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Identification and expression analysis of *WRKY* transcription factor genes in canola (*Brassica napus* L.) in response to fungal pathogens and hormone treatments

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Abstract

Background: Members of plant WRKY transcription factor families are widely implicated in defense responses and various other physiological processes. For canola (*Brassica napus* L.), no WRKY genes have been described in detail. Because of the economic importance of this crop, and its evolutionary relationship to *Arabidopsis thaliana*, we sought to characterize a subset of canola *WRKY* genes in the context of pathogen and hormone responses.

Results: In this study, we identified 46 *WRKY* genes from canola by mining the expressed sequence tag (EST) database and cloned cDNA sequences of 38 *BnWRKYs*. A phylogenetic tree was constructed using the conserved WRKY domain amino acid sequences, which demonstrated that BnWRKYs can be divided into three major groups. We further compared *BnWRKYs* to the 72 *WRKY* genes from *Arabidopsis* and 91 *WRKY* from rice, and we identified 46 presumptive orthologs of *AtWRKY* genes. We examined the subcellular localization of four BnWRKY proteins using green fluorescent protein (GFP) and we observed the fluorescent green signals in the nucleus only.

The responses of 16 selected *BnWRKY* genes to two fungal pathogens, *Sclerotinia sclerotiorum* and *Alternaria brassicae*, were analyzed by quantitative real time-PCR (qRT-PCR). Transcript abundance of 13 *BnWRKY* genes changed significantly following pathogen challenge: transcripts of 10 *WRKYs* increased in abundance, two *WRKY* transcripts decreased after infection, and one decreased at 12 h post-infection but increased later on (72 h). We also observed that transcript abundance of 13/16 *BnWRKY* genes was responsive to one or more hormones, including abscisic acid (ABA), and cytokinin (6-benzylaminopurine, BAP) and the defense signaling molecules jasmonic acid (JA), salicylic acid (SA), and ethylene (ET). We compared these transcript expression patterns to those previously described for presumptive orthologs of these genes in *Arabidopsis* and rice, and observed both similarities and differences in expression patterns.

Conclusion: We identified a set of 13 *BnWRKY* genes from among 16 *BnWRKY* genes assayed, that are responsive to both fungal pathogens and hormone treatments, suggesting shared signaling mechanisms for these responses. This study suggests that a large number of BnWRKY proteins are involved in the transcriptional regulation of defense-related genes in response to fungal pathogens and hormone stimuli.

Background

Canola (*Brassica napus*) is an economically important crop in Canada and other temperate regions, and is susceptible to adverse effects by fungal pathogens. Among these, *Sclerotinia sclerotiorum*, causing stem rot and *Alternaria brassicae*, causing Alternaria black spot, have potential to cause significant crop losses [1]. Considerable efforts are underway to develop canola varieties that are better able to tolerate these pathogens. We have previously used proteomics and genomics to survey the global changes in gene expression that occur as a result of pathogen challenge in canola [2-5].

Plant defense responses include the transcriptional control of expression of stress-responsive genes [6-9], including a number of transcription factors (TFs) whose abundance is altered as a result of the pathogen challenge. These TFs are presumably involved in regulating the expression of defense-related genes, and specifically include those containing Ethylene Response Factor (ERF)/ Apetala2 (AP2)-domain, homeodomain, basic Leucine Zipper (bZIP), MYB, WRKY families and other zinc-finger factors, all of which have been observed to increase in response to pathogen challenge [10]. These defense-associated TFs can regulate downstream defense-related genes, and may themselves be regulated by phosphorylation [11-14].

The name of the WRKY family itself is derived from the most prominent feature of these proteins, the WRKY domain, which constitutes 60 amino acids [11]. In this WRKY domain, a conserved WRKYGQK heptapeptide is followed by a C_2H_2- or C_2HC-type of zinc finger motif [11]. One or two WRKY zinc-finger motifs may be present, which can bind to the W-box DNA motif (C/T)TGAC(C/T) [15-19]. Furthermore, *cis*-elements other than TTGAC(C/T) have also been identified as a target of the WRKY domain of a barley WRKY TF [20,21]. The Group I WRKY TFs contain two WRKY domains: the C-terminal domain that plays a major role in binding to the W-box, while the N-terminal WRKY domain affects the binding affinity [15,16].

WRKY proteins belong to a super-family of zinc finger proteins [WRKY-Glial Cell Missing (GCM1)] containing six members [22]. For example, genes coding WRKY proteins were found not only in plants but also in the slime mold *Dictyostelium discoideum* and the protist *Giardia lamblia*, which indicates that WRKYs may have evolved prior to the evolution of plant phyla [23-25]. Some WRKY functions are thought to be conserved between phylogenetically distant species [26].

WRKY TF genes form large families in plants, with 72 members in *Arabidopsis* and close to 100 in *Oryza sativa* (rice) [27]. Previous studies have demonstrated that *WRKY* TFs are implicated in plant defense responses [14], sugar signaling [21] and chromatin remodeling [28]. Furthermore, *WRKY*s have been found to play essential roles in various normal physiological processes, including embryogenesis, seed coat and trichome development, senescence, regulation of biosynthetic pathways, and hormonal signaling [29-34]. As alluded to earlier, abiotic and biotic stresses are among the major external factors influencing the expression of *WRKY* genes in plants [11,23,35-38] and have been demonstrated to be involved in the defense against phytopathogens such as bacteria [25,39-42]; fungi [43-45]; and viruses [46,47].

The responses of *Arabidopsis* to pathogens have been observed to be mediated by signaling pathways [48-50]. For example, salicylic acid (SA) plays a positive role in plants against biotrophic pathogens, whereas jasmonic acid/ethylene (JA/ET) appears to be important in the case of necrotrophic pathogens [50-53]. It is also known that these (SA and JA/ET) signaling pathways are mutually antagonistic [54]. In *Arabidopsis*, it was observed that 49 out of 72 *AtWRKY* genes are regulated by *Pseudomonas syringae* or SA treatment [42]. On the other hand, of JA-responsive TF in *Arabidopsis*, AtWRKY TFs are one of the greatest numbers of induced [45]. Moreover, it is observed that cross-talk of SA- and JA-dependent defense response could be mediated by AtWRKY70, which is downstream of nonexpressor of pathogenesis-related gene 1 (*NPR1*) [55].

Previous studies have shown that abscisic acid (ABA), a negative factor in the SA and JA/ET signaling defense response, did not increase disease resistance [56-60]. However, recent research has demonstrated that ABA has a positive effect on callose deposition, which could lead to increased resistance of plants towards some pathogens [61-63]. Although WRKY TFs have been demonstrated to be involved in abiotic stress and ABA signaling [31,35,64-66], there are no reports available on the role of WRKYs in ABA-mediated biotic stress responses. The role of other hormones, such as cytokinins, has been investigated by many groups and it was observed that cytokinins, serving as endogenous inducers for distinct classes of pathogenesis-related (PR) proteins, are necessary for the biosynthesis of SA and JA [67-69]. Others have observed that the effect of cytokinins is mediated through the stimulation of ET production [70]. However, whether cytokinins induce the expression of PR genes through WRKYs is not presently clear.

Despite the obvious importance of WRKYs in responses to pathogens and hormone signaling, there are no reports as of yet, describing *WRKY* TFs in canola and their role(s) in mediating responses to pathogens. In our previous micro-

array analysis of canola response to *S. sclerotiorum*, we identified three WRKY genes whose transcript abundance was significantly affected by this fungus [5]. These results prompted us to systematically identify and examine *WRKY* TF genes in canola using the large set of available expressed sequence tags (ESTs). In this study, we analyzed ESTs from publicly available sequence information of canola and identified 46 sequences with similarities to *Arabidopsis WRKY* TFs. We investigated the evolutionary relationship of canola WRKY TFs with their counterparts from *Arabidopsis* and rice. We examined the subcellular localization of four BnWRKY proteins using green fluorescent protein (GFP). Subsequently, we studied the responses of representative members of monophyletically distinct WRKY clades to two fungal pathogens, as well as five plant hormones, in order to gain further insights into their roles in canola defense responses.

Results

Identification of 46 WRKY transcription factor genes in B. napus

Although the complete sequence of the *B. napus* genome has not yet been determined, the number of publicly available ESTs was 593,895 as of May 30, 2008. It is well known that gene discovery and genome characterization through the generation of ESTs is one of the most widely used methods [71]. A keyword search in NCBI "nr" dataset, returned only two previously annotated *BnWRKY* sequences. We used BLAST alignments to search the dbEST database and identified 343 unique GenBank EST accessions from *B. napus* that showed significant similarity to the 72 *AtWRKY* genes and 36 other *WRKY* sequences. We then used ESTpass to remove four chimerical ESTs and clustered the remaining 339 ESTs into 69 contigs and 66 singlets. For subsequent analyses, we also identified the largest open reading frame of each of the 135 contigs or singlets using OrfPredictor [72,73]. We also searched the DFCI oilseed rape gene index (BnGI, release 3.1) and identified 70 tentative consensuses (TC) and 79 singlets, which consisted of 314 ESTs. We found that all 314 of the BnGI ESTs were present within the 339 dbESTs we extracted from Genbank. The differences in numbers of WRKY ESTs from these databases can be explained by the fact that the number of entries in these databases are different, based on their release frequency. The Shanghai database http://rapeseed.plantsignal.cn/, [74]) is more recent, with a greater number of entries, and produced an additional number of WRKY EST's that were incorporated in the current study. We note that the EST information available for canola is biased towards seed coat and embryo tissues, which likely limited our ability to identify a complete set of WRKY genes for this species. As the contigs/singlets output from ESTpass were annotated based on their similarity to *Arabidopsis WRKY* genes, we were able to identify the presumptive orthologs of the respective canola *WRKY* genes. Therefore, we assigned names to each *BnWRKY* (Additional file 1) based on the name of the corresponding *Arabidopsis WRKY*s.

We noted that among all the *BnWRKY* genes we annotated, *BnWRKY11* has the largest number (40) of ESTs, followed by *BnWRKY32* with a total of 26 ESTs, while *BnWRKY*26, 30, 36, and 66 have only one EST each (Additional file 1 and additional file 2). To facilitate subsequent phylogenetic, GFP fusion, and qRT-PCR analyses, we designed primers based on the identified ESTs for each of the 46 *BnWRKY* genes to obtain full length cDNA sequences, at least for each of the coding regions, employing RT-PCR together with 3'RACE. As a result, we succeeded in cloning the cDNA sequences of 38 of these 46 *BnWRKY* genes, among which we identified two different alleles (or possibly homeoalleles) for each of 13 *BnWRKY* genes (Additional file 1). We were also able to identify putative orthologs of these *BnWRKY* genes in both *Arabidopsis* and rice using the program InParanoid [75] (Additional file 1).

Although WRKY proteins have a conserved heptapeptide WRKYGQK motif [11], many studies have reported slight variations of the sequence for some WRKY proteins in *Arabidopsis*, rice, tobacco and barley [24,26,31,76]. Similarly, a number of the BnWRKYs we identified have amino acid sequence substitutions in their conserved WRKY signatures. For example, the following variations were noted: WRKYGKK in BnWRKY50, and WRKYGRK in BnWRKY51 (Additional file 3). We also observed a 25 amino acid insertion in the C-terminal WRKY domain of BnWRKY26, compared to AtWRKY26 (Additional file 3). An examination of the cDNA sequence of *Bn WRYKY26* revealed that the insert starts with TT and ends with GG, suggesting that it is most probably not an intron. Usually the nucleotide sequence of the predominant class of introns begins with GT ends with AG and that of a minor class begins with AT and ends with AC [77,78], neither of which are true in this particular instance. Our results thus suggest that BnWRKY26 has diverged considerably during the evolutionary process.

