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Overexpression of *BnWRKY33* in oilseed rape enhances resistance to *Sclerotinia sclerotiorum*

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SUMMARY

Sclerotinia sclerotiorum causes a devastating disease in oilseed rape (*Brassica napus*) resulting in a tremendous yield loss worldwide. Studies on various host–pathogen interactions have shown that plant WRKY transcription factors are essential for defence. For the *B. napus*–*S. sclerotiorum* interaction, little direct evidence has been found with regard to the biological roles of specific *WRKY* genes in host resistance. In this study, we isolated a *B. napus WRKY* gene, *BnWRKY33*, and found that the gene is highly responsive to *S. sclerotiorum* infection. Transgenic *B. napus* plants overexpressing *BnWRKY33* showed markedly enhanced resistance to *S. sclerotiorum*, constitutive activation of the expression of *BnPR1* and *BnPDF1.2*, and inhibition of H_2O_2 accumulation in response to pathogen infection. Further, we isolated a mitogen-activated protein (MAP) kinase substrate gene, *BnMKS1*, and found that not only can BnWRKY33 interact with BnMKS1, which can also interact with BnMPK4, using the yeast two-hybrid assay, consistent with their collective nuclear localization, but also BnWRKY33, BnMKS1 and BnMPK4 are substantially and synergistically expressed in response to *S. sclerotiorum* infection. In contrast, the three genes showed differential expression in response to phytohormone treatments. Together, these results suggest that *BnWRKY33* plays an important role in *B. napus* defence to *S. sclerotiorum*, which is most probably associated with the activation of the salicylic acid (SA)- and jasmonic acid (JA)-mediated defence response and inhibition of H_2O_2 accumulation, and we propose a potential mechanism in which BnMPK4–BnMKS1–BnWRKY33 exist in a nuclear localized complex to regulate resistance to *S. sclerotiorum* in oilseed rape.

Keywords: *Brassica napus*, disease resistance, *Sclerotinia sclerotiorum*, WRKY33 transcription factor.

INTRODUCTION

Oilseed rape (*Brassica napus*) is an agriculturally important oilseed crop. Its contribution to global oilseed production is considerable: approximately 64 million metric tonnes (MMT) were produced worldwide in 2012, with China producing about 14 MMT and Canada, the European Union, India and Australia being the other major contributors [Statistics Division of the Food and Agriculture Organization (FAOSTAT) data 2012, http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor]. In oilseed rape farming, Sclerotinia disease, caused by *Sclerotinia sclerotiorum*, is one of the most devastating diseases worldwide. It causes rotting of leaves, stems and pods, resulting in a tremendous loss in seed yield: for example, 10%–20% of yield losses every year, and up to 80% in severely infected fields in China (Wu *et al*., 2013). No immune or highly resistant germplasm in *B. napus* has been reported to date, and few genetic sources of host resistance to the pathogen are available to breeders (Liu S *et al*., 2005). Disease management depends heavily on the application of fungicides to the crop, but this may cause environmental contamination, increase farming costs and may be ineffective because of the difficulties associated with the application of sprays to thick canopies and the lack of suitable forecasting methods to enable the timely application of fungicides. Attempts have been made to engineer disease resistance in important crop plants. However, the molecular mechanisms of host defence to *S. sclerotiorum* remain poorly understood in the *B. napus*–*S. sclerotiorum* interaction, which restricts the engineering of resistance by transgenic approaches.

Plant defence responses include the transcriptional control of the expression of stress-responsive genes (Chen and Chen, 2002; Chen *et al*., 2002; Maleck *et al*., 2000; Mysore *et al*., 2002), including a number of transcription factors (TFs) whose abundance is altered as a result of pathogen challenge. Plant WRKY TFs, proteins containing WRKY zinc-finger motifs, regulate many plant defence responses to diverse biotic and abiotic stresses (Chen and Chen, 2002; Eulgem and Somssich, 2007). The WRKY domain is defined by the conserved amino acid sequence WRKYGQK, and WRKY proteins can be classified into three groups according to the number of WRKY domains and the characteristic of their zinc-finger-like motif (Eulgem *et al*., 2000). There is a large body of indirect evidence implicating plant WRKY proteins in plant defence responses to pathogens. For example, in *Arabidopsis thaliana*, it has been observed that 49 *AtWRKY* genes are regulated by *Pseudomonas syringae* or treatment with salicylic acid (SA), a defence signalling molecule (Chen and Chen, 2002; Dong *et al*.,

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2003). In *Brassica napus*, the transcript abundance of 13 tested *BnWRKY* genes is responsive to *S. sclerotiorum* and one or more hormones, including SA, jasmonic acid (JA) and abscisic acid (ABA) (Yang *et al*., 2009).

Transgenic approaches have provided further direct evidence that plant *WRKY* genes are involved in plant defence. In *A. thaliana*, transient expression of *AtWRKY29* in leaves leads to reduced disease symptoms (Asai *et al*., 2002), and overexpression of *AtWRKY18* and *AtWRKY70* results in increased resistance to virulent pathogens (Chen and Chen, 2002; Li *et al*., 2004). In addition, the recessive RRS1-R gene *RRS1*, which encodes the AtWRKY52 protein, confers resistance to the bacterial pathogen *Ralstonia solanacearum* (Deslandes *et al*., 2002; Lahaye, 2002). In particular, a study has shown that *AtWRKY33* is required for resistance to *Botrytis cinerea* (Zheng *et al*., 2006). More recently, the overexpression of *AtWRKY28* and *AtWRKY75* in *Arabidopsis* has been shown to enhance resistance to *S. sclerotiorum* (Chen *et al*., 2013).

In rice, plants overexpressing *OsWRKY13* showed enhanced resistance to both bacterial *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and fungal *Magnaporthe grisea* infections (Qiu D *et al*., 2007, 2008). Similarly, overexpression of *OsWRKY53* and *OsWRKY45* led to enhanced resistance to *M. grisea* (Chujo *et al*., 2007; Shimono *et al*., 2007). Transgenic rice plants overexpressing *OsWRKY03* and *OsWRKY71* showed enhanced resistance to *Xoo* (Chujo *et al*., 2008; Liu XQ *et al*., 2005). In contrast, overexpression of *OsWRKY62* compromised Xa21-mediated resistance to *Xoo* (Peng *et al*., 2008). In tobacco, virus-induced silencing of three *NtWRKY* genes compromises N gene-mediated resistance to *Tobacco mosaic virus* (Liu *et al*., 2004).

Despite the obvious importance of plant WRKYs in various host–pathogen interactions, there have been no reports to date investigating the biological functions of WRKY TFs directly involved in host defence in the *B. napus*–*S. sclerotiorum* interaction, one of the most important host–pathogen interactions, by a transgenic approach. In *Arabidopsis*, *AtWRKY33* plays an important role in resistance to *Botrytis cinerea*, a necrotrophic fungus closely related to *S. sclerotiorum* (Zheng *et al*., 2006), and AtWRKY33 has been found to interact with AtMKS1, an AtMPK4 substrate (Andreasson *et al*., 2005). Further, a previous study has shown that *B. napus* plants overexpressing *BnMPK4*, a *B. napus* homologous gene of *AtMPK4*, show enhanced resistance to *S. sclerotiorum* (Wang *et al*., 2009). Therefore, we investigated the involvement of *BnWRKY33* in defence responses against *S. sclerotiorum* infection. We found that *B. napus* transgenic lines overexpressing *BnWRKY33* showed enhanced resistance to *S. sclerotiorum*, and that both BnMPK4 and BnWRKY33 interact with BnMKS1. Mitogen-activated protein (MAP) kinase signalling plays a central role in signal transduction mechanisms, linking upstream receptors and downstream targets and leading to the rapid activation of defence responses on recognition of invading pathogens. Our data suggest a potential mechanism in which BnMPK4–BnMKS1–BnWRKY33 exist in a nuclear localized complex to regulate resistance to the pathogen in oilseed rape.

