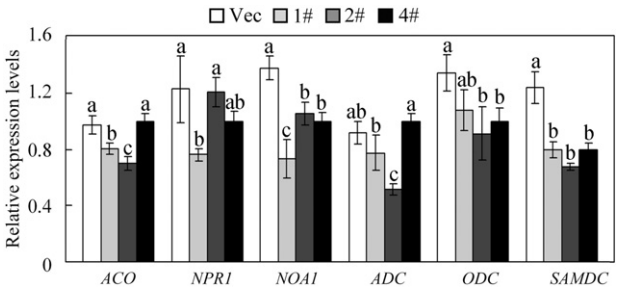


Fig. 9 Quantitative real-time polymerase chain reaction (qPCR) analysis of the expression of signalling-related genes in overexpressing (OE) and vector control (Vec) plants under *Ralstonia solanacearum*-inoculated conditions. Different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. *ACO*, *1-aminocyclopropane-1-carboxylate oxidase*; *ADC*, *arginine decarboxylase*; *NOA1*, *nitric oxide-associated 1*; *NPR1*, *NONEXPRESSER OF PR GENES1*; *ODC*, *ornithine decarboxylase*; *SAMDC*, *S-adenosylmethionine decarboxylase*; 1#, 2#, 4#, overexpressing lines.

than in Vec plants. The expression of *NONEXPRESSER OF PR GENES1* (*NPR1*), which is essential for SA-mediated signal transduction, did not show a significant difference between OE and Vec plants (except for line 1#). In addition, the polyamine synthesis-related genes *arginine decarboxylase* (*ADC*), *ornithine decarboxylase* (*ODC*) and *S-adenosylmethionine decarboxylase* (*SAMDC*) were tested. There was a slightly lower expression of *ADC* in OE plants than in Vec plants after treatment (except line 4#), whereas the transcriptional levels of *ODC* and *SAMDC* were much lower in OE plants than in Vec plants. These results suggest that *GhMKK4* may have negative effects on pathogen-induced polyamine synthesis.

DISCUSSION

As the key node of MAPK cascades, MAPKKs may integrate multiple signal transduction pathways and may be involved in various biological processes. In this study, we identified a novel group C MAPKK gene, *GhMKK4*, from cotton. As a result of the difficulty of genetic transformation in cotton, we performed the initial functional analyses of the gene by ectopic expression in *N. benthamiana*.

Most of the plant MAPKKs reported recently have shown positive roles in pathogen resistance, whereas negative effects on resistance are rare. For example, *AtMKK1* can be activated by pathogen elicitors and confers plant resistance to both virulent and avirulent bacterial pathogens (Meszaros *et al*., 2006; Teige *et al*., 2004). The overexpression of *Arabidopsis AtMKK7* contributes to the activation of plant basal resistance and SAR in plants (Zhang *et al*., 2007). Furthermore, *N. benthamiana NbMKK1* mediates the nonhost resistance to *Pseudomonas cichorii* (Takahashi *et al*., 2007b). As a rarely negative event, AtMKK1 and AtMKK2 can interact with MPK4 to negatively regulate the innate immune response in plants (Gao *et al*., 2008). In particular, the group C MAPKKs reported to date, including AtMKK4/MKK5 and rice OsMKK4, confer pathogen-associated molecular pattern (PAMP)-triggered defence responses (Asai *et al*., 2002; Kishi-Kaboshi *et al*., 2010). Our previous study has revealed that *GhMKK5*, belonging to group C MAPKKs, confers resistance to the bacterial pathogen *R. solanacearum* in transgenic plants, but increases the susceptibility to *P. parasitica* var. *nicotianae* Tucker (Zhang *et al*., 2012). In this study, *GhMKK4*-overexpressing plants were more susceptible to both bacterial and fungal pathogens than control (Vec) plants (Figs 4 and 5). These results provide further evidence of the negative roles of MAPKKs in disease resistance.