Phylogenetic analysis of BnWRKY proteins

From the 46 canola WRKY genes identified, we were able to extract 53 WRKY domains that were each approximately 60 amino acids in length. In 11 BnWRKY TF proteins, we identified two separate WRKY domains (Additional file 3), and both N- and C-terminal WRKY domains of these proteins were included in the phylogenetic analysis. The WRKY domain amino acid sequences were aligned with each other (Additional file 3) and a consensus maximum parsimony (MP) tree was inferred (Figure 1). Subsequently, we reconstructed a rooted MP tree using a WRKY protein from the world's smallest uni-

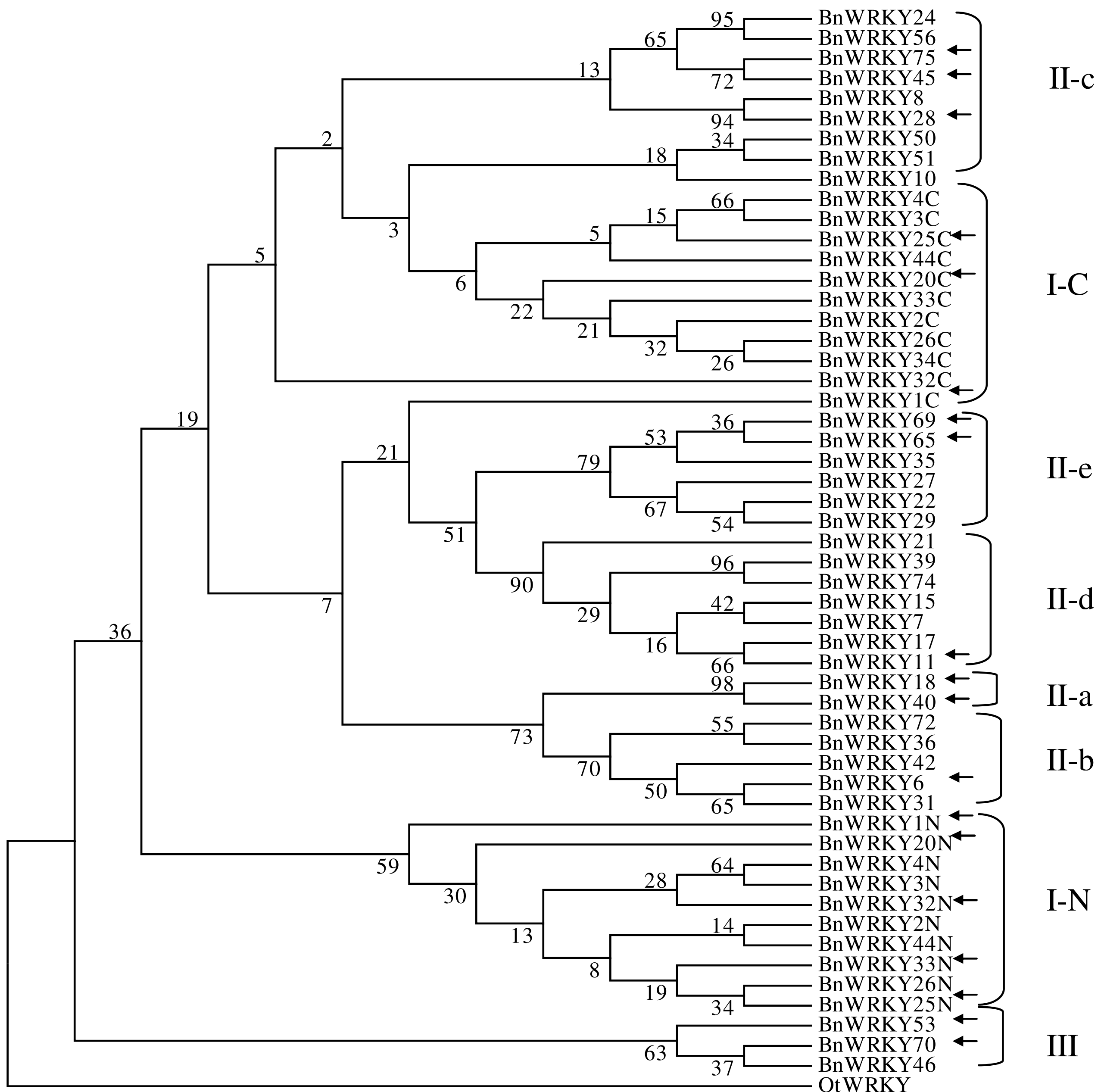

Figure 1

A bootstrap consensus maximum parsimony tree of WRKY TFs in canola. The phylogenetic tree was based on the amino acid sequences from WRKY domains only. Only the ~60 amino acid residues in the WRKY domain were aligned using ClustalX (v1.83) and were further examined manually for optimal alignment. The parsimony tree was drawn using MEGA4. The percentage of replicate trees is shown on the branches and it is calculated in the bootstrap test (500 replicates) for the associated taxa being clustered together. The two letters N and C after group I represent the N-terminal and the C-terminal WRKY domains of group I proteins, respectively.

cellular green algae *Ostreococcus tauri* WRKY as the outgroup (Figure 1). This tree demonstrates the polyphyletic nature of BnWRKY TFs, which is consistent with previous studies [22,23,26].

We next classified the BnWRKY TFs we identified into three major groups using criteria that had been previously described for this family [11]. Accordingly, the Group II proteins were further divided into five subgroups. From our study, at least two representatives for each subgroup

of WRKY proteins were identified in the canola genome (Figure 1). For example, twelve *BnWRKYs* (*BnWRKY1*, 2, 3, 4, 19, 20, 25, 26, 32, 33, 34 and 44) code for proteins with two WRKY domains and clearly cluster with Group I of the AtWRKYs. The N- and C-terminal domains of these twelve BnWRKY form two different clusters named Group IN and Group IC (Figure 1). The 28 identified Group II WRKY members of canola were distributed unevenly among the five subgroups (subgroups IIa-e, Figure 1) and this is in agreement with previous studies in *Arabidopsis*, rice and barley [11,26,31]. Two BnWRKYs (BnWRKY18, 40) formed a distinct subclade, IIa, similar to the observations in *A. thaliana* [11]. Five canola WRKYs (BnWRKY6, 31, 36, 42, 72) belong to Group IIb; eight (BnWRKY8, 24, 28, 45, 50, 51, 56, 75) belong to Group IIc; seven (BnWRKY7, 11, 15, 17, 21, 39, 74) belong to Group IId; and six canola WRKY (BnWRKY22, 27, 29, 35, 65, 69) belong to Group IIe. Group III is represented by four single WRKY domain canola proteins (BnWRKY46, 53, 66 and 70). The comparison of number of WRKY genes in *Arabidopsis* (AtWRKY), rice (OsWRKY), barley (HvWRKY) and canola (BnWRKY) within each of the WRKY group/subgroups (Table 1) showed that about 53–59% of the expected WRKY genes of canola have been identified. It appears that within group IId, the same number of *WRKY* genes from *A. thaliana* and canola have been identified whereas for other subgroups, additional *BnWRKY* genes remain to be identified (Table 1). Our observations are similar to the study on the barley WRKY gene family in which approximately 50% of the expected *HvWRKY* genes were identified [26].

To further explore the phylogenetic relationships between *WRKY*s from canola and other species, we generated a phylogenetic tree incorporating all the *WRKY*s we identified from *Arabidopsis*, rice, and canola (Additional file 4; [27]). These results are consistent with our proposed classification of the newly characterized *WRKY*s from canola.

Table 1: Comparison of number of WRKY proteins of Arabidopsis (AtWRKY), rice (OsWRKY), barley (HvWRKY) and canola (BnWRKY) in each of the WRKY group/subgroups.

WRKY group	AtWRKY[a]	OsWRKY[b]	HvWRKY[c]	BnWRKY
I	15	13	8	12
IIa	3	4	4	2
IIb	8	7	1	5
IIc	17	20	11	8
IId	7	6	5	7
IIe	8	8	3	6
III	14	32	13	3
IV		6		
Total	72	96	45	43

According to a) Eulgem et al. [11], b) Xie et al. [31], Ross et al., [27], and c) Mangelsen et al. [26].

However, in rice, there are four major groups of *WRKY*s, I, II, III and IV [27,31] and it can be observed that some members of the rice WRKY family are scattered throughout the phylogenetic tree, an observation that has also been made previously [27,31]. For example, OsWRKY57 (a group II WRKY) is clustered with those from group I-N and OsWRKY61 (a group Ib WRKY) is clustered with those of group III members (Additional file 4). Similarly, OsWRKY 9 and 83 (group Ia WRKYs) are clustered with group II members (groups IIb and d, respectively; Additional file 4). The three group IVa WRKYs (OsWRKY52, 56, and 58; Additional file 4) are scattered within branches of group II and III. Interestingly, we observed that OsWRKY86 (a group I member) is clustered with group III instead of group II as previously reported by others [27] and, OsWRKY84 is clustered within group III in our study, contrary to a previous report of this WRKY being clustered within group I ([38]; additional file 4). These discrepancies may be due to the use of different algorithms (neighbor-joining versus MP) to generate the phylogenetic trees.

Nuclear localization of four BnWRKY proteins

The function of a TF normally requires that it is localized in the nucleus, although TFs targeting chloroplasts, mitochondria, or endoplasmic reticulum (ER) have also been identified [79]. To confirm that the BnWRKY TFs we identified are indeed targeted to the nucleus, we selected four *BnWRKY* genes based on their known functions in mediating defense responses in *Arabidopsis* [25,45,80-82] for analysis *in vivo*. We fused the coding regions of *BnWRKY6*, 25, 33, and 75 to the N-terminus of synthetic green fluorescent protein (sGFP) [83] and expressed them in *Arabidopsis* under the control of the constitutive cauliflower mosaic virus (CaMV) 35*S* promoter. Analysis of conceptually translated *BnWRKY6*, 25, and 33 coding sequences revealed the presence of a monopartite nuclear localization signal (NLS) (prediction program of protein localization sites, http://psort.nibb.ac.jp), however, no NLS was detected in the translated BnWRKY75 sequence. We analyzed transgenic *Arabidopsis* seedlings harboring the respective four constructs. In all four cases, green fluorescent signals were observed only in the nucleus (Figure 2A–D). With the control vector alone, GFP signals were distributed in both the cytoplasm and nucleus (Figure 2E). Our results indicate that *BnWRKY6*, *25*, 33, and 75 are indeed nuclear-localized proteins, which is consistent with their predicted function as transcription factors.