RESULTS

Sequence analysis of *BnWRKY33* and its response to *S. sclerotiorum* infection in oilseed rape

A full-length cDNA was cloned from a cDNA library of *B. napus* cv. Zhongshuang9 by a homology cloning approach, was consequently identified as a homologous gene of *AtWRKY33* and was designated as *BnWRKY33* (GenBank accession no. KF712488). *BnWRKY33* encodes 483 deduced amino acid residues with a calculated molecular mass of 53 kDa and a predicted pI of 8.37.

The deduced amino acid sequence of *BnWRKY33* was used in a BLASTP search of the National Center for Biotechnology Information (NCBI) database. As shown in Fig. S3 (see Supporting Information), alignment of the 10 top-scoring matches with the BnWRKY33 sequence demonstrated that all 11 proteins are highly similar and that BnWRKY33 belongs to group 1 of the WRKY superfamily of plant TFs based on the presence of two WRKY domains (Eulgem *et al*., 2000). The two WRKY domains with their $Cx_4Cx_{22/23}HxH$ zinc-finger motifs are highly conserved in these sequences and the C-terminal WRKY domain contains a consensus aspartic acid (Asp) residue at the same position, which may be a critical residue for interaction with proteins containing a conserved VQ motif (Cheng *et al*., 2012). In contrast, at the relatively conserved position of N-terminal regions, these proteins contain four to five serine–proline (Ser-Pro) residues that are potential MAP kinase phosphorylation sites (Liu and Zhang, 2004; Sharrocks *et al*., 2000). In addition, as expected from TFs, all these proteins contain highly conserved putative nuclear localization signals (NLSs) predicted by the PSORT II program.

In order to determine whether *BnWRKY33* functions in defence responses to *S. sclerotiorum*, we used the *B. napus* cv. Zhongshuang9, with higher resistance to *S. sclerotiorum*, to detect the expression of *BnWRKY33* in response to pathogen infection, employing quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The results showed that *BnWRKY33* transcription was induced rapidly and strongly by pathogen infection (Fig. 1). In particular, the transcript level of *BnWRKY33* increased by an average of nine-fold over noninoculated controls at 24 and 48 h after pathogen inoculation, indicating that *BnWRKY33* is highly responsive to *S. sclerotiorum* in *B. napus*. These primary results led us to conduct further studies on *BnWRKY33*.

Transgenic plants overexpressing *BnWRKY33* show enhanced resistance to *S. sclerotiorum*

To further functionally characterize *BnWRKY33*, we overexpressed the gene in *B. napus* and investigated its possible function in

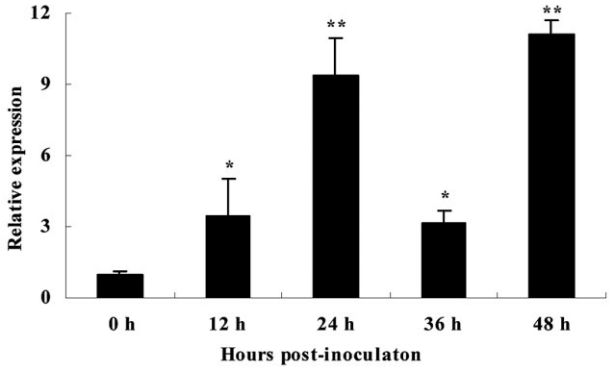

Fig. 1 Response of *BnWRKY33* to *Sclerotinia sclerotiorum* infection. Relative expression levels of *BnWRKY33* in *Brassica napus* were determined by real-time quantitative polymerase chain reaction at 0, 12, 24, 36 and 48 h post *S. sclerotiorum* inoculation. Values are means of three replicates. The error bars show the standard deviation. The significances of the gene expression differences between each time point and the 0-h time point are indicated: **(Student's *t*-test, $P > 0.01$) or *(Student's *t*-test, $P > 0.05$).

resistance to *S. sclerotiorum*. The full-length *WRKY33* cDNA was cloned behind the cauliflower mosaic virus (CaMV) 35S promoter and transformed into *B. napus* plants (Fig. 2a). Hygromycin and PCR were used to screen *BnWRKY33* transgenic lines. Three independent transgenic lines (6, 8 and 10) showed much higher levels of expression of the *BnWRKY33* gene than the untransformed control by semi-quantitative RT-PCR analysis (Fig. 2b), and were used for further analysis.

To test the effect of the transgene on resistance to *S. sclerotiorum*, T2 generation transgenic plants were selected by hygromycin screening and confirmed by PCR for the presence of the *BnWRKY33* transgene. Leaves from these PCR-positive plants and their untransformed controls at the six-true-leaf stage were used for inoculation with *S. sclerotiorum* mycelial plugs. The results indicated that *BnWRKY33*-overexpressing plants developed less severe disease symptoms and sustained less tissue damage than untransformed controls (Fig. 2c). Investigation of disease progression showed that soft-rotting necrosis occurred as early as 24 h post-inoculation (hpi), and the lesion size differences between the transgenic plants and untransformed controls became apparent at 36 hpi and up to a maximum at 48 hpi [Figs 2d and S4 (see Supporting Information)], suggesting that transgenic plants did not support rapid lesion expansion when compared with the untransformed controls. The difference in the rate of lesion expansion suggests that BnWRKY33-mediated defences appear to inhibit or delay the spread of the pathogen. Ultimately, disease symptoms were limited to leaves that had been inoculated and fungal spread virtually stopped after 72 hpi. These results show that overexpression of *BnWRKY33* triggers plant resistance to *S. sclerotiorum* infection.

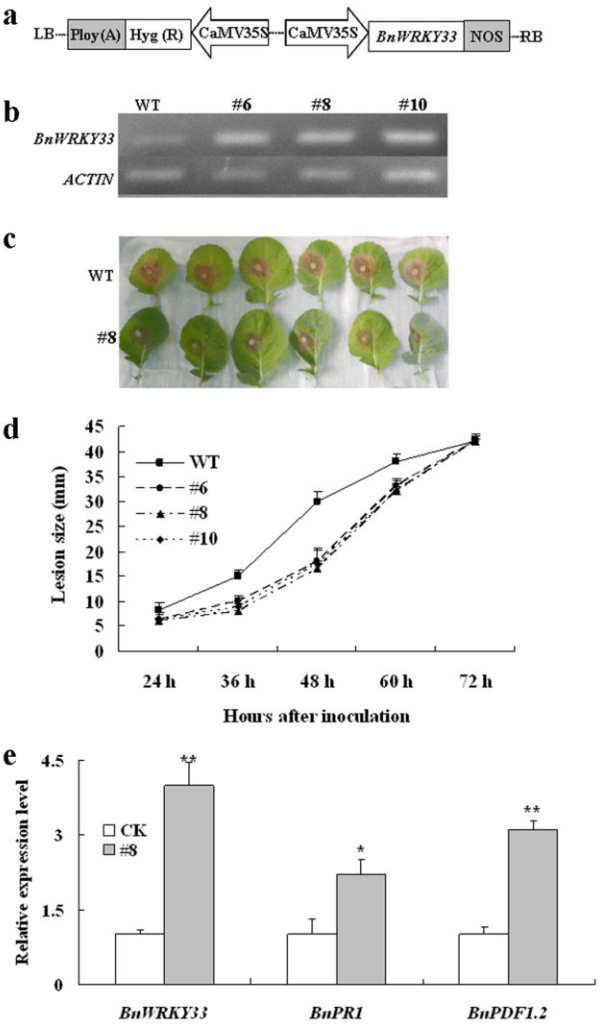

Fig. 2 Characterization of *BnWRKY33*-overexpressing lines. WT, untransformed wild-type control; #6, #8 and #10, three independent *BnWRKY33* transgenic T2 lines. (a) Diagram of the plasmids used in this study. CaMV35S, cauliflower mosaic virus 35S promoter; NOS, terminator. (b) Validation of *BnWRKY33*-overexpressing lines at transcription levels revealed by reverse transcription-polymerase chain reaction (RT-PCR). (c) Disease responses of inoculated plants at 48 h post-inoculation (hpi). Photographs were taken of leaves from three plants of WT and three hygromycin- and PCR-positive plants of line 8. (d) Disease progression is shown from 24 to 72 hpi. Error bars indicate standard deviations. Differences in susceptibility between WT and the transgenic lines were significant ($P < 0.05$) from 36 to 60 hpi. (e) Relative expression levels of *BnWRKY33*, *BnPDF1.2* and *BnPR1* were quantified by real-time qPCR. Values are means of three replicates. The error bars show the standard deviation. The significances of the gene expression differences between line 8 and WT (CK) are indicated: **(Student's *t*-test, $P > 0.01$) or *(Student's *t*-test, $P > 0.05$).