Emerging evidence has revealed that ABA plays a negative role in disease resistance. The resistance to the biotrophic pathogens *Hyaloperonospora parasitica* and *Blumeria graminis* was enhanced in the ABA biosynthesis mutants (Jensen *et al*., 2008; Mohr and Cahill, 2003). Similarly, ABA suppresses the resistance to necrotrophic pathogens, including *Botrytis cinerea*, *Fusarium oxysporum*, *Plectosphaerella cucumerina* and *Erwinia chrysanthemi* (Anderson *et al*., 2004; Asselbergh *et al*., 2008; Audenaert *et al*., 2002; Hernandez-Blanco *et al*., 2007). The gain-of-function mutation *cds2-1D* leads to the constitutive accumulation of high levels of endogenous ABA, which enhances the susceptibility to *Pseudomonas syringae* (Fan *et al*., 2009). However, some reports have also revealed that ABA may positively regulate the resistance to some pathogens (Adie *et al*., 2007; Kaliff *et al*., 2007; Ton and Mauch-Mani, 2004; Wawrzynska *et al*., 2008). In this study, OE plants showed higher ABA levels than Vec plants. Therefore, we infer that the relatively higher ABA levels may be involved in the regulation of the enhanced pathogen susceptibility of OE plants. Furthermore, OE plants exhibited relatively lower pathogen-induced expression of *ODC* and *SAMDC*, which are rate-limiting enzymes in polyamine synthesis, suggesting that there is a lower pathogen-induced polyamine level in OE plants. Polyamines have been suggested to play important roles in pathogen resistance (Walters, 2003). Together, these mechanisms may explain the decreased disease resistance in OE plants. The resistance mechanisms of plants to pathogens are very sophisticated. The molecular mechanisms through which *GhMKK4* is involved in the negative regulation of the pathogen response have still not been elucidated fully and further investigation is ongoing.

A large body of evidence has shown that exogenous SA treatment can induce the accumulation of H_2O_2 in plant tissues (Agarwal *et al*., 2005; Chao *et al*., 2010; Harfouche *et al*., 2008; Rao *et al*., 1997). In this study, OE plants exhibited greater H_2O_2 accumulation after SA treatment. One possible explanation is that SA inhibits the H_2O_2-degrading activity of CAT in OE plants. However, the activity of POD, the other major enzyme involved in the scavenging of H_2O_2, was elevated in OE plants after SA treatment. The elevated activity of POD may be a result of induction by increased H_2O_2. These results are consistent with those of previous studies (Fang *et al*., 2009). SA treatment can initiate lipid peroxidation, cell death and the activation of several other defence mechanisms through H_2O_2 (Rao *et al*., 1997). In this study, SA treatment induced greater accumulation of H_2O_2 and more serious cell death in OE plants than in Vec plants. Thus, *GhMKK4* may be involved in an H_2O_2-mediated defence mechanism, which may compensate for the enhanced susceptibility to the pathogens in OE plants.

It is noteworthy that GhMKK4 has high homology (77.11%) to GhMKK5, which has been identified previously. However, their functions are somewhat different. According to the amino acid alignment, although most subdomains are the same, some amino acids in subdomains between GhMKK4 and GhMKK5 are different, including subdomains I, VIa, VII and XI (Fig. 1A). In particular, several amino acids in the docking sites and one amino acid in the S/TXXXXXS/T motif of GhMKK4 and GhMKK5 are different. This information suggests that GhMKK4 and GhMKK5 may have

different interactional substrates, and may be activated by different upstream MAPKKKs. In addition, several amino acids in the docking sites and A-loops of GhMKK4 and GhMKK5 are different from those of AtMKK4 and AtMKK5. This suggests that GhMKK4 and GhMKK5 may have somewhat different roles from AtMKK4 and AtMKK5. Although there is high homology of GhMKK4 and GhMKK5 with AtMKK4 and AtMKK5, their genetic backgrounds are quite different. Furthermore, their overexpression in plants may result in various biochemical or physiological changes. MAPKKs classified into the same subgroups based on sequence similarity may not necessarily have identical functions (Lee *et al*., 2004). This may also explain the contrary role of the other group C member NtMEK2 from GhMKK4 in polyamine synthesis during pathogen resistance (Jang *et al*., 2009). Although the cascades in which GhMKK4 is involved are currently unknown, this should be the subject of future studies.