Expression analysis of BnWRKY *genes in response to fungal pathogens*-S. sclerotiorum *and* A. brassicae

Because the divergence of paralogous genes is often after the sub-functionalization [84], we employed qRT-PCR to investigate the responses of representatives of each of the three major *WRKY* clades. We selected 16 *BnWRKY* genes,

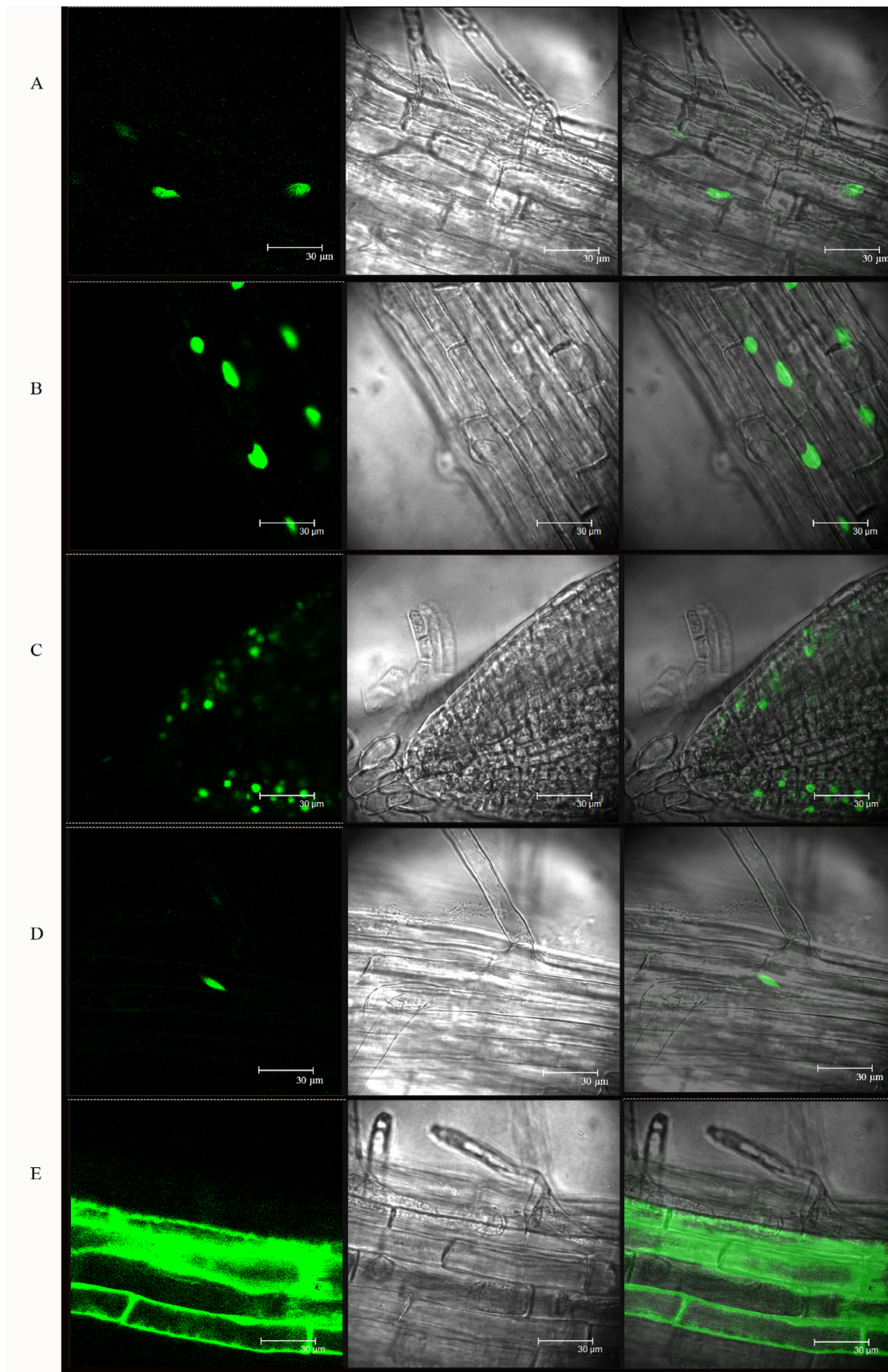

Figure 2
Nuclear localization of four BnWRKY proteins. Transgenic (T_2) Arabidopsis roots of five-day old seedlings were observed under confocal microscope. Panels A-E represent the subcellular localization of BnWRKY6-sGFP, BnWRKY25-sGFP, BnWRKY33-sGFP, BnWRKY75-sGFP and pCsGFPBT vector control, respectively. In each case, the extreme left panel is GFP fluorescence, the middle bright field and the right represents an overlay of the two images.

WRKY1, 6, 11, 18, 20, 25, 28, 32, 33, 40, 45, 53, 65, 69, 70 and 75, as representatives of each clade (Additional file 1, Figure 1). After challenge with the fungal pathogen *S. sclerotiorum*, transcript abundance of 13 *BnWRKY* genes was observed to be significantly (t-test, $P < 0.05$) modulated with 10 being increased, two being decreased and one being decreased at 12 h but subsequently increased at 72 h (Figure 3A). *BnWRKY6*, 25, 28, 33, 40, 45, 53,65, 69 and75 were highly induced at 48 h after the inoculation. However, *BnWRKY20* and 32 were repressed by *S. sclerotiorum* infection. *BnWRKY1* was observed to be repressed at an earlier time point (12 h) but induced later (72 h, Figure 3A).

We then examined the changes in transcript abundance of these 16 *BnWRKY* genes in response to a second fungal pathogen, *A. brassicae*, which is also a necrotrophic pathogen. The symptom development in these two pathosystems (*S. sclerotiorum* and *A. brassicae*) is different with respect to time required, with *A. brassicae* requiring a much longer period before visible disease symptoms could be observed (data not shown). Accordingly, the transcript abundance of only four *BnWRKY* genes were significantly affected by *A. brassicae* with two (*BnWRKY33* and 75) being significantly increased at 48 h post-pathogen challenge and two (*BnWRKY70* at both 48 and 72 h and *BnWRKY69* only at 72 h) with decreased transcript abundance (Figure 3B). In summary, our results indicate that *BnWRKY33* and 75 are induced by both *S. sclerotiorum* and *A. brassicae* with *BnWRKY75* exhibiting a similar temporal pattern of changes in transcript abundance between the two fungi. However, *BnWRKY69* and 70 had different responses to *S. sclerotiorum* and *A. brassicae*. Our results suggest that although both pathogens investigated in this study are necrotrophic, they elicit slightly different responses with respect to changes in transcript abundance of *BnWRKY* genes.

Response of selected BnWRKY *genes to hormone treatments*

To investigate the hormonal control mechanisms underlying *BnWRKY* gene expression, we treated canola plants with five phytohormones, JA, SA, ABA, BAP and ET and analyzed the changes in transcript abundance of these 16 *BnWRKY* genes using qRT-PCR. To ensure that the hormone applications were eliciting expected responses in plants, we first examined the responses of a few additional canola genes that are proposed to be orthologs of *Arabidopsis* genes previously reported to respond to these hormones. These *Arabidopsis* genes were two *bZIP* transcription factors, *TGA5*, *TGA6* for SA [85-87]; allene oxide cyclase (*AOC*) [88] and plant defensin 1.2 (*PDF1.2*) for JA [50]; ethylene insensitive 2 (*EIN2*) [89] and ethylene responsive factor (*ERF2* and *ERF4*) [90] for ET; ABA insensitive 5 (*ABI5*) [91-93] for ABA, and *Arabidopsis* response regulator 6 (*ARR6*) [94] and cytokinin response 1 (*CRE*) [95] for BAP. We observed that the abundance of transcripts for all of these genes was significantly increased in response to the hormone treatments (data not shown), confirming the efficacy of our hormone treatments.

Our results demonstrated that among the 16 *BnWRKY* genes studied, *BnWRKY40*, 69 and 75 were induced by ET and *BnWRKY53* was repressed by ABA at 6 h (Figure 4A, Table 2). In contrast, *BnWRKY25*, 32, 45, 69 and 70 were repressed by BAP at 6 h (Figure 4A, Table 2). At 24 h, *BnWRKY1*, 28, 32, 33, 45, and 75 were specifically induced by ET and *BnWRKY70* was repressed by ET (Figure 4B, Table 2). Three *BnWRKY* genes exhibited modulation of expression in response to two hormones (Table 2). At 6 h, both JA and ET repressed *BnWRKY11* and both ET and BAP repressed *BnWRKY1*, 20 and32 (Figure 4C, Table 2). However, none of the genes were observed to be affected by the two hormones at 24 h. In addition, both ABA and BAP repressed *BnWRKY69* (Figure 4C, Table 2). None of these *BnWRKY* genes were affected by three or more hormones (Table 2).

As indicated earlier, JA and SA are important signaling molecules which are implicated in plant defense responses [96,97]; and other phytohormones, through their effect on SA or JA signaling, may influence disease outcomes [98]. *BnWRKY11* was observed to be repressed by JA at 6 h although no significant change was observed at 24 h (Table 2). In response to SA treatment, we observed that the transcript abundance for seven genes (*BnWRKY6*, 18, 33, 40, 53, 70 and 75) exhibited modulation at 6 h and three (*BnWRKY53*, 70 and 75) at 24 h (Table 2), however, these observed changes were not statistically significant.

In summary, SA did not significantly affect the transcript abundance of any of the *BnWRKYs* tested, whereas ET, ABA, JA and the cytokinin BAP did affect the transcript abundance of various *BnWRKY* genes investigated in this study (Table 2). Although the 16 genes tested did not show significant changes in expression levels after exogenous treatments with SA, there is the possibility that other *BnWRKY* genes may be responsive to SA.

Discussion

In this study, we describe the identification and annotation of cDNA sequences of 46 members of the *WRKY* gene family in canola and their classification into groups I to III (Figure 1, Additional file 1). Among the 46 *BnWRKY* genes identified, both the hallmark WRKYGQK motif (43) and its variants (two variants, WRKYGKK for BnWRKY50 and WRKYGRK for BnWRKY51) were identified in the translated amino acid sequences while that of

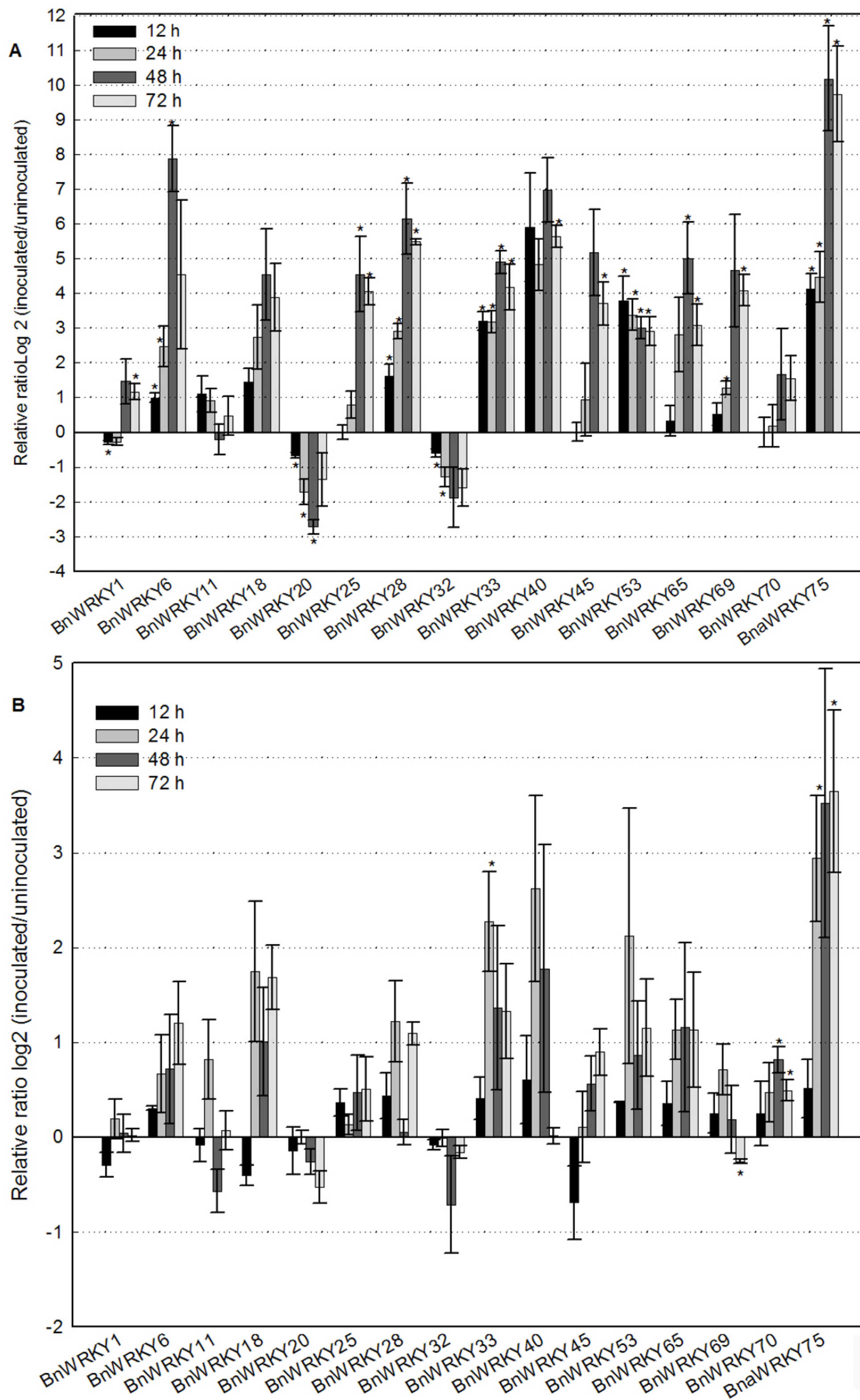

Figure 3

Expression analyses of *BnWRKY* genes in response to fungal challenge. Changes in *BnWRKY* transcript abundance in response to (A) *S. sclerotiorum* and (B) *A. brassicae* infection. Data is the mean of three biological replicates ± S.E.