To investigate whether overexpression of *BnWRKY33* leads to expression changes in defence marker genes, we detected the transcript abundance of *BnPDF1.2* and *BnPR1* using quantitative RT-PCR. Although expression levels of *BnWRKY33* in the transgenic plants were significantly elevated by about four-fold,

the levels of *BnPR1* and *BnPDF1.2* increased by about 2.5-fold and 3.3-fold, respectively (Fig. 2e). These results show that overexpression of *BnWRKY33* in *B. napus* leads to activation of *BnPR1* and *BnPDF1.2* in the absence of induction by chemicals or pathogen treatment. The two genes *PDF1.2* and *PR1* are considered as marker genes for the JA-mediated and SA-mediated defence pathways, respectively (Brodersen *et al*., 2006; Penninckx *et al*., 1996; Thomma *et al*., 1998). Thus, our results suggest that *BnWRKY33* overexpression may activate the SA- and JA-mediated defence responses.

Transgenic plants overexpressing *BnWRKY33* inhibit H_2O_2 accumulation in response to *S. sclerotiorum* infection

The generation of reactive oxygen species (ROS, e.g. O_2^- and H_2O_2) is well documented as one of the earliest and most universal responses of plants to biotic stress, and H_2O_2 in particular, as a major component of ROS, is involved in a broad range of plant physiological processes, including disease defence. To further understand the resistance mechanism of transgenic plants overexpressing *BnWRKY33*, we collected inoculated leaves of transgenic plants and untransformed wild-type (WT) controls at time points of 0, 6, 12, 18 and 24 hpi and stained them with 3,3-diaminobenzidine (DAB) to visualize H_2O_2 accumulation. As shown in Fig. 3, a red–brown precipitate in inoculated leaves occurred in the untransformed WT control as early as 6 hpi but, in *BnWRKY33* transgenic plants, a red–brown precipitate occurred at 18 hpi, suggesting that the overexpression of *BnWRKY33* delayed the production of H_2O_2. The results demonstrate that transgenic plants overexpressing *BnWRKY33* show inhibited H_2O_2 accumulation in response to *S. sclerotiorum* infection.

Isolation of *BnMKS1* from *B. napus* and its sequence analysis

A previous study has demonstrated that *B. napus* plants overexpressing *BnMPK4* show enhanced resistance to *S. sclerotiorum* (Wang *et al*., 2009). In *Arabidopsis*, AtWRKY33 was found to interact with AtMKS1, a substrate of AtMPK4, and a homologue of BnMPK4. Thus, we investigated whether BnWRKY33 is capable of interacting with BnMKS1, which may, in turn, interact with BnMPK4. For *B. napus*, however, there is no information on the sequence of *BnMKS1*. To continue our studies, we first set out to clone the gene *BnMKS1*.

Brassica napus originated from a spontaneous hybridization of *Brassica rapa* L. (syn. *campestris*; AA, 2*n* = 20) and *Brassica oleracea* L. (CC, 2*n* = 18). Here, based on the *MKS1* homologous sequence from *B. oleracea*, a full-length cDNA was cloned from a cDNA library of *B. napus* by PCR, consequently identified as the homologous gene of *AtMKS1* and designated as *BnMKS1* (GenBank accession no. KF712487). *BnMKS1* encodes 217

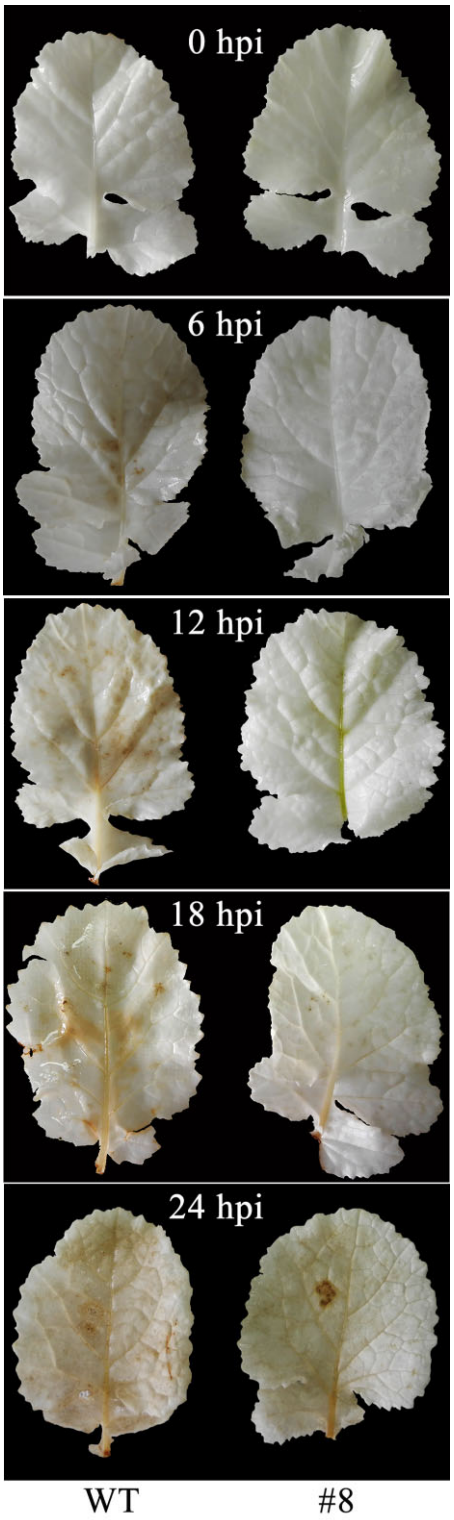

Fig. 3 Transgenic plants overexpressing *BnWRKY33* inhibit H_2O_2 accumulation in response to *Sclerotinia sclerotiorum* infection. *In situ* detection of H_2O_2 was performed using 3,3-diaminobenzidine staining in the untransformed wild-type (WT) control and the transgenic line 8 at 0, 6, 12, 18 and 24 h post-inoculation (hpi).

deduced amino acid residues with a calculated molecular mass of 23 kDa and a predicted pI of 6.29.

The deduced amino acid sequence of *BnMKS1* was used in a BLASTP search of the NCBI database. As shown in Fig. S5 (see Supporting Information), alignment of the 11 top-scoring matches with the BnMKS1 sequence showed that these sequences contain four conserved regions (domains I, II, III and IV). In the highly conserved domain II region, there is a conserved short FxxxVQxLTG motif, indicating that these proteins belong to members of the VQ protein family. The function of the VQ motif is unknown, but loss-of-function mutants and/or overexpressing lines for VQ genes are altered in their tolerance to abiotic stress or resistance to pathogen infection, suggesting that members of this protein family play important roles in responses to environmental conditions (Cheng *et al*., 2012). As would be expected from MAP kinase substrates, these proteins contain numerous Ser/tryptophan (Trp)-Pro residues, the potential MAP kinase phosphorylation sites. BnMKS1 contains 10 Ser-Pro sites, one each in domains I and II, two each in domains III and IV and three in the C-terminal region. PSORT prediction showed that the protein sequence of BnMKS1 lacks putative NLSs and other predicted subcellular targeting sequences.