MAPKKs are the central components in plant MAPK cascades and play important roles in abiotic stresses, such as salt, drought and low temperatures. In this study, OE plants did not show significant tolerance to salt or drought stress. Under NaCl treatment, although there was no significant difference between the overall germination rates of the transgenic seeds during germination, a greater number of OE seeds germinated slightly earlier at the very beginning of treatment. Under mannitol treatment, although the difference was not significant, OE seeds showed a relatively higher germination rate than Vec seeds. Therefore, we cannot conclude that there is no response to salt or drought stress in OE plants. We assume that the potentially enhanced salt or drought stress tolerance may be impaired by a negative regulatory mechanism existing in the response to biotic stress. Furthermore, previous studies have reported that the overexpression of heterologous *SAMDC* in plants generally results in an improvement in the tolerance to abiotic stress, including salt (Roy and Wu, 2002) and drought (Waie and Rajam, 2003). Here, OE plants showed decreased expression of *SAMDC* and *ODC*, which may be another explanation for the lack of significant salt or drought stress tolerance. In addition, OE plants showed relatively lower GA levels than Vec plants, which means that OE seeds should show stunted germination or seedling growth relative to Vec seeds. However, there were no significant differences in germination or seedling growth between OE and Vec plants under stress conditions. Therefore, *GhMKK4* may have potential effects on tolerance to salt or drought stress.

As a result of the different genetic background between *N. benthamiana* and cotton, altered expression of *GhMKK4* in cotton itself may result in different phenotypes. In this study, overexpression of *GhMKK4* did not enhance the tolerance to stresses, which probably limits its application in genetic improvement. However, these results may provide good reference, and the potential defence mechanism of *GhMKK4* may provide clues on cotton genetic improvement.

EXPERIMENTAL PROCEDURES

Plant material, growth conditions and treatments

Cotton (*G. hirsutum* L. cv. lumian 22) seeds were germinated in a wet cloth, and the seedlings were maintained in hydroponic culture for growth under glasshouse conditions at 26 ± 1 °C with a 16-h light/8-h dark cycle (relative humidity of 60%–75%). *Nicotiana benthamiana* seeds were placed on moist filter paper under glasshouse conditions for germination. Three- or four-leaf-stage *N. benthamiana* seedlings were transplanted into pots with soil and maintained under glasshouse conditions. For NaCl or mannitol treatments, uniformly developed cotton seedlings were sprayed with and dipped in these two solutions. For H_2O_2, ABA, SA, MeJA, ethephon and GA3 treatment, the uniformly developed cotton seedlings were sprayed with each chemical and then transferred to a box to maintain humidity. For pathogen treatment, 7-day-old cotton seedlings were inoculated with conidial suspensions of *R. solani* (10^5 conidia/mL) using the root dip method. The treated cotyledons were collected for RNA extraction. Each treatment was repeated at least twice.

Gene isolation, vector construction and genetic transformation

The *GhMKK4* gene was isolated as described previously (Shi *et al*., 2011). The primer sequences used are provided in Table S1 (see Supporting Information). The vector construction and genetic transformation were performed as described by Zhang *et al*. (2012). Plants transformed with vector pBI121 were used as controls. The T3 progeny of OE and Vec plants were used for further experiments.

Semi-quantitative RT-PCR and qPCR analyses

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol, and used for first-strand cDNA synthesis with a reverse transcriptase system (TransGen Biotech, Beijing, China). Semi-quantitative RT-PCR was used to detect the expression levels of *GhMKK4* under different treatments. The expression of the cotton *18S rRNA* gene was used as a control. qPCR was performed using the SYBR PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China) in a 25-μL volume on a CFX96TM Real-time System (Bio-Rad, Hercules, CA, USA), as described previously (Yu *et al*., 2012). The house-keeping gene *N. benthamiana β-actin* was used as control gene for qPCR analysis. The relative expression levels of the stress-related genes were determined using the $2^{-\Delta\Delta CT}$ method.