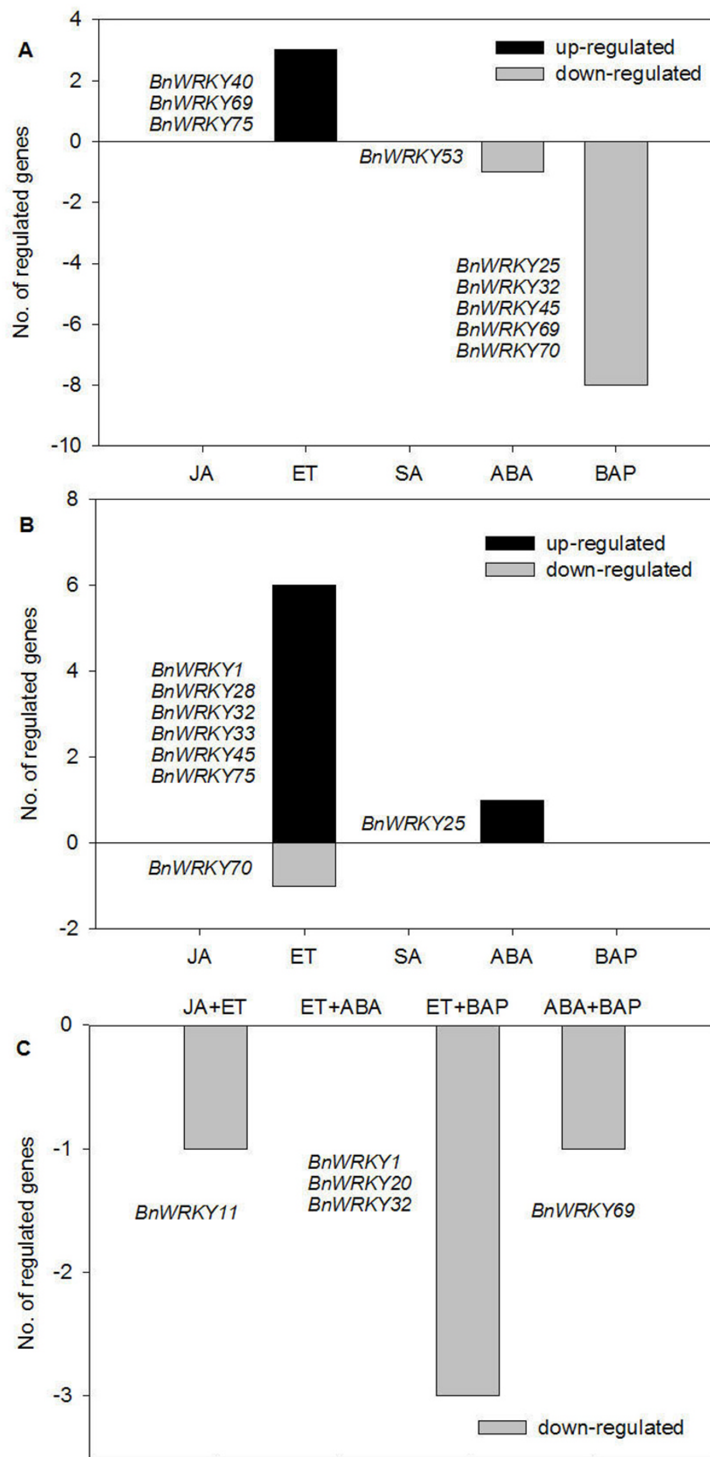

Figure 4

Expression analyses of *BnWRKY* genes in response to different hormone treatments. Changes in *BnWRKY* transcript abundance as a result of hormone application at (A) 6 h, (B) 24 h and (C) those that respond to more than one hormone at 6 h.

Table 2: Expression analyses of *BnWRKY* genes to five plant defense-related hormone treatments assayed by qRT-PCR.

gene	JA		ET		SA		ABA		BAP	
	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h
BnWRKY1	2.47 (± 1.40)	1.11 (± 0.06)	0.83 (± 0.03)*	1.13 (± 0.03)*	1.53 (± 0.26)	1.10 (± 0.2)	2.54 (± 1.31)	1.03 (± 0.08)	0.59 (± 0.02)**	0.58 (± 0.08)*
BnWRKY6	0.64 (± 0.14)	0.88 (± 0.29)	1.07 (± 0.59)	3.98 (± 0.81)	1.99 (± 0.39)	1.29 (± 0.37)	2.17 (± 0.17)	1.23 (± 0.13)	0.72 (± 0.13)	0.64 (± 0.22)
BnWRKY11	0.63 (± 0.01)**	0.85 (± 0.12)	0.70 (± 0.00)**	1.02 (± 0.10)	1.63 (± 0.21)	1.22 (± 0.29)	1.16 (± 0.03)	1.06 (± 0.37)	0.64 (± 0.11)	0.97 (± 0.05)
BnWRKY18	1.99 (± 0.64)	1.31 (± 0.33)	1.74 (± 0.20)	1.60 (± 0.33)	11.65 (± 4.02)	6.48 (± 0.59)	3.01 (± 0.62)	1.07 (± 0.39)	0.71 (± 0.27)	0.38 (± 0.11)*
BnWRKY20	0.85 (± 0.24)	0.95 (± 0.12)	0.68 (± 0.04)*	1.38 (± 0.13)	1.38 (± 0.35)	1.19 (± 0.15)	0.77 (± 0.15)	0.87 (± 0.03)	0.61 (± 0.07)*	0.59 (± 0.14)
BnWRKY25	1.71 (± 0.54)	1.53 (± 0.16)	1.82 (± 0.16)	2.34 (± 0.50)	1.69 (± 0.39)	0.92 (± 0.09)	2.51 (± 1.45)	1.83 (± 0.19)*	0.55 (± 0.07)*	0.42 (± 0.13)*
BnWRKY28	0.75 (± 0.11)	1.55 (± 0.25)	0.86 (± 0.26)	1.49 (± 0.05)**	1.37 (± 0.45)	3.64 (± 3.00)	1.00 (± 0.09)	1.75 (± 1.02)	1.00 (± 0.38)	1.32 (± 0.73)
BnWRKY32	1.11 (± 0.15)	1.14 (± 0.15)	0.81 (± 0.04)*	1.33 (± 0.06)*	1.44 (± 0.34)	0.88 (± 0.08)	1.27 (± 0.17)	0.97 (± 0.12)	0.73 (± 0.04)*	0.82 (± 0.14)
BnWRKY33	0.68 (± 0.30)	0.76 (± 0.09)	3.89 (± 0.09)	2.38 (± 0.31)*	4.80 (± 1.23)	1.40 (± 0.36)	0.50 (± 0.15)	1.00 (± 0.21)	1.09 (± 0.17)	0.87 (± 0.18)
BnWRKY40	0.84 (± 0.2)	1.17 (± 0.44)	4.74 (± 0.05)*	6.49 (± 1.63)	2.17 (± 0.47)	0.99 (± 0.24)	1.28 (± 0.47)	1.69 (± 0.74)	0.61 (± 0.05)*	0.56 (± 0.16)
BnWRKY45	1.5 (± 0.51)	0.91 (± 0.21)	1.29 (± 0.17)	3.41 (± 0.39)*	1.39 (± 0.17)	1.11 (± 0.20)	2.35 (± 1.07)	1.71 (± 0.21)	0.63 (± 0.01)**	0.94 (± 0.21)
BnWRKY53	0.39 (± 0.14)	0.89 (± 0.50)	2.14 (± 0.07)	0.75 (± 0.10)	8.14 (± 1.69)	2.33 (± 1.05)	0.45 (± 0.00)**	0.81 (± 0.37)	1.43 (± 0.19)	2.08 (± 0.69)
BnWRKY65	1.41 (± 0.36)	1.58 (± 0.51)	1.82 (± 0.70)	1.46 (± 0.33)	1.64 (± 0.40)	1.84 (± 0.71)	0.77 (± 0.19)	1.16 (± 0.42)	0.78 (± 0.17)	0.50 (± 0.05)**
BnWRKY69	0.71 (± 0.10)	1.04 (± 0.27)	1.29 (± 0.01)**	1.76 (± 0.29)	1.29 (± 0.16)	1.15 (± 0.19)	0.42 (± 0.08)*	1.17 (± 0.41)	0.65 (± 0.08)*	0.56 (± 0.10)*
BnWRKY70	0.84 (± 0.16)	1.12 (± 0.21)	1.43 (± 0.24)	0.52 (± 0.00)**	13.98 (± 6.01)	3.66 (± 2.00)	0.85 (± 0.12)	1.01 (± 0.36)	0.46 (± 0.04)**	0.83 (± 0.26)
BnWRKY75	1.55 (± 0.36)	2.30 (± 0.93)	2.39 (± 0.03)*	9.21 (± 0.63)*	17.58 (± 12.54)	8.19 (± 4.61)	4.53 (± 2.14)	2.03 (± 0.43)	0.50 (± 0.24)	0.60 (± 0.30)

Results are presented as a ratio of transcript abundance in treatment/mock on a linear scale. Data were mean of three biological replicates ± S.E. The asterisk indicates that the corresponding gene was significantly up- or down-regulated under a stress treatment by *t*-test (* for $p < 0.05$ and ** for $p < 0.01$).

BnWRKY10 waits to be identified (Additional file 3). A recent study demonstrated that AtWRKY TFs bearing the WRKYGQK motif exhibit binding site preferences, which are partly dependent on the adjacent DNA sequences outside of the TTGACY-core motif [20]. For those WRKY TFs that do not contain the canonical WRKYGQK motif, a binding sequence other than the W-box element ((C/T)TGAC(C/T)) may exist. For instance, the binding sequence of tobacco (*Nicotiana tabacum*) NtWRKY12 with a WRKYGKK motif is TTTTCCAC, which deviates significantly from W-box [76]. Moreover, soybean (*Glycine max*) GmWRKY6 and GmWRKY21 lose the ability to bind to a W-box containing the variant WRKYGKK motif [66]. It seems likely that the BnWRKY TFs that lack the canonical WRKYGQK motif might not be able to interact with W-box and therefore may have different target genes and possibly divergent roles, a proposal that must be verified in future studies. Finally, mutation of amino acid Q to K of AtWRKY1 was observed to affect binding activity with the consensus W-box [99]. Furthermore, the second characteristic feature of WRKY proteins is a unique zinc-finger motif C-X_{4-5}-C-X_{22-23}-H-X-H [11]. Of the 53 BnWRKY domains, most of them contain this unique zinc-finger motif while BnWRKY46, 53 and 70 (group III) have an extended zinc-finger motif which is C-X_7-C-X_{23}-H-X-C. This observation is consistent with previous study in *Arabidopsis* [11], barley [26] and rice [100].