Interaction of *BnMKS1*, *BnWRKY33* and *BnMPK4*

We used a Gal4 transcription activation-based yeast two-hybrid system to investigate the interaction of BnMKS1, BnWRKY33 and BnMPK4. We first fused whole BnWRKY33 protein with the DNA binding domain (BD) of Gal4 (BD-W33), but found that the fusion protein itself had transcription-activating activity and activated reporter genes in yeast cells. Thus, we fused Gal4 BD with whole BnMKS1 protein and did not find activation of reporter genes when the fusion protein was expressed in yeast cells. Then, we fused *BnWRKY33* and *BnMPK4*, respectively, with the Gal4 activation domain (AD) in the vector pGADT7. Double transformed cells were selected on –Leu/–Trp dropout medium (medium lacking leucine and tryptophan), and interaction of proteins was checked on high-stringency –Ade/–His/–Leu/–Trp dropout medium (medium lacking adenine, histidine, leucine and tryptophan) with β-galactosidase. The results showed that BnMKS1 was capable of interacting with both BnWRKY33 and BnMPK4 (Fig. 4a). We also fused Gal4 BD with whole BnMPK4 protein to investigate the interaction between BnMPK4 and BnWRKY33, but found that they cannot interact (data not shown). The results suggest that BnMPK4–BnMKS1–BnWRKY33 exist in a complex to regulate the plant defence response.

Subcellular localization of *BnWRKY33*, *BnMKS1* and *BnMPK4*

As BnWRKY33, BnMKS1 and BnMPK4 exist in a complex, the three proteins should co-localize. Further, the function of BnWRKY33, as a TF, normally requires that it is localized in the nucleus, although TFs targeting chloroplasts, mitochondria or endoplasmic reticulum (ER) have also been identified (Schwacke *et al*., 2007). Thus, we verified the possible nuclear localization of the three proteins using a tobacco transient expression system.

Three constructs expressing gene fusions between the coding region of BnWRKY33, BnMKS1 or BnMPK4 and a sequence encoding an enhanced green fluorescent protein (eGFP) were produced. Each of the three constructs, together with another construct expressing a gene fusion of the *red fluorescent protein* (*RFP*) gene and a nuclear-localized marker gene, was transiently co-transferred by injection of transformed *Agrobacterium tumefaciens* cells into tobacco leaves. Five days after injection, GFP and RFP fluorescence in leaf cells was associated with regions of nuclei autofluorescence, and co-transformation of each of the three genes and the marker gene led to yellow fluorescence (Fig. 4b). Our results indicate that BnWRKY33, BnMKS1 and BnMPK4 are primarily located in the nucleus, which is consistent with their ability to interact *in vivo*. Unexpectedly, BnMPK4 and BnMKS1, but not BnWRKY33, also appear to be located in the cytomembrane to some extent (Fig. 4b), suggesting that both BnMPK4 and BnMKS1 are involved in other plant physiological processes without the involvement of BnWRKY33.

Expression of *BnWRKY33*, *BnMKS1* and *BnMPK4* during the activation of plant defence responses

We also compared the expression of *BnWRKY33*, *BnMKS1* and *BnMPK4* in response to *S. sclerotiorum* infection in *B. napus*. As shown in Fig. 5, the three genes were induced by the pathogen and showed similar expression profiles. In particular, the expression of the three genes was substantially and synergistically enhanced at 24 and 48 hpi.

In contrast, in response to treatments with SA and methyl jasmonate (MeJA), two important defence signalling molecules that exhibit antagonistic interactions in their expression of mediated defence genes, the three genes showed different expression profiles, although their expression was up-regulated together at certain time points. Interestingly, although the expression levels of *BnWRKY33* during treatment with MeJA were elevated significantly by more than an average of 2.6-fold over noninoculated controls at 3 h, the levels of *BnMKS1* at the corresponding time points increased by more than 4.8-fold during treatment with SA (Fig. 5). In addition, for the gene *BnMPK4*, involved in the repression of SA signalling (Wang *et al*., 2009), SA appeared to be more effective than MeJA in the activation of its expression. Its induction peak was at 3 hpi with 6.9-fold greater expression than in non-inoculated controls with SA treatment, whereas, with MeJA treatment, the peak was at 9 h with 2.6-fold greater expression. During treatment with ABA, another defence signalling molecule that antagonizes JA in the mediation of defence gene expression

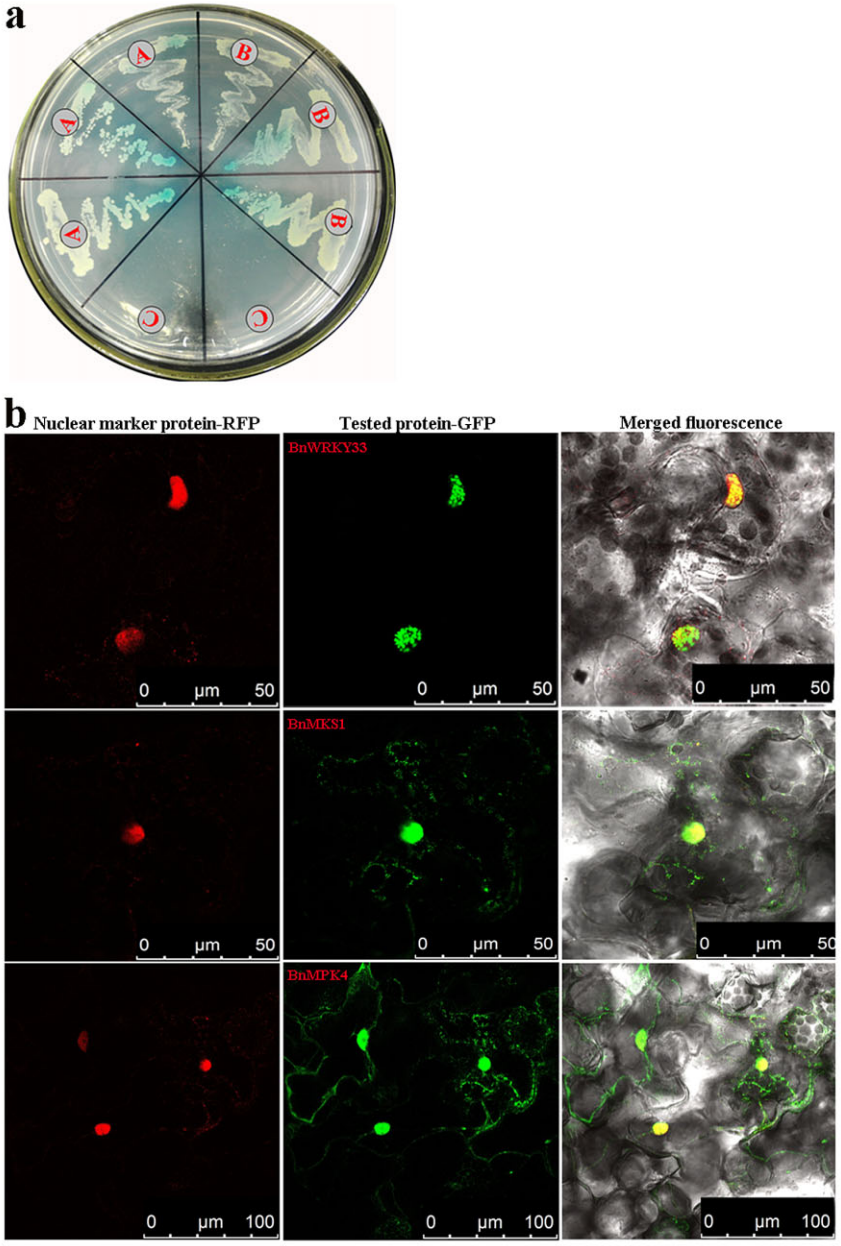

Fig. 4 Interaction and subcellular localization of BnWRKY33, BnMKS1 and BnMPK4 proteins. (a) Interaction of BnWRKY33, BnMKS1 and BnMPK4 in yeast cells. The Gal4 DNA binding domain (BD)-BnMKS1 fusion bait vectors were co-transformed with the activation domain (AD)-BnWRKY33 or BnMPK4 fusion prey vectors into yeast cells, and the transformant cells were assayed for LacZ reporter gene expression on high-stringency (SD/–Ade/–His/–Leu/–Trp) plates: A, pGBKT7-BnMKS1 + pGDAT7-BnWRKY33; B, pGBKT7-BnMKS1 + pGDAT7-BnMPK4; C, BnMKS1 (pGBKT7-BnMKS1) and SV40 large T-antigen (pGADT7-T) co-transformants were used as negative controls. (b) Subcellular localization of BnWRKY33, BnMKS1 and BnMPK4. *In planta* localization in *Nicotiana benthamiana* leaves of protein-green fluorescent protein (GFP), nuclear marker protein-red fluorescent protein (RFP) and merged fluorescence from RFP and GFP.