Pathogen infection assay

The bacterial pathogen *R. solanacearum* was cultured overnight at 37 °C in Luria–Bertani (LB) broth, harvested by centrifugation and resuspended in sterile tap water. The fungal pathogen *R. solani* was cultured on potato dextrose agar (PDA) medium at 28 °C for 2 weeks, and the spores were then suspended in 1% glucose. For the infection assay, detached uniform leaves from 2-month-old T3 generation OE and Vec plants were inoculated with suspensions of *R. solanacearum* bacteria [optical density at 600 nm (OD_{600}) = 0.6] or *R. solani* spores (10^5 spores/mL). At least three leaves

from each line were detached for the infection assay. The infected leaves were kept in a transparent box under glasshouse conditions. Furthermore, the infection was confirmed by inoculation with the above suspensions of *R. solanacearum* bacteria or *R. solani* spores using the trickle irrigation method. The inoculated plants were kept in a moist chamber. Bacterial growth was monitored by performing serial dilutions onto King's B agar medium. The disease resistance analysis was repeated three times.

3,3-Diaminobenzidine (DAB), nitroblue tetrazolium (NBT) and trypan blue staining assays

The DAB, NBT and trypan blue staining assays were performed as described previously (Zhang *et al*., 2011, 2012).

Salt and drought stress analyses

For the germination analysis, T3 generation OE and Vec seeds were placed on filter papers moistened with NaCl (50 and 200 mM) or mannitol (100 and 200 mM) solutions. The germination percentage was measured daily. For salt treatment, 2-month-old OE and Vec plants were irrigated with NaCl solutions (50 and 200 mM) for over 2 weeks, and the survival rate (the number of surviving plants relative to the total number of treated plants) was recorded. For the drought treatment, water was withheld completely from the plants for 12 days, and the plants were then watered regularly for 2 days to allow them to recover. For the water loss measurements, detached leaves from OE and Vec plants were incubated at 37 °C and weighed on an electronic balance at the indicated times. The rate of water loss was calculated as the percentage of the initial fresh weight at each time point. The salt and drought stress analyses were repeated at least three times.

Seed germination and growth in response to exogenous ABA and GA

For the germination analysis, the seeds were placed on filter papers moistened with ABA (5 μM) or GA3 (5 μM) solutions. The germination percentage was measured daily. To determine the seedling growth rate, the seeds were placed on filter papers moistened with ABA (0.1 and 0.3 μM) or GA3 (1 and 3 μM) solutions for germination and growth. The plates were maintained under glasshouse conditions for more than 10 days, and the root length and fresh weight (weight of 10 seedlings) were recorded.

Quantification of endogenous ABA and GA by enzyme-linked immunosorbent assay (ELISA)

The samples were homogenized in liquid nitrogen and extracted in ice-cold phosphate-buffered saline (PBS, pH 7.4). After centrifugation at 4000 ***g*** (4 °C) for 20 min, the supernatant was stored at −20 °C for ELISA. ELISA was performed on a 96-well microtitration plate, as described previously (Yang *et al*., 2001).

Antioxidative enzyme activity assays

For the enzyme activity assays, 0.5 g of leaf tissue was homogenized in 5 mL of ice-cold extraction buffer (50 mM PBS, pH 7.8) using a prechilled mortar and pestle on ice. The homogenate was centrifuged at 12 000 ***g*** for 20 min at 4 °C. The resulting supernatant was collected, and the enzymatic activities of SOD, POD and CAT were measured, as described previously (Kong *et al*., 2011; Yang *et al*., 2008).

Statistical analysis

The results are expressed as the mean ± standard error (SE) of triplicate experiments. Statistical significance between measurements for different treatments or times was subjected to Duncan's multiple range test with an analysis of variance (ANOVA) using Statistical Analysis System (SAS) version 9.1 software (SAS Institute, Cary, NC, USA).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 The accumulation of superoxide anion (O_2^-) induced by salicylic acid (SA) in the transgenic plants. The content of superoxide anion was visualized by nitroblue tetrazolium (NBT) staining under normal and SA-induced conditions. Leaves were treated with 10 mM SA for 2 h, and three leaves at least of each line were used. Vec, vector control; 2#, overexpressing lines.

Fig. S2 The expression patterns of genes related to reactive oxygen species (ROS) production and scavenging in transgenic plants under normal and salicylic acid (SA)-induced conditions analysed by quantitative real-time polymerase chain reaction (qPCR). Leaves were treated with 10 mM SA for 2 h, and three leaves at least of each line were used. Vec, vector control; 1#, 2#, 4#, overexpressing lines.

Table S1 The primers used in this study.