Complete or partial WRKY domains are found in ESTs from many species of land plants [24]. Recently, 37 *WRKY* genes were identified in the moss, *Physcomitrella patens* [101]. So far, no *WRKY* genes have been identified in the archaea, eubacteria, fungi, or animal lineages [24]. However, in the genomes of the protist, *Giardia lamblia* and the slime mold, *Dictyostelium discoideum*, a single *WRKY* gene with two *WRKY* domains were recently identified [23,24]. Further examination of the two WRKY domains existing in the two organisms indicates that *G. lamblia* WRKY TF

has a WRKYGSK heptapeptide at its N-terminal and a WKKYGHK at its C-terminal, whereas in *D. discoideum*, both WRKY domains have a classical WRKYGQK heptapeptide [24,101]. This suggests an ancient origin of the canonical WRKYGQK heptapeptide and its variants. In the green algae, *Chlamydomonas reinhardtii*, a WRKY TF containing two WRKY domains (Acc. XM_001692290) was also identified [24,101]. In the genome of the recently sequenced, world's smallest free-living eukaryote, the unicellular chlorophytic algae, *Ostreococcus tauri*, a WRKY gene containing a single WRKY domain and a WRKYGCK heptapeptide is also present (Acc. CAL54953).

The identification of WRKY genes in primitive eukaryotes suggests an ancient origin of the WRKY family, and this family had emerged before the evolution and diversification of the plant phyla [24]. During the long evolutionary history, the WRKY gene family greatly expanded, as demonstrated by the increased numbers of *WRKY* genes in higher plants [101], and this expansion may be primarily due to segmental duplications of genomic fragments as a result of independent polyploidy events [24,102-104]. Comparison of a genomic region harboring five genes, one of which is *WRKY10*, between tomato, *Arabidopsis* and *Capsella rubella*, has revealed a great degree of microsynteny between closely and distantly related dicotyledonous species [105]. In addition, AtWRKY10, with one WRKY domain, is clustered within group I AtWRKYs possessing two-domains [11]. The ortholog of AtWRKY10 in tomato or rice (OsWRKY35, Os04g39570) contains two WRKY domains, which may suggest that during evolution, the N-terminal domain has been lost and the occurrence of the loss of the N-terminal WRKY domain of AtWRKY10 is after the divergence of monocots and dicots [27,105].

An overall genomic duplication event has been identified to exist in the tribe *Brassiceae* after a comparative genomic analysis, and many genomic units that are conserved between canola and *Arabidopsis* have also been identified [106-108]. We observed Brassicaeceae-specific clades and rice-specific clades based on our current analysis. In group III, *Arabidopsis* WRKY domains (six AtWRKYs) form Brassicaeceae-specific clade while rice WRKY domains (23 OsWRKYs) also form rice-specific clade (Additional file 4). Similar to that observed by [26], some monocot-specific clades were observed in groups IIc and III WRKY domains of rice and barley. This further supports the conclusion by Mangelsen et al. [26] about the occurrence of this diversification after the divergence of mono- and dicotyledonous plants. Possibly more Brassicaeceae-specific WRKY clades could be indentified from future phylogenetic analysis after the whole genome of canola has been determined. A further comparative genomic analysis of the *WRKY*-containing regions between canola and *Arabidopsis* should enable us to reveal the extent of microcolinearity between these closely related species and a better understanding of the expansion of the *WRKY* gene family in canola.

WRKY TFs are involved in the regulation of various biological processes, including pathogen responses and hormone signaling [109]. A previous expression analysis of *AtWRKY* genes demonstrated that nearly 70% are differentially expressed by pathogen infection or SA treatment, suggesting important roles for *WRKY* in defense responses [42]. Recently, two studies of the rice *WRKY* genes also demonstrated that many are responsive to JA, SA and ABA treatments [37,38]. Increased transcript abundance of SA- and JA-responsive genes is essential for the induced resistance conferred by the two signaling pathways [97,110,111]. *WRKY* TFs are also reported to participate in disease resistance in *Arabidopsis* and tobacco through modulation of SA- or JA-responsive gene expression similar to that induced by the TGA class of basic leucine-zipper transcription factors [39,40,45,81,112].

Previous studies from our laboratory as well as those of others revealed that few genes related to SA-signaling were modulated by infection of canola with *S. sclerotiorum* [2,5], suggesting that SA does not play a crucial role in mediating responses of canola to this pathogen. The responses of *Arabidopsis* to *A. brassicicola*, which causes black spot in canola as *A. brassicae* does, also appear to be mediated through JA instead of SA [113], which is similar to responses to other necrotrophic fungi, including *Pythium* species [80,114]. Hence, it is possible that WRKY TFs may play an important role in suppressing the involvement of SA in response to those pathogens. This suggestion is consistent with the conclusion that AtWRKY33, which is induced by many pathogens, acts as a positive regulator of JA- and ET-mediated defense signaling but as a negative regulator of SA-mediated responses [45]. As mentioned earlier, both *A. brassicae* as well as *S. sclerotiorum* are able to induce *BnWRKY33*, one of the genes belonging to group I. Moreover, it has been demonstrated that pathogen-induced *AtWRKY33* expression does not require SA signaling [80]. Similar to *AtWRKY33*, *AtWRKY25* acts as a negative regulator of the SA-mediated signaling pathway [25]. The increased abundance of *BnWRKY25* due to the infection by *S. sclerotiorum*, but not in the case of *A. brassicae* challenge, also suggests that it might also work as a negative regulator of SA-related signaling pathways in the canola-*S. sclerotiorum* pathosystem, but not in the canola-*A. brassicae* pathosystem. Of the other group I members investigated in our study, (*BnWRKY1, 20 and 32*), *BnWRKY1* was observed to be significantly induced by *S. sclerotiorum* only at 72 h (Figure 3), and *BnWRKY20* and 32 were repressed by *S. sclerotiorum* (Figure 3), indicating the differences in behavior of

group I BnWRKYs in response to fungal pathogens (*S. sclerotiorum* and *A. brassicae*).

It is possible that several BnWRKY TFs may also be involved in signaling the responses of canola to the pathogens *S. sclerotiorum* and *A. brassicae*. For instance, group IIa members have been demonstrated to play both positive and negative roles in plant defense [112,115-117]. The transcript levels of two genes of the group IIa: *BnWRKY18*, and 40, orthologs of which are known to act as negative regulators of plant defense in *Arabidopsis* [95], were observed to increase in response to *S. sclerotiorum* and *A. brassicae* challenge. For *A. brassicae*, the differences in transcript abundance between controls and inoculated plants were not statistically significant (Figure 3). In addition, it has been reported that *AtWRKY6*, one member of the group IIb, acts as a positive regulator of the senescence- and pathogen defense-associated *PR1* promoter activity, and is also induced by SA and bacterial infection [114]. Since leaf senescence is often linked to plant defense [118], the induction of *BnWRKY6* by *S. sclerotiorum*, ABA and SA at an early time-point (6 h) but not *A. brassicae*, may suggest a role in leaf senescence, which is observed very early in the *S. sclerotiorum*-canola pathosystem (Figure 5).

We also observed that group IIc (*BnWRKY28*,45) and III (*BnWRKY75*) BnWRKYs in our study were all induced by the infection of *S. sclerotiorum* and ET whereas *BnWRKY75* was induced only by *A. brassicae* (Figure 3, Table 2). Changes in expression of *BnWRKY75* induced by both pathogens suggest that an ET-mediated signaling pathway may be involved in mediating the responses of canola to necrotrophic pathogens. In *Arabidopsis* growing in 1/2 × MS liquid media supplemented with 10 μM of 1-aminocyclopropane-1-carboxylate (ACC), it has been previously observed that *AtWRKY45* and *AtWRKY75* were induced; while *AtWRKY28* was repressed compared to untreated controls [119]. This differences between the expression of *AtWRKY28* [116] and *BnWRKY28* (this study) in response to ET treatment may be the result of subtle differences in experiments including the use of different tissues (seedlings versus leaves), and/or the ethylene-generating reagents (ACC versus ET) used. Further studies on the role of these three BnWRKY TFs in mediating defense responses are ongoing in our laboratory.

Although *BnWRKY11* (Group IId) was not affected by either *S. sclerotiorum* or *A. brassicae* in this study, our previous microarray profiling of transcriptome changes in canola as a result of *S. sclerotiorum* infection revealed that transcript levels of *BnWRKY11* and 15 increased while that of *BnWRKY17* decreased at specific time points, although the magnitude of response was less than twofold [5]. *Arabidopsis AtWRKY11* and *AtWRKY17* are both known to act as negative defense regulators and *WRKY11* appears to act upstream of JA [120] since it does not respond to JA [119,121]. However, the expression of *AtWRKY11* has been reported to correlate with the induction of the JA biosynthesis enzymes AOS and LOX 2 h after challenge with *P. syringae* [120]. Incidentally, the accumulation of JA also occurs within the first hour of the interaction with *P. syringae* [122]. Taken together with our observations that *BnWRKY11* was repressed by JA and ET treatments at 6 h, and was not induced by the pathogens, it is possible that the pathogen-induced accumulation of JA might modulate the expression of *BnWRKY11*.

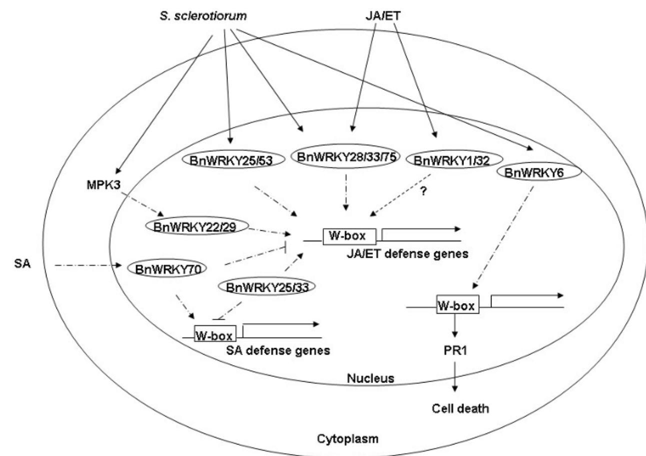

Figure 5

Hypothetical model of WRKY network in mediating canola responses to *S. sclerotiorum* and phytohormones. JA/ET-responsive, but not SA-responsive genes were observed in the necrotrophic pathogen *S. sclerotiorum*-canola interaction [2,5], and these induced *BnWRKY* genes, such as *BnWRKY25*, *28*, *33*, *53* and *75*, could potentially activate the downstream JA/ET signaling pathway or cell death. Moreover, BnWRKY70 might also negatively regulate JA/ET signaling pathway while BnWRKY6 possibly modulates the expression of *PR1* gene, culminating in cell death, which would benefit the infection and growth of necrotrophic fungi [114,118]. MPK3, one of mitogen-activated protein kinases, was also observed to be induced by *S. sclerotiorum* in our previous microarray study [5], and MPK3 in Arabidopsis is a positive regulator of AtWRKY22/29 [39]. Solid and dashed arrows represent likely or putatively positive regulation of the downstream targets while open blocks indicate negative regulation of the downstream genes.

Given the recently emerging role for ABA in defense responses [61-63], it is possible that ABA exerts some of these effects through the modulation of *BnWRKY* genes, specifically *BnWRKY53* and *BnWRKY69*. Similarly, the cytokinin BAP has been implicated in both alleviating and exacerbating the hypersensitive response (HR), which is characterized by tissue necrosis and is frequently accom-

panied by the subsequent induction of systemic acquired resistance (SAR) throughout the plant [123]. Furthermore, cytokinins can promote the susceptibility of biotrophs by inducing the necrotroph resistance pathway, which is responsive to JA/ET [98]. As suggested for ABA mediated plant defense responses, it is possible that the BnWRKYs, which were observed to be modulated by exogenous BAP application, may be responsible, at least in part, for mediating the observed effects with the necrotrophic pathogens.