(Anderson *et al*., 2004), the *BnMPK4* gene was induced strongly at 6–9 h after ABA treatment. The expression of *BnWRKY33* increased modestly within 6–12 h after ABA treatment. In contrast, the expression of *BnMKS1* was reduced persistently by 0.33-fold at 6–12 h after ABA treatment.

DISCUSSION

In *Arabidopsis* and other plants, loss-of-function and gain-of-function studies have demonstrated that the *WRKY* genes are involved in plant defence against pathogens, but little direct evidence has been found for the biological roles of specific *WRKY* genes from *B. napus* in defence against the pathogen *S. sclerotiorum*. In this study, our results provide new data indicating that *BnWRKY33* plays an important role in resistance to *S. sclerotiorum* in *B. napus*. Not only is the expression of *BnMPK4* increased strongly (up to 11-fold) by *S. sclerotiorum* infection, but it is also induced by SA and JA, two phytohormones associated with defence against the pathogen in oilseed rape (Wang *et al*., 2009). Further, overexpression of *BnWRKY33* in transgenic plants significantly enhances the resistance to *S. sclerotiorum*. These new data, which indicate enhanced resistance to necrotrophic *S. sclerotiorum* in oilseed rape plants, are complementary to the positive effects observed in a study on the *AtWRKY33* gene,

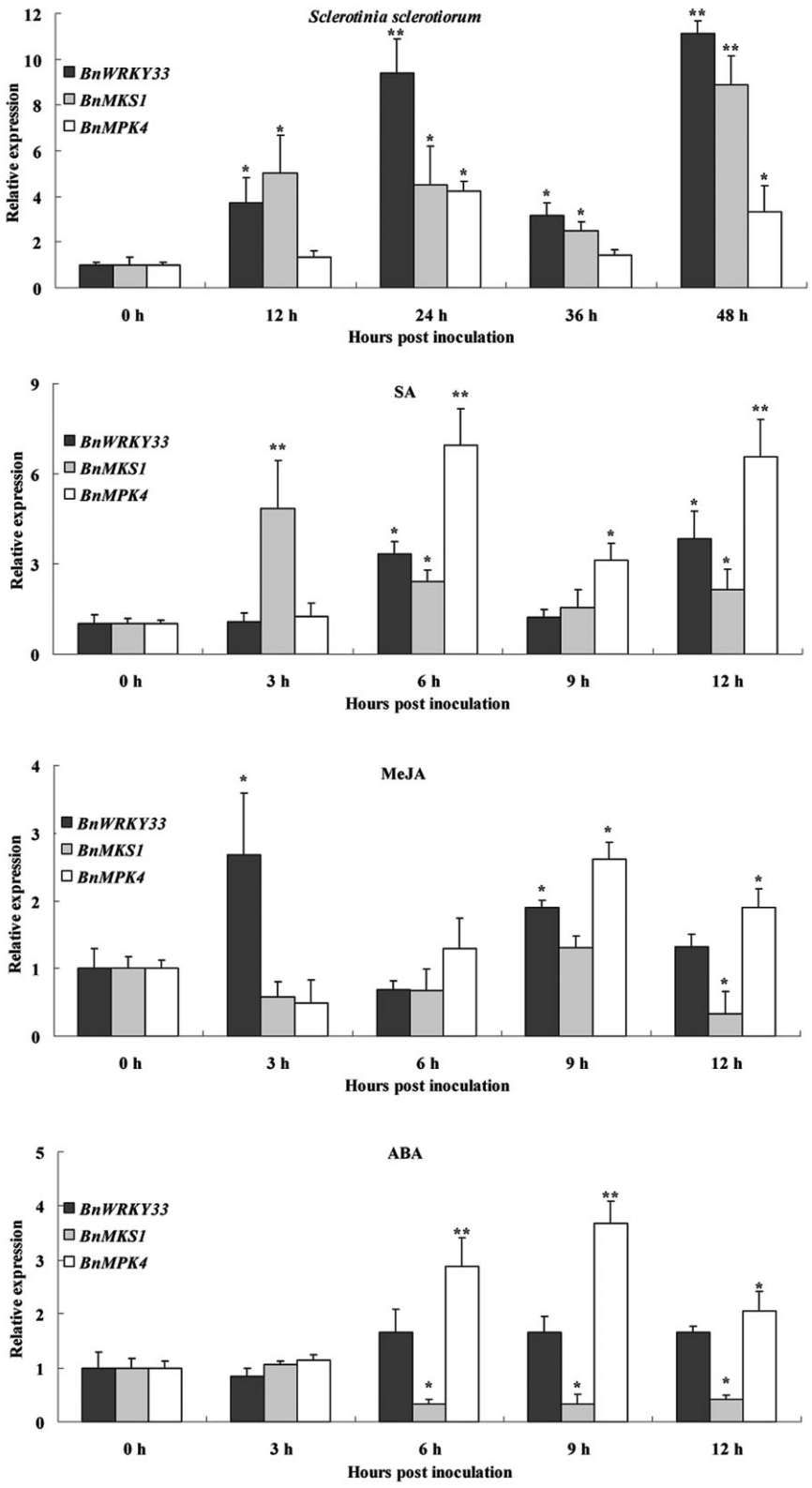

Fig. 5 Expression of *BnWRKY33*, *BnMKS1* and *BnMPK4* during activation of plant defence responses. Expression of *BnWRKY33*, *BnMKS1* and *BnMPK4* in response to *Sclerotinia sclerotiorum* and treatment with chemicals. Plants were inoculated or treated with the various chemicals as described in Experimental procedures. SA, salicylic acid; MeJA, methyl jasmonate; ABA, abscisic acid. Values are means of three replicates. Error bars indicate standard deviations. The significances of the gene expression differences between each time point and the 0-h time point are indicated: **(Student's *t*-test, $P > 0.01$) or *(Student's *t*-test, $P > 0.05$).

which is required for resistance to two other necrotrophic fungi, *Alternaria brassicicola* and *Botrytis cinerea*, in *Arabidopsis* plants (Zheng *et al*., 2006). We observed that *BnWRKY33* transgenic plants showed enhanced expression of *PR1* and *PDF1.2*, suggesting the activation of both SA and JA signalling. These observations are similar to those reported for *AtWRKY75*, which plays a role in defence against *S. sclerotiorum* in *Arabidopsis* plants (Chen *et al*., 2013). It has been shown that SA- and JA-mediated defence responses are involved in defence against *S. sclerotiorum* in *B. napus* (Wang *et al*., 2012), suggesting that the activation of the two signalling pathways may explain, at least in part, the positive effect of *BnWRKY33* in *B. napus* resistance to *S. sclerotiorum*.

Overexpression of *BnWRKY33* may involve redox control to enhance resistance to the necrotrophic pathogen *S. sclerotiorum* at the early stage. Previous studies have shown that a decrease in H_2O_2 levels in the host reduces the susceptibility to necrotrophic *Botrytis cinerea* (Govrin and Levine, 2000), and the disease severity of the pathogen increases when spraying various crops with H_2O_2 (Elad, 1992). In this study, *BnWRKY33* transgenic plants showed inhibition of H_2O_2 accumulation in response to *S. sclerotiorum* infection and exhibited enhanced resistance to the pathogen. This is consistent with results showing that exposure of *B. napus* plants to H_2O_2 solution dramatically decreases the resistance to *S. sclerotiorum* (Wang *et al*., 2009). In the case of *BnMPK4*, BnMPK4 transgenic plants with decreased H_2O_2 levels exhibited enhanced resistance to *S. sclerotiorum* (Wang *et al*., 2009). Likewise, the *Atmpk4* mutant with increased H_2O_2 levels (Nakagami *et al*., 2006) exhibits increased susceptibility to necrotrophic *Alternaria brassicicola* (Brodersen *et al*., 2006). The oxidative burst, the controlled release of O_2^- and H_2O_2, is one of the earliest and most universal responses observed in plants following pathogen challenge (Apel and Hirt, 2004). This response occurs in both compatible and incompatible responses, and a resulting frequent outcome is programmed cell death (PCD), commonly known as the hypersensitive response (HR), which may deprive biotrophic pathogens of a food source, but may be beneficial to necrotrophic pathogens (Kim *et al*., 2008). For example, in biotrophic pathogen–host interactions, an increase in H_2O_2 can, in general, lead to enhanced host resistance. By contrast, in necrotrophic pathogen–host interactions, the cell death driven by ROS does not seem to enhance host resistance. Further, host cell death inhibition leads to markedly enhanced resistance to necrotrophic *S. sclerotiorum* in transgenic tobacco plants expressing negative regulators of mammalian apoptosis (Dickman *et al*., 2001); when ROS induction is inhibited, apoptotic-like cell death induced by oxalic acid does not occur and the PCD response is required for disease development (Kim *et al*., 2008). More recently, it has been shown that the control of cell death governs the outcome of the *S. sclerotiorum*–plant interaction (Kabbage *et al*., 2013) and, once infection is established, the necrotrophic *S. sclerotiorum* induces the generation of plant ROS, leading to PCD of host tissue, the result of which is of direct benefit to the pathogen (Williams *et al*., 2011). Thus, taking these data together, it is suggested that the inhibition of H_2O_2 accumulation in transgenic plants overexpressing *BnWRKY33* is another defence mechanism against necrotrophic *S. sclerotiorum*.

The positive role of *BnWRKY33* in *B. napus* defence against *S. sclerotiorum* is also consistent with the possible regulation of *BnWRKY33* by *BnMPK4*, a positive regulator of resistance to the pathogen (Wang *et al*., 2009). The C-terminal WRKY domain of BnWRKY33, a member of group 1, contains a consensus Asp residue, a potential residue for interaction with VQ proteins (Cheng *et al*., 2012), such as BnMKS1, which contains a conserved VQ motif, representing the core of a protein–protein interaction domain (Cheng *et al*., 2012; Morikawa *et al*., 2002), referred to here as domain II. Simultaneously, in the N-terminal region of BnMKS1 and throughout the sequence of BnWRKY33, there are four Ser-Pro sites and 10 Ser-Pro sites, respectively, indicating that the two proteins may be phosphorylated by MAP kinases, such as BnMPK4. Further, consistent with the collective nuclear localization of BnWRKY33, BnMKS1 and BnMPK4, yeast two-hybrid analysis showed that BnWRKY33 can interact with BnMKS1, which, in turn, interacts with BnMPK4, and that BnMPK4 and BnWRKY33 do not interact directly with each other; this is in good agreement with the results of studies on their homologues in *Arabidopsis* (Qiu JL *et al*., 2008; Zheng *et al*., 2006). This could imply that BnMPK4 and BnWRKY33 exist in a complex that depends on BnMKS1, and that BnMKS1 may function as an adaptor linking BnMPK4 activity to the TF BnWRKY-regulated defence responses. The interactions may also explain the nuclear localization of BnMKS1, which lacks predicted NLSs. Furthermore, the three genes *BnWRKY33*, *BnMKS1* and *BnMPK4* were synergistically expressed in response to *S. sclerotiorum* infection. Thus, these results indicate that BnWRKY33 functions downstream of BnMPK4 via BnMKS1 to regulate *S. sclerotiorum* defence responses in oilseed rape.

Qiu JL *et al*. (2008) have shown the existence of MKS1–WRKY33 complexes both before and after phosphatase treatments of MKS1, and that phospho-mimics, non-phosphorylatable and wild-type forms of MKS1 bind WRKY33 equally well in yeast, suggesting that the complex exists independently of MKS1 phosphorylation. Interestingly, MPK4 also interacts equally well with these forms of MKS1 in yeast. Thus, the interaction may not involve MKS1 phosphorylation. One possible explanation could be that other unknown factors are involved in the interaction. The precise mechanism remains to be determined.

Sclerotinia sclerotiorum is considered to be one of the most damaging pathogens; it is capable of causing disease on at least 408 described species of plant from 278 genera in 75 families, including important crops, such as cotton, tomato, sunflower, dry bean, soybean and oilseed rape (Boland and Hall, 1994). The expression of plant *WRKY* genes associated with pathogen infec-

tion has been investigated in *Arabidopsis* and *B. napus*. In *Arabidopsis* plants, *AtWRKY28* and *AtWRKY75* were up-regulated on challenge with oxalic acid, an important pathogenicity determinant of *S. sclerotiorum*. Moreover, overexpression of *AtWRKY28* or *AtWRKY75* in *Arabidopsis* enhances host resistance to the pathogen (Chen *et al*., 2013). In *B. napus*, Yang *et al*. (2009) investigated the expression profiles of 16 selected *WRKY* genes during the plant defence response and showed that 10 genes, including *BnWRKY33*, were induced after infection with *S. sclerotiorum* in the susceptible *B. napus* cv. Westar using qPCR. Using a *B. napus* oligonucleotide microarray, Zhao *et al*. (2007) investigated the patterns of gene expression in *B. napus* infected by *S. sclerotiorum*, and showed that *BnWRKY33* was induced in a partially resistant cv. ZY821. These results are consistent with our observations in cv. Zhongshuang 9 with higher resistance than cv. ZY821 (Wang *et al*., 2004). The biphasic response of *BnWRKY33* expression to *S. sclerotiorum* infection can be explained by the recent observation that defence against the pathogen in oilseed rape is associated with the sequential activation of SA and JA signalling (Wang *et al*., 2012), as the expression of *BnWRKY33* can be induced by both SA and JA. Consequently, we further identified its role in the resistance to *S. sclerotiorum* through a transgenic approach in *B. napus*. The results from our and previous studies suggest that many *WRKY* genes are involved in host resistance to *S. sclerotiorum*.

It is interesting that *BnMKS1* was induced rapidly and strongly at the earlier time point (3 h) after treatment with SA, whereas *BnWRKY33* was induced by JA at the same time point. Further, the expression differences between *BnMKS1* and *BnWRKY33* were more obvious during treatment with ABA, another plant hormone showing antagonistic interactions with JA in defence gene expression. The expression of *BnMKS1* was significantly down-regulated by ABA, whereas *BnWRKY33*, together with *BnMPK4*, were induced at the corresponding time point. It has been shown that the SA-mediated signalling pathway provides protection from biotrophic fungi, oomycetes and bacteria, such as *Erysiphe orontii*, *Hyaloperonospora* (formerly *Peronospora*) *parasitica* and *Pseudomonas syringae*, whereas the JA-mediated signalling pathway activates defence responses against many necrotrophic fungi, such as *Alternaria brassicicola* and *Botrytis cinerea* (Glazebrook, 2005; Penninckx *et al*., 1996; Thomma *et al*., 1998). The role of ABA seems to depend not only on the specific interaction, but also on the pathogen's lifestyle (Adie *et al*., 2007). Thus, the different induction of *BnWRKY33* and *BnMKS1* by these defence signalling molecules suggests that the two genes are subject to different defence signal regulation, as BnMKS1, but not BnWRKY33, is also located in the cytomembrane (Fig. 4b). Indeed, in contrast with the resistance role of *AtWRKY33* in defence against *B. cinerea* in *Arabidopsis*, constitutive expression of *AtMKS1* confers susceptibility to the necrotrophic pathogen infection (Fiil and Petersen, 2011; Petersen *et al*., 2010) and loss-of-function *Atmks1* mutants exhibit increased susceptibility to biotrophic *P. syringae* (Petersen *et al*., 2010).