Based on our previous and current studies, we propose a model outlining the possible roles of BnWRKY TFs in mediating the responses of canola to *S. sclerotiorum* and phytohormones (Figure 5). Both *S. sclerotiorum* and JA/ET can induce *BnWRKY28*, 33, and 75 at 24 h post inoculation and possibly activate the downstream JA/ET signaling pathway at later time points (Figure 5). *S. sclerotiorum* specifically induces *BnWRKY6*, 25, and 53, and JA/ET specifically induce *BnWRKY1* and 32 at 24 h post inoculation (Figure 5). This may be explained by the more complicated molecular patterns generated by *S. sclerotiorum* during the infection compared to the application of JA or ET only. BnWRKY70 in this study is also found to negatively regulate JA/ET defense genes, and it is possibly positively regulated by SA (Figure 5), which could lead to interference with the JA/ET signaling pathway. This is consistent with previous report of AtWRKY70 being the node of convergence of JA and SA signaling [55]. It is also possible that BnWRKY6 modulates the expression of *PR1* gene, culminating in cell death (Figure 5), which is conducive for the growth of necrotrophic fungi [114,118], a hypothesis that must be tested in the future. A mitogen-activated protein kinase (MAPK) gene, *MPK3*, was also observed to be induced at 24 h and 48 h post inoculation in our previous study [5]. MPK3 in *Arabidopsis* has been demonstrated to positively regulate *AtWRKY22/29* [39], (Figure 5). Similar to AtWRKY25 and 33, BnWRKY25 and 33, may act as a positive regulators of JA- and ET-mediated defense signaling pathways and as negative regulators of the SA-mediated signaling pathway [25,45] (Figure 5). Further work needs to be performed to characterize orthologs of AtWRKY22 and 29 in canola, and to examine the relationship between MAPK signaling cascade and BnWRKYs. Moreover, a detailed functional characterization of BnWRKY TFs, using a variety of reverse genetic techniques, in the context of *S. sclerotiorum* response, will help to better delineate their physiological roles.

As discussed above for *B. napus*-*S. sclerotiorum* pathosystem and function of related *AtWRKY* gene, several WRKY factors act as negative regulators of plant defense whereas others positively modulate this response implying their association with distinct regulatory complexes. Functional redundancy in defense programs is an inherent feature of WRKY genes [109] and it may reflect a strong need to backup essential regulatory functions [22]. Still, we can expect exciting novel revelations about WRKY TFs in the very near future on the basis of the enormous progress made within the past two years.

Conclusion

In summary, we identified 46 BnWRKY TFs based on the publicly available EST resources of canola and cloned the cDNA sequences for 38 of them. We characterized the responses of 16 selected genes, based on their phylogenetic relationship in response to two fungal pathogens and five hormone treatments. Based on our data, we propose that BnWRKY TFs might play an important role in plant defense response, possibly by acting as positive or negative regulators of plant defense, and canola may respond differently to *S. sclerotiorum* and *A. brassicae* from BnWRKY mediated plant defense system. Our results also confirm that there is cross-talk between biotic stress and hormone signaling. Functional redundancy in defense programs is an inherent feature of WRKY genes [109] and future studies will be directed towards delineating the specific roles of individual WRKY TFs in those and related pathosystems in order to explore the possibility that manipulation of abundance of one or several of these proteins may lead to durable and robust resistance to the pathogen, apart from contributing to our understanding of the molecular processes that occur during host-pathogen interactions.

Methods

BnWRKY *gene identification*

Thirty-six WRKY domain sequences (WRKY-seed) downloaded from Pfam http://pfam.sanger.ac.uk/family?acc=PF03106 were used to search the dbEST http://www.ncbi.nlm.nih.gov/dbEST/index.html datasets (release 053008) for WRKY genes in *B. napus* (oilseed rape and canola) using the tBlastn program. The significant hits (E < 1e-4) were retrieved and Microsoft Excel 2003 was then used to obtain unique sequences based on the GenBank Accession numbers. 177 unique ESTs were retrieved and organized into a FASTA format file before input into ESTpass program [124] for cleansing, clustering, and assembling of the unique ESTs. To confirm that the obtained contigs and singlets encode WRKY proteins, the nucleotide sequences were translated in six possible reading frames using OrfPredictor into amino acid sequences, which were then examined for the existence of the heptapeptide WRKYGQK and its variants. The resulting 36 contigs and 38 singlets were used as query sequences in a BLASTn search against *B. napus* EST dataset in NCBI dbEST and Shanghai RAPESEED database (http://rapeseed.plantsignal.cn/, [74]) in order to obtain maximum sequence length for each *BnWRKY*, and 339 unique ESTs were retrieved. We also used a key word search of

WRKY genes in *B. napus* in the non-redundant (nr) database of NCBI and obtained two cDNA sequences (GenBank Acc. DQ539648 and DQ209287), which were annotated to be *BnWRKY40*. Altogether we obtained 341 unique sequences based on the accession numbers. We then used the ESTpass program for cleansing, clustering, and assembling of the unique ESTs. The resultant contigs and singletons were then used as query sequences in a Blastn search against *Arabidopsis* to find the best hit (putative orthologs) among the 72 *AtWRKY* genes. Afterwards, the putative transcripts were analyzed using OrfPredictor to predict open reading frames (ORFs) and obtain the translated amino acid sequences. The amino acid sequence of the largest ORF for each putative transcript was filtered out and entered into the SMART program http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1 to predict the WRKY domain. In case of the absence of the characteristic features of the WRKY domain for a particular transcript, it was translated in six possible reading frames in DNAMAN (V4.0, Lynnon BioSoft) and manually checked to output the amino acid sequences. At this step, we obtained 46 unique *BnWRKY* genes and identified those *BnWRKY* genes that contain incomplete or no WRKY domain and therefore we used RT-PCR together with 3'RACE to extend the WRKY domain sequences.

Plant growth and gene cloning

Wild type canola (Westar) plants were grown in Sunshine soil mix 4 (Sungro, Vancouver, BC, Canada) in the greenhouse with a photoperiod of 16 h light (combination of natural light and T5 fluorescent tubes with a light intensity of approximately 200 μE m^{-2} s^{-1})/8 h dark, and a temperature of 21°C day/18°C night for 18 days. Young leaves were harvested for RNA isolation using the RNeasy Plant Mini kit (Qiagen, Mississauga, ON, Canada). RNA integrity was checked by electrophoresis on a formaldehyde agarose gel and quantified using the NanoDrop 1000 (NanoDrop Technologies, Inc., Wilmington, DE, USA). First-strand cDNA was synthesized from 2 μg of total RNA using Superscript II (Invitrogen, Burlington, ON, Canada) and Oligo$(dT)_{18}$ primers (Fermentas, Burlington, ON, Canada). PCR primers were designed using PrimerSelect (DNAStar Inc.) or Primer 3 (v0.4.0, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi and are listed in additional file 5. PCR was conducted in a 50-μL final volume including 0.5 μL of cDNA template, 1× *Pfx* buffer, 200 μM deoxynucleotide triphosphates (dNTPs) (Fermentas), 400 nM of each primer, and 2 units of Platinum *Pfx* polymerase (Invitrogen). The PCR conditions included an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, 68°C for 1 min per kb, with a final extension at 68°C for 5 min. PCR products were gel purified using the QIAquick gel extraction kit (Qiagen) and cloned into pJET1.2 vector supplied with the CloneJET PCR cloning kit (Fermentas) and sequenced from the two ends using BigDye reagent on an ABI3700 sequencer (Applied Biosystems, Foster city, CA, USA).

For rapid amplification of cDNA ends (3'RACE), first-strand cDNA was made from 2 μg of total RNA extracted from wild-type canola (cv. Westar) using Superscript II and an oligo$(dT)_{17}$ adaptor sequence [125], and 0.5 μL of cDNA template was used for 3' RACE. Reactions were conducted in a 50-μL final volume including 1× Taq buffer, 0.2 mM dNTPs, 0.4 μM of each primer, and 0.2 μL (1 unit) of Platinum *Taq* polymerase (Invitrogen). The primers designed by PrimerSelect (DNAstar) are outlined in the additional file 5 and the adaptor sequence was 5'-GACTCGAGCGACATCGAT-3' [125]. The PCR conditions included an initial denaturation of 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products were purified and cloned into pGEM-T vector (Promega, Madison, WI, USA) or pJET1.2 vector (Fermentas) and sequenced. Sequences were analyzed and translated using DNAStar. Based on the sequenced cDNA sequences from 3'RACE, new primers were designed, which were then used to clone the full-length cDNAs of some *BnWRKY* genes. At least two independent clones were sequenced from both ends.

Phylogenetic tree construction and bioinformatics

The WRKY domain boundary was defined as previously described [11]. The peptide sequences of the domains were aligned using ClustalX (v1.83) with a gap opening penalty of 35 and gap extension penalty of 0.75 in pairwise alignment, and a gap opening penalty of 15 and gap extension penalty of 0.30 in multiple alignment parameters settings. The multiple alignments were adjusted with gaps manually inserted for optimal alignment based on the conserved features of the WRKY domains. The maximum parsimony algorithm implemented in MEGA4 [126] for amino acid sequences were used for phylogenetic tree reconstruction according to [127,128]. One hundred bootstrapped data sets were used to estimate the confidence of each tree clade. The protein sequences of *Arabidopsis* WRKY TFs were retrieved from TAIR http://www.arabidopsis.org and rice WRKY TFs from the Database of Rice Transcription Factors (DRTF, http://drtf.cbi.pku.edu.cn/). The nomenclature of rice (*Oryza sativa*. cv japonica) WRKY TFs was as previously described [27]. Putative orthologs of *BnWRKY* genes were identified in both *Arabidopsis* and rice using the translated amino acid sequences in InParanoid [75].

Subcellular localization and confocal microscopy

The coding regions (CDS) of *BnWRKY6*, *BnWRKY25*, *BnWRKY33*, and *BnWRKY75* were amplified by RT-PCR

from canola (Westar) cDNAs using the primers listed in additional file 5. PCR products were purified using a QIAquick PCR purification kit (Qiagen), restricted by *Nco* I (New England Biolabs, Ipswich, MA, USA) and/or *Bsp* HI (Fermentas), purified again and cloned into *Nco* I digested pCsGFPBT (GenBank: DQ370426) vector with a Gly-Ala-rich peptide linker between CDSs and sGFP. All constructs were sequenced and mobilized into *Agrobacterium tumefaciens* GV3101 through the freeze-thaw method and transformed into wild-type *A. thaliana* (Col-0) employing the floral dip method (Clough and Bent, 1998). Resistant lines were selected on 1/2 × MS containing 1% (w/v) sucrose and 50 mg/L hygromycin B (Sigma-Aldrich) for 7 d before being transferred into soil to grow the plants to maturity and to harvest T_2 seeds, which were further sown on the same type of hygromycin-containing medium. Five-day-old seedlings from ten independent T_2 lines were mounted on slides for GFP observation under confocal microscope (Carl Zeiss). At least five cells were screened for each line.

Fungal pathogen inoculation and hormone treatments

Wild type canola (cv. Westar) plants were grown as described previously in a greenhouse for 18 days. Potato dextrose agar (PDA) agar plugs of *S. sclerotiorum* were prepared as described earlier [5] and placed on the first and second true leaves, which were wounded slightly. The preparation of spores of *A. brassicae* and inoculation of canola leaves were performed as described previously [4]. Leaves of uninoculated/mock plants were treated similarly with PDA agar plugs without the mycelia or with water in the case of *A. brassicae*. Plants were placed in a humidity chamber for 24 h before being placed in the greenhouse. Tissues were harvested 12, 24, 48 and 72 h post inoculation and kept at -80°C after being flash-frozen in liquid nitrogen. JA, SA, BAP and ABA were applied by spraying 50 μM JA, 1 mM SA, 20 μM BAP or 50 μM (±)-ABA (Sigma-Aldrich, St. Louis, MO, USA). A stock solution (500 μM) of JA in water was first prepared and then diluted with 0.1% (v/v) ethanol to 50 μM. ABA was first dissolved in absolute ethanol to prepare a 20 mM stock solution and then diluted with 0.1% (v/v) ethanol to the final 50 μM solution. SA was dissolved in water to prepare a 100 mM stock solution with the adjustment of pH to 6.5 using 1 M KOH before dilution in water to the 1 mM working solution and BAP was dissolved in 1 M NaOH to prepare a 1 mM stock solution after which it was diluted with water to the 20 μM working solution. The mock treatments were 0.1% (v/v) ethanol for JA and ABA, water adjusted to pH 6.5 with 1 M KOH or 1 M NaOH for SA or BAP treatments, respectively. Ethylene treatment was carried out in an airtight clear acrylic chamber (1.5 m × 0.6 m × 0.6 m) placed in the same greenhouse, into which 100 ppm ethylene gas in air (Praxair, Mississauga, ON, Canada) was passed at a rate of 2 L/min. Mock plants were placed in a separate chamber into which air (Praxair) was passed at the same rate. Leaves from mock and hormone treated plants were harvested at 6 and 24 h time points, flash frozen in liquid nitrogen and stored at -80°C. The entire sample preparation was independently repeated three times.