In summary, this study demonstrates that transgenic oilseed rape overexpressing *BnWRKY33* shows significantly enhanced resistance to *S. sclerotiorum*, which is most probably associated with the activation of the SA- and JA-mediated defence responses and the inhibition of H_2O_2 accumulation. A potential mechanism is proposed in which BnMPK4–BnMKS1–BnWRKY33 exist in a nuclear localized complex to regulate resistance to *S. sclerotiorum* in oilseed rape. Data from this study may help to shed light on the mechanisms underlying the resistance to *S. sclerotiorum* and may provide critical clues to help curb crop diseases caused by *S. sclerotiorum*.

EXPERIMENTAL PROCEDURES

Plant and fungal materials

The *B. napus* cultivar Zhongshuang9 was used in this study. Plants were grown in a plant growth room under the following growth conditions: 20 ± 2 °C with a photoperiod of 16 h light and 8 h dark at a light intensity of 44 μmol/m^2/s and 60%–90% relative humidity. Fresh sclerotia of the fungus *S. sclerotiorum*, collected from oilseed rape stems in the field in Zhenjiang, China, were germinated to produce hyphal inoculum on potato dextrose agar (PDA).

Isolation of *BnWRKY33* and *BnMKS1* cDNA

Total RNA from *B. napus* leaf tissues was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After removal of genomic DNA contamination by DNase I (TaKaRa, Dalian, China), 200 ng of poly(A)$^+$ mRNA was converted into cDNA by MMLV Reverse Transcriptase (Promega, Madison, WI, USA). The cDNA template was used for PCR analysis subsequently. For the *BnWRKY33* cDNA, a full-length cDNA was obtained using the primers 5'-ATGGATCCGTCGGAGTCTTT-3' and 5'-TTATCCCCAAATATGACTGAACAA-3' according to the sequence of a *B. napus* WRKY33 homologue (Yang *et al*., 2009). *BnMKS1* cDNA was acquired using the primers 5'-ATGGCTGCTTCTTCCCTTC-3' and 5'-TCAAGACAAAAACGAATCAAAG-3' based on the *MKS1* homologue sequence from *B. oleracea* (Andreasson *et al*., 2007). The PCR products were inserted into pMD19-T simple vector (TaKaRa) and then sequenced (Invitrogen, Shanghai, China). Subcellular localization of the deduced polypeptides was predicted by WOLF PSORT (http://wolfpsort.org) (Horton *et al*., 2007). The molecular mass and theoretical pI (isoelectric point) of the polypeptides were determined using the Compute pI/MW tool (http://web.expasy.org) (Wilkins *et al*., 1999). Multiple-aligned sequences were determined by CLUSTALX (Thompson *et al*., 1997), and GENEDOC was used to manually edit the results.

Plasmid construction for transgenic plant generation

To construct a vector for the constitutive expression of *BnWRKY33*, the vector pCAMBIA1300-35S-Nos was generated by inserting two fragments from the vector pEGAD containing the CaMV 35S promoter and CaMV Nos

terminator into the *Eco*RI/*Kpn*I and *Bam*HI/*Hin*dIII sites of pCAMBIA1300, respectively. The PCR primers were 5'-gaattcTTAATTAAGAGCTCGCA TGCC-3' and 5'-ggtaccGTCCCCGTGTTCTCTCCAA-3' for the insertion of the CaMV 35S promoter. The primers were 5'-ggatccGAATTTCCCCGA TCGTTCAA' and 5'-aagcttGATCTAGTAACATAGATGACACCGC-3' for the insertion of the CaMV Nos terminator. pCAMBIA1300-35S-Nos contains a hygromycin-resistant gene in its T-DNA region for selection of transgenic plants by hygromycin. Then, a 1.473-kb full-length *BnWRKY33* cDNA was PCR amplified from its cDNA clone with the primers 5'-ggtaccATGG CTGCTTCTTCCCTTC-3' and 5'-ggatccTCAAGACAAAAACGAATCAAAG-3', and inserted into the *Kpn*I/*Bam*HI sites of pCAMBIA1300-35S-Nos, creating a *BnWRKY33*-expressing vector 1300-35S-W33-NOS. The inserted sequences were confirmed by restriction enzyme digestion and sequencing, and the 1300-35S-W33-NOS vector was transformed into *Agrobacterium tumefaciens* (LBA4404). The plants were then grown in a protected field in Zhenjiang, China, and transformed by *in planta Agrobacterium*-mediated transformation according to the procedure described by Wang *et al*. (2009). Three independent transformants overexpressing *BnWRKY33* were examined as described in the Results section. Hygromycin-resistant T2 generation plants were identified by PCR from the progeny of the primary transformants.

Plant inoculation

Mycelia of *S. sclerotiorum* were cultured on PDA. Agar plugs (3 mm in diameter) were excised from the edges of growing colonies and upended onto the adaxial surface of plant leaves of seedlings at the six-leaf stage. Three independent T2 lines were screened with hygromycin (100 mg/L), and leaves of hygromycin-resistant plants were collected for PCR detection. The PCR-positive plants were used for inoculation. Inoculation of the detached leaves was performed as described previously (Dong *et al*., 2008). The experiment was in a randomized complete block design and was repeated three times, with 16 leaves from eight plants used in each replicate. Twenty hours after inoculation and at intervals thereafter, the lesion size was determined as the diameter of the lesion after *S. sclerotiorum* infection.

H_2O_2 detection by DAB staining

Leaves were soaked in DAB solution (1 mg/mL, pH 3.8) for 8 h in the dark at room temperature and then placed in acetic acid–glycerol–ethanol (1 : 1 : 1, v/v/v) and boiled for 5 min in a water bath. Subsequently, the solution was exchanged and the leaves were maintained in 60% glycerol. DAB was polymerized locally in the presence of H_2O_2, giving a visible brown stain.

Chemical treatments

Detached leaves were treated with SA (Mallinckrodt Baker, Deventer, the Netherlands) by dipping the leaves into a solution of 0.015% (v/v) Silwet L77 (Van Meeuwen Chemicals BV, Weesp, the Netherlands) containing 100 μM SA, as described by Leon-Reyes *et al*. (2010). Control treatments were dipped in a solution containing 0.015% (v/v) Silwet L77. The MeJA solution was diluted to 100 μM and used to treat detached leaves as described by Thomma *et al*. (2000). Treatments without MeJA were used as controls.

RNA extraction, cDNA synthesis, semi-quantitative reverse transcription and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using Trizol reagent. Total RNA (2 μg) was treated with RNase-free DNase (Invitrogen, Carlsbad, CA, USA), and first-strand cDNA was synthesized with oligo-dT using a first-strand cDNA Synthesis Kit (Invitrogen). For semi-quantitative reverse transcription, *B. napus BnACTIN* was used as reference gene for internal control. For qRT-PCR, *BnACTIN* and *BnUBC21* (ubiquitin-conjugating enzyme 21) were used as reference genes. The expression of the target genes and reference genes in plants was analysed by qRT-PCR using SYBR green real-time PCR master mix in an Eppendorf Mastercycler ep Realplex2 PCR system. Quantification of the threshold cycle (Ct) values in quantitative PCR analysis was achieved using the $2^{[-\Delta\Delta C(t)]}$ method (Livak and Schmittgen, 2001). Primers for *BnACTIN* (AF111812) were 5'-GCTGACCGTATGAGCAAAG-3' and 5'-AAGATGGATGGACCCGAC-3'; primers for *BnUBC21* (EV086936) were 5'-CCTCTGCAGCCTCCTCAAGT-3' and 5'-CATATCTCCCCTGTCTTGAAATGC-3'; primers for *BnWRKY33* were 5'-AGAGGACGGTTACAACTGGAGAAA-3' and 5'-TGTCGGACAGCTTGGGAAAG-3'; primers for *BnPDF1.2* (AY884023) were 5'-CATCACCCTTCTCTTCGCTGC-3' and 5'-ATGTCCCACTTGACCTCT CGC-3'; primers for *BnPR1* (AY623008) were 5'-ATGCCAACGCTCA CAACCA-3' and 5'-CACGGGACCTACGCCTACT-3'; primers for *BnMKS1* were 5'-GGAAGAAGCAGCCGAGT-3' and 5'-GCGAGAACATCCCACCT-3'; primers for *BnMPK4* (EU581868) were 5'-GCACGAAAGGATTGGCTAC-3' and 5'-CGATGGGACGAAGAGGAG-3'. These primer sets were tested by dissociation curve analysis and verified for the absence of nonspecific amplification (Fig. S1, see Supporting Information), and the efficiencies (*E*) of the primer-specific PCR amplifications were tested (Fig. S2, see Supporting Information).

Yeast two-hybrid assays

A yeast two-hybrid assay was performed using the GAL4-based two-hybrid system adopted from Clontech (Clontech, Changping District, Beijing, China). *BnMKS1* was PCR amplified from its cDNA clone with the gene-specific primers 5'-CGgaattcATGGATCCGTCGGAGTCT-3' and 5'-AActgcagTTATCCCCAAATATGACTGAAC-3', and inserted into the *Eco*RI/*Pst*I sites of pGBKT7, creating a pGBKT7-BnMKS1 fusion construct. *BnWRKY33* was cloned into the *Eco*RI/*Xho*I sites of pGDAT7 using PCR primers 5'-CGgaattcATGGCTGCTTCTTCCCTT-3' and 5'-AActcgagcTCAAGA CAAAAACGAATCAAAG-3', creating a pGDAT7-BnWRKY33 fusion construct. Likewise, the pGDAT7-BnWMPK4 fusion construct was generated using PCR primers 5'-CGgaattcATGTCGGCGGAGAACTGT-3' and 5'-AActcgagcttaCTGAGGATTGAACTTGACTGTTT-3'. The pGBKT7-BnMKS1 construct was transformed into AH109 cells on selective medium (minimal SD base supplemented with 2% glucose and the required dropout solution, –Trp in this case), and it was verified that they did not autonomously activate reporter genes. Singly transformed strains were used as a yeast stock in which to transform the pGDAT7-protein constructs. Double transformed cells were selected on –Leu/–Trp auxotrophy medium and the interaction of proteins was checked on high-stringency –Ade/–His/–Leu/–Trp auxotrophy medium with β-galactosidase. BnMKS1 (pGBKT7-BnMKS1) and SV40 large T-antigen (pGADT7-T) co-transformants were used as negative controls.

Subcellular localization of *BnWRKY33*, *BnMKS1* and *BnMPK4*

The *BnWRKY33*, *BnMKS1* and *BnMPK4* genes were cloned into the destination vector pK7FWG2.0 to create a C-terminal GFP fusion (Karimi *et al*., 2002) using the Gateway LR recombinase (Invitrogen, China). The nuclear marker gene *INDEHISCENT* (*IND*) was cloned into pK7FWG2.0 to create a C-terminal RFP fusion (Tan *et al*., 2009). These constructs were introduced into *Agrobacterium tumefaciens* (LBA4404).

Agrobacterium infiltration into *Nicotiana benthamiana* leaves was performed as described previously (Wood *et al*., 2009). Cells of *Agrobacterium tumefaciens* strain were cultured at 28 °C for 2 days on Luria–Bertani (LB) agar medium containing 50 μg/mL kanamycin and 2.5 μg/mL tetracycline. The recombinant agrobacteria were grown in 10 mL LB liquid medium supplemented with appropriate antibiotics at 28 °C, and then harvested by centrifugation. The cell pellet was resuspended in buffer [10 mM 2-(*N*-morpholino)ethanesulphonic acid (MES), pH 5.6, 10 mM $MgCl_2$ and 150 μM acetosyringone], adjusted to a final optical density at 600 nm (OD_{600}) of 0.6, and incubated for 3 h at room temperature before inoculation. *Nicotiana benthamiana* leaves were hand infiltrated using a needleless 1-mL syringe with the transformed *Agrobacterium* mixed with *Agrobacterium* containing the gene-silencing suppressor p19 (Voinnet *et al*., 2003). Inoculated plants were incubated at 26 °C in a growth chamber for 1–2 days. Five days after transformation, fluorescence was monitored by confocal microscopy. Fluorescence emission was at 510–540 nm for GFP and 580–584 nm for RFP, and excitation was at 488 nm for GFP and 543 nm for RFP.

Statistical analysis

Statistical analysis was performed using the SAS program (SAS Institute Inc., Cary, NC, USA). The data relating to lesion size were subjected to one-way analysis of variance followed by a comparison of the means according to a significant difference test at $P < 0.05$. Using ΔCt values (target – reference), pairwise comparisons relating to PCR were conducted according to Student's *t*-test at $P < 0.05$ or $P < 0.01$ under the assumption that variances are unequal.

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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 Specificity of quantitative reverse transcription-polymerase chain reaction (qRT-PCR) amplicons. (a) 1% agarose

gel electrophoresis showing amplification of a single product of the expected size for all tested genes. M represents DL2000 DNA marker. (b) Dissociation curves with single peaks generated for all amplicons.

Fig. S2 The efficiencies (*E*) of the primer-specific polymerase chain reaction (PCR) amplifications.

Fig. S3 BnWRKY33 and homologues. The amino acid sequence of BnWRKY33 was aligned with those of the 10 closest matching proteins from a BLAST search. Identical amino acids are shown in black boxes, and similar amino acids are shown in grey boxes. The WRKYGQK peptide stretch is shown in pink. The zinc-finger-like motifs in the two WRKY domains are shown in blue. Putative nuclear localization signals are shown in yellow. Putative mitogen-activated protein (MAP) kinase phosphorylation sites (S/P) are shown in green. Critical residues for interaction with VQ proteins are shown in brown. Species abbreviations are as follows: Bn, *Brassica napus*; Bo, *Brassica oleracea*; Cr, *Capsella rubella*; ThUn-protein, *Thellungiella halophila* unnamed protein; AtPu-protein, *Arabidopsis thaliana* putative WRKY33; Al, *Arabidopsis lyrata*; Cr, *Capsella rubella*; RcTF, *Ricinus communis* transcription factor; Jc, *Jatropha curcas*.

Fig. S4 Disease progression of *Sclerotinia sclerotiorum* in *BnWRKY33*-overexpressing lines and the untransformed wild-type control. Photographs were taken of leaves from three wild-type plants and three hygromycin- and PCR-positive plants of line 8. WT, untransformed wild-type control; #8, *BnWRKY33* transgenic T2 line; hpi, h post-inoculation.

Fig. S5 BnMKS1 and homologues. The amino acid sequence of BnMKS1 was aligned with those of the 10 closest matching proteins from a BLAST search. Identical amino acids are shown in black boxes, and similar amino acids are shown in grey boxes. Putative domains I, II, III and IV are underlined in the consensus. The FxxxVQxLTG motif is shown in pink. Putative mitogen-activated protein (MAP) kinase phosphorylation sites (S/P) are shown in green. Species abbreviations are as follows: Bn, *Brassica napus*; Bo, *Brassica oleracea*; At, *Arabidopsis thaliana*; AlHy-protein, *Arabidopsis lyrata* hypothetical protein; CrHy-protein, *Capsella rubella* hypothetical protein; TcPu-MKS1, *Theobroma cacao* putative MKS1; Gm, *Glycine max*; PtPr-protein, *Populus trichocarpa* predicted protein; Ca, *Cicer arietinum*; Mt, *Medicago truncatula*; Fv, *Fragaria vesca*; RcPu-MKS1, *Ricinus communis* putative MKS1.