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from mock, inoculated or hormone treated leaf tissue using the RNeasy Plant Mini kit (Qiagen) with on-column DNA digestion. RNA was quantified by NanoDrop ND-1000 (NanoDrop Technologies, Inc.) and the integrity of the RNA was assessed on a 1% (w/v) agarose gel. Primers were designed using PrimerExpress3.0 (Applied Biosystems) targeting an amplicon size of 80–150 bp. The primers used are listed in the additional file 5. The specificity of all primers designed was submitted to BLASTn search against NCBI *B. napus* nr and EST databases and any nonspecific primers were eliminated or redesigned. Hence, the results from qRT-PCR analysis might represent the response of specific *BnWRKY* genes. The qRT-PCR assay was performed as described previously [5]. qRT-PCR for each gene was performed in duplicate for each of the three independent biological replicates. Significance was determined with SAS software version 9.1 (SAS Institute Inc.) (p value < 0.05).

Accession numbers

The cDNA sequences of 38 BnWRKY TF genes cloned in this study were deposited in public database [GenBank: EU912389–EU912407, EU912409–EU912418, FJ012166–FJ012171, FJ210288–FJ210290 and FJ384101 – FJ384114].

Authors' contributions

BY designed, carried out all the experiments and drafted the manuscript. YQJ participated in data analysis, and confocal microscopy. MHR provided assistance. NNVK and MKD provided research facility/tools. NNVK designed and supervised the research. All authors contributed to the writing and editing of the manuscript and approved the manuscript.

Additional material

Additional file 1

Supplementary Table 1. *B. napus (canola) WRKY transcription factors identified in this study.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2229-9-68-S1.xls]

Additional file 2

Supplementary table 2. *Expression sequence tags (ESTs) identified for* BnWRKY *genes.*
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Additional file 3

Alignment of Sequences of 53 WRKY domains of BnWRKY transcription factors*. Identical amino acids are shaded in black, and similar amino acids are shaded in gray. The conserved WRKYGQK heptapeptide or its variants are underlined at the top of the alignment and, the cysteines and histidines of the C2H2- or C2HC-type zinc finger motif are indicated by arrows. The consensus amino acids are shown at the bottom of the alignment. This alignment was produced by BOXSHADE 3.21 http://www.ch.embnet.org/software/BOX_form.html.*
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Additional file 4

A bootstrap consensus maximum parsimony tree of WRKY TFs in canola, Arabidopsis and rice (**japonica*****)****. Only the WRKY domain residues were aligned using ClustalX (v1.83) and the evolutionary history was inferred using the maximum parsimony method in MEAG4. The percentage of replicate trees is shown on the branches and it is calculated in the bootstrap test (500 replicates) for the associated taxa being clustered together. All alignment gaps were treated as missing data. There were a total of 150 positions in the final dataset, out of which 66 were parsimony informative. The two letters N and C after group I represents the N-terminal and the C-terminal WRKY domains of group I proteins, respectively. A chlorophyte alga,* Ostreococcus tauri *(Ot) WRKY (Acc. CAL54953) is used as the outgroup.*
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Additional file 5

Primers used in this study*. F, forward primer for RT-PCR; R, reverse primer for RT-PCR; QF, qRT-PCR forward primer; QR, qRT-PCR reverse primer; RACE-F, 3'RACE forward primer; GFP-F, forward primer for N-terminal GFP fusion; GFP-R, reverse primer for N-terminal GFP fusion.*
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References

1. Bolton MD, Thomma B, Nelson BD: ***Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen.** *Mol Plant Patho* 2006, **7(1):**1-16.
2. Liang Y, Srivastava S, Rahman MH, Strelkov SE, Kav NN: **Proteome changes in leaves of *Brassica napus* L. as a result of *Sclerotinia sclerotiorum* challenge.** *J Agric Food Chem* 2008, **56(6):**1963-1976.
3. Sharma N, Hotte N, Rahman MH, Mohammadi M, Deyholos MK, Kav NNV: **Towards identifying Brassica proteins involved in mediating resistance to *Leptosphaeria maculans* : A proteomics-based approach.** *Proteomics* 2008, **8(17):**3516-3535.
4. Sharma N, Rahman MH, Strelkov S, Thiagarajah M, Bansal VK, Kav NNV: **Proteome-level changes in two *Brassica napus* lines exhibiting differential responses to the fungal pathogen *Alternaria brassicae*.** *Plant Sci* 2007, **172(1):**95-110.
5. Yang B, Srivastava S, Deyholos MK, Kav NNV: **Transcriptional profiling of canola (*Brassica napus* L.) responses to the fungal pathogen Sclerotinia sclerotiorum.** *Plant Sci* 2007, **173(2):**156-171.
6. Chen W, Provart N, Glazebrook J, Katagiri F, Chang H-S: **Expression profile matrices of Arabidopsis transcription factor genes predict their putative functions in response to environmental stresses.** *Plant Cell* 2002, **14:**559-574.
7. Durrant W, Rowland O, Piedras P, Hammond-Kossak K, Jones J: **cDNA-AFLP reveales a striking overlap in the race-specific resistance and wound response expression profiles.** *Plant Cell* 2000, **12:**963-977.
8. Maleck K, Levine A, Eulgem T, Morgan A, Schmid J, Lawton KA, Dangl JL, Dietrich RA: **The transcriptome of Arabidopsis thaliana during systemic acquired resistance.** *Nat Genet* 2000, **26(4):**403-410.
9. Mysore K, Crasta O, Tuori R, Folkerts O, Swirsky P, Martin G: **Comprehensive transcript polling of the PToand Prf-mediated host defense responses to infection by Pseudomonas syringae pv. tomato.** *Plant J* 2002, **32:**299-316.
10. Singh KB, Foley RC, Onate-Sanchez L: **Transcription factors in plant defense and stress responses.** *Curr Opin in Plant Biol* 2002, **5(5):**430-436.
11. Eulgem T, Rushton PJ, Robatzek S, Somssich IE: **The WRKY superfamily of plant transcription factors.** *Trends in Plant Sci* 2000, **5(5):**199-206.
12. Korfhage U, Trezzini GF, Meier I, Hahlbrock K, Somssich IE: **Plant Homeodomain Protein Involved in Transcriptional Regulation of a Pathogen Defense-Related Gene.** *Plant Cell* 1994, **6(5):**695-708.
13. Rushton PJ, Torres JT, Parniske M, Wernert P, Hahlbrock K, Somssich IE: **Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley PR1 genes.** *EMBO J* 1996, **15(20):**5690-5700.
14. Zhou JM, Tang XY, Martin GB: **The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a cis-element of pathogenesis-related genes.** *EMBO J* 1997, **16(11):**3207-3218.
15. dePater S, Greco V, Pham K, Memelink J, Kijne J: **Characterization of a zinc-dependent transcriptional activator from Arabidopsis.** *Nucleic Acids Res* 1996, **24(23):**4624-4631.
16. Eulgem T, Rushton PJ, Schmelzer E, Hahlbrock K, Somssich IE: **Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors.** *EMBO J* 1999, **18(17):**4689-4699.
17. Rushton PJ, Macdonald H, Huttly AK, Lazarus CM, Hooley R: **Members of a new family of DNA-binding proteins bind to a conserved cis-element in the promoters of alpha-Amy2 genes.** *Plant Mol Biol* 1995, **29(4):**691-702.
18. Wang ZP, Yang PZ, Fan BF, Chen ZX: **An oligo selection procedure for identification of sequence-specific DNA-binding activities associated with the plant defence response.** *Plant J* 1998, **16(4):**515-522.
19. Yang PZ, Chen CH, Wang ZP, Fan BF, Chen ZX: **A pathogen- and salicylic acid-induced WRKY DNA-binding activity recognizes the elicitor response element of the tobacco class I chitinase gene promoter.** *Plant J* 1999, **18(2):**141-149.
20. Ciolkowski I, Wanke D, Birkenbihl RP, Somssich IE: **Studies on DNA-binding selectivity of WRKY transcription factors lend structural clues into WRKY-domain function.** *Plant Mol Biol* 2008, **68(1–2):**81-92.
21. Sun CX, Palmqvist S, Olsson H, Boren M, Ahlandsberg S, Jansson C: **A novel WRKY transcription factor, SUSIBA2, participates in sugar signaling in barley by binding to the sugar-responsive elements of the iso1 promoter.** *Plant Cell* 2003, **15(9):**2076-2092.
22. Babu MM, Iyer LM, Balaji S, Aravind L: **The natural history of the WRKYGCM1 zinc fingers and the relationship between transcription factors and transposons.** *Nucleic Acids Res* 2006, **34(22):**6505-6520.
23. Ulker B, Somssich IE: **WRKY transcription factors: from DNA binding towards biological function.** *Curr Opin in Plant Biol* 2004, **7(5):**491-498.
24. Zhang YJ, Wang LJ: **The WRKY transcription factor superfamily: its origin in eukaryotes and expansion in plants.** *BMC Evol Biol* 2005, **5:**1.
25. Zheng ZY, Mosher SL, Fan BF, Klessig DF, Chen ZX: **Functional analysis of Arabidopsis WRKY25 transcription factor in plant defense against Pseudomonas syringae.** *BMC Plant Biol* 2007, **7:**2.

26. Mangelsen E, Kilian J, Berendzen KW, Kolukisaoglu UH, Harter K, Jansson C, Wanke D: **Phylogenetic and comparative gene expression analysis of barley (Hordeum vulgare) WRKY transcription factor family reveals putatively retained functions between monocots and dicots.** *BMC Genomics* 2008, **9**:17.
27. Ross CA, Liu Y, Shen QXJ: **The WRKY gene family in rice (Oryza sativa).** *J Integr Plant Biol* 2007, **49(6)**:827-842.
28. Kim YK, Li D, Kolattukudy PE: **Induction of Ca2Þcalmodulin signaling by hard surface contact primes Colletotrichum gloeosporioides conidia to germinate and form appressoria.** *J Bacteriol* 1998, **180**:5144-5150.
29. Johnson CS, Kolevski B, Smyth DR: **TRANSPARENT TESTA GLABRA2, a trichome and seed coat development gene of Arabidopsis, encodes a WRKY transcription factor.** *Plant Cell* 2002, **14(6)**:1359-1375.
30. Lagace M, Matton DP: **Characterization of a WRKY transcription factor expressed in late torpedo-stage embryos of Solanum chacoense.** *Planta* 2004, **219(1)**:185-189.
31. Xie Z, Zhang ZL, Zou XL, Huang J, Ruas P, Thompson D, Shen QJ: **Annotations and functional analyses of the rice WRKY gene superfamily reveal positive and negative regulators of abscisic acid signaling in aleurone cells.** *Plant Physiol* 2005, **137(1)**:176-189.
32. Xu YH, Wang JW, Wang S, Wang JY, Chen XY: **Characterization of GaWRKY1, a cotton transcription factor that regulates the sesquiterpene synthase gene (+)-delta-cadinene synthase-A.** *Plant Physiol* 2004, **135(1)**:507-515.
33. Zhang ZL, Xie Z, Zou XL, Casaretto J, Ho THD, Shen QXJ: **A rice WRKY gene encodes a transcriptional repressor of the gibberellin signaling pathway in aleurone cells.** *Plant Physiol* 2004, **134(4)**:1500-1513.
34. Zou XL, Seemann JR, Neuman D, Shen QXJ: **A WRKY gene from creosote bush encodes an activator of the abscisic acid signaling pathway.** *J Biol Chem* 2004, **279(53)**:55770-55779.
35. Jiang YQ, Deyholos MK: **Comprehensive transcriptional profiling of NaCl-stressed Arabidopsis roots reveals novel classes of responsive genes.** *BMC Plant Biol* 2006, **6**:25.
36. Jiang Y-Q, Deyholos MK: **Functional characterization of Arabidopsis WRKY25 and WRKY33 transcription factors in abiotic stresses.** *Plant Mol Biol* 2009, **69(1–2)**:91-105.
37. Ramamoorthy R, Jiang SY, Kumar N, Venkatesh PN, Ramachandran S: **A comprehensive transcriptional profiling of the WRKY gene family in rice under various abiotic and phytohormone treatments.** *Plant Cell Physiol* 2008, **49(6)**:865-879.
38. Ryu HS, Han M, Lee SK, Cho JI, Ryoo N, Heu S, Lee YH, Bhoo SH, Wang GL, Hahn TR, *et al.*: **A comprehensive expression analysis of the WRKY gene superfamily in rice plants during defense response.** *Plant Cell Rep* 2006, **25(8)**:836-847.
39. Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J: **MAP kinase signalling cascade in Arabidopsis innate immunity.** *Nature* 2002, **415(6875)**:977-983.
40. Chen CH, Chen ZX: **Potentiation of developmentally regulated plant defense response by AtWRKY18, a pathogen-induced Arabidopsis transcription factor.** *Plant Physiol* 2002, **129(2)**:706-716.
41. Dellagi A, Heilbronn J, Avrova AO, Montesano M, Palva ET, Stewart HE, Toth IK, Cooke DEL, Lyon GD, Birch PRJ: **A potato gene encoding a WRKY-like transcription factor is induced in interactions with Erwinia carotovora subsp atroseptica and Phytophthora infestans and is coregulated with class I endochitinase expression.** *Mol Plant Microbe Interact* 2000, **13(10)**:1092-1101.
42. Dong J, Chen C, Chen Z: **Expression profiles of the Arabidopsis WRKY gene superfamily during plant defense response.** *Plant Mol Biol* 2003, **51(1)**:21-37.
43. Marchive C, Mzid R, Deluc L, Barrieu F, Pirrello J, Gauthier A, Corio-Costet MF, Regad F, Cailleteau B, Hamdi S, *et al.*: **Isolation and characterization of a Vitis vinifera transcription factor, VvWRKY1, and its effect on responses to fungal pathogens in transgenic tobacco plants.** *J Exp Bot* 2007, **58(8)**:1999-2010.
44. Shimono M, Sugano S, Nakayama A, Jiang CJ, Ono K, Toki S, Takatsuji H: **Rice WRKY45 plays a crucial role in benzothiadiazole-inducible blast resistance.** *Plant Cell* 2007, **19(6)**:2064-2076.
45. Zheng ZY, Abu Qamar S, Chen ZX, Mengiste T: **Arabidopsis WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens.** *Plant J* 2006, **48(4)**:592-605.
46. Oh SK, Bek KH, Park JM, Yi SY, Yu SH, Kamoun S, Choi D: **Capsicum annuum WRKY protein CaWRKY1 is a negative regulator of pathogen defense.** *New Phytol* 2008, **177(4)**:977-989.
47. Yoda H, Ogawa M, Yamaguchi Y, Koizumi N, Kusano T, Sano H: **Identification of early-responsive genes associated with the hypersensitive response to tobacco mosaic virus and characterization of a WRKY-type transcription factor in tobacco plants.** *Mol Genet Genomics* 2002, **267(2)**:154-161.
48. Liu RH, Zhao JW, Xiao Y, Meng JL: **Identification of prior candidate genes for Sclerotinia local resistance in Brassica napus using Arabidopsis cDNA microarray and Brassica-Arabidopsis comparative mapping.** *Sci China C Life Sci* 2005, **48(5)**:460-470.
49. McDowell JM, Dangl JL: **Signal transduction in the plant immune response.** *Trends Biochem Sci* 2000, **25(2)**:79-82.
50. Thomma B, Eggermont K, Penninckx I, Mauch-Mani B, Vogelsang R, Cammue BPA, Broekaert WF: **Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens.** *Proc Natl Acad Sci USA* 1998, **95(25)**:15107-15111.
51. Berrocal-Lobo M, Molina A: **Ethylene response factor 1 mediates Arabidopsis resistance to the soilborne fungus Fusarium oxysporum.** *Mol Plant Microbe Interact* 2004, **17(7)**:763-770.
52. Berrocal-Lobo M, Molina A, Solano R: **Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi.** *Plant J* 2002, **29(1)**:23-32.
53. Staswick PE, Yuen GY, Lehman CC: **Jasmonate signaling mutants of Arabidopsis are susceptible to the soil fungus Pythium irregulare.** *Plant J* 1998, **15(6)**:747-754.
54. Kunkel BN, Brooks DM: **Cross talk between signaling pathways in pathogen defense.** *Curr Opin in Plant Biol* 2002, **5(4)**:325-331.
55. Li J, Brader G, Palva ET: **The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense.** *Plant Cell* 2004, **16(2)**:319-331.
56. Audenaert K, De Meyer GB, Hofte MM: **Abscisic acid determines basal susceptibility of tomato to Botrytis cinerea and suppresses salicylic acid-dependent signaling mechanisms.** *Plant Physiol* 2002, **128(2)**:491-501.
57. Henfling J, Bostock R, Kuc J: **Effect of Abscisic-Acid on Rishitin and Lubimin Accumulation and Resistance to Phytophthora-Infestans and Cladosporium-Cucumerinum in Potato-Tuber Tissue-Slices.** *Phytopathology* 1980, **70(11)**:1074-1078.
58. Koga H, Dohi K, Mori M: **Abscisic acid and low temperatures suppress the whole plant-specific resistance reaction of rice plants to the infection of Magnaporthe grisea.** *Physiol Mol Plant Pathol* 2004, **65(1)**:3-9.
59. Mohr PG, Cahill DM: **Abscisic acid influences the susceptibility of Arabidopsis thaliana to Pseudomonas syringae pv. tomato and Peronospora parasitica.** *Funct Plant Biol* 2003, **30(4)**:461-469.
60. Thaler JS, Bostock RM: **Interactions between abscisic-acid-mediated responses and plant resistance to pathogens and insects.** *Ecology* 2004, **85(1)**:48-58.
61. Mauch-Mani B, Mauch F: **The role of abscisic acid in plant-pathogen interactions.** *Curr Opin in Plant Biol* 2005, **8(4)**:409-414.
62. Ton J, Jakab G, Toquin V, Flors V, Iavicoli A, Maeder MN, Metraux JP, Mauch-Mani B: **Dissecting the beta-aminobutyric acid-induced priming phenomenon in arabidopsis.** *Plant Cell* 2005, **17(3)**:987-999.
63. Ton J, Mauch-Mani B: **beta-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose.** *Plant J* 2004, **38(1)**:119-130.
64. Pnueli L, Hallak-Herr E, Rozenberg M, Cohen M, Goloubinoff P, Kaplan A, Mittler R: **Molecular and biochemical mechanisms associated with dormancy and drought tolerance in the desert legume Retama raetam.** *Plant J* 2002, **31(3)**:319-330.
65. Xie Z, Zhang ZL, Zou XL, Yang GX, Komatsu S, Shen QXJ: **Interactions of two abscisic-acid induced WRKY genes in repressing gibberellin signaling in aleurone cells.** *Plant J* 2006, **46(2)**:231-242.
66. Zhou QY, Tian AG, Zou HF, Xie ZM, Lei G, Huang J, Wang CM, Wang HW, Zhang JS, Chen SY: **Soybean WRKY-type transcrip-**

tion factor genes, GmWRKY13, GmWRKY21, and GmWRKY54, confer differential tolerance to abiotic stresses in transgenic Arabidopsis plants. *Plant Biotech J* 2008, **6(5):**486-503.

67. Sano H, Ohashi Y: **Involvement of Small Gtp-Binding Proteins in Defense Signal-Transduction Pathways of Higher-Plants.** *Proc Natl Acad Sci USA* 1995, **92(10):**4138-4144.
68. Sano H, Seo S, Koizumi N, Niki T, Iwamura H, Ohashi Y: **Regulation by cytokinins of endogenous levels of jasmonic and salicylic acids in mechanically wounded tobacco plants.** *Plant Cell Physiol* 1996, **37(6):**762-769.
69. Sano H, Seo S, Orudgev E, Youssefian S, Ishizuka K, Ohashi Y: **Expression of the Gene for a Small Gtp-Binding Protein in Transgenic Tobacco Elevates Endogenous Cytokinin Levels, Abnormally Induces Salicylic-Acid in Response to Wounding, and Increases Resistance to Tobacco Mosaic-Virus Infection.** *Proc Natl Acad Sci USA* 1994, **91(22):**10556-10560.
70. Cary AJ, Liu WN, Howell SH: **Cytokinin Action Is Coupled to Ethylene in Its Effects on the Inhibition of Root and Hypocotyl Elongation in *Arabidopsis thaliana* Seedlings.** *Plant Physiol* 1995, **107(4):**1075-1082.
71. Rudd S: **Expressed sequence tags: alternative or complement to whole genome sequences?** *Trends in Plant Sci* 2003, **8(7):**321-329.
72. Iseli C, Jongeneel CV, Bucher P: **ESTScan: a program for detecting, evaluating, and reconstructing potential coding regions in EST sequences.** *Proc Int Conf Intell Syst Mol Biol* 1999:138-148.
73. Min XJ, Butler G, Storms R, Tsang A: **OrfPredictor: predicting protein-coding regions in EST-derived sequences.** *Nucleic Acids Res* 2005, **33:**W677-W680.
74. Wu GZ, Shi QM, Niu Y, Xing MQ, Xue HW: **Shanghai RAPESEED Database: a resource for functional genomics studies of seed development and fatty acid metabolism of Brassica.** *Nucleic Acids Res* 2008, **36:**D1044-D1047.
75. Remm M, Storm CEV, Sonnhammer ELL: **Automatic clustering of orthologs and in-paralogs from pairwise species comparisons.** *J Mol Biol* 2001, **314(5):**1041-1052.
76. van Verk MC, Pappaioannou D, Neeleman L, Bol JF, Linthorst HJM: **A novel WRKY transcription factor is required for induction of PR-1a gene expression by salicylic acid and bacterial elicitors.** *Plant Physiol* 2008, **146(4):**1983-1995.
77. Lewin B: **Genes VII.** Oxford University Press, London; 1999:699.
78. Schuler MA: **Splice Site Requirements and Switches in Plants.** In *Nuclear Pre-Mrna Processing in Plants Volume 326*. Berlin: Springer-Verlag Berlin; 2008:39-59.
79. Schwacke R, Fischer K, Ketelsen B, Krupinska K, Krause K: **Comparative survey of plastid and mitochondrial targeting properties of transcription factors in Arabidopsis and rice.** *Mol Genet Genomics* 2007, **277(6):**631-646.
80. Lippok B, Birkenbihl RP, Rivory G, Brummer J, Schmelzer E, Logemann E, Somissich IE: **Expression of AtWRKY33 encoding a pathogen- or PAMP-responsive WRKY transcription factor is regulated by a composite DNA motif containing W box elements.** *Mol Plant Microbe Interact* 2007, **20(4):**420-429.
81. Robatzek S, Somssich IE: **Targets of AtWRKY6 regulation during plant senescence and pathogen defense.** *Genes Dev* 2002, **16(9):**1139-1149.
82. Zhang ZQ, Li Q, Li ZM, Staswick PE, Wang MY, Zhu Y, He ZH: **Dual regulation role of GH3.5 in salicylic acid and auxin signaling during Arabidopsis-Pseudomonas syringae interaction.** *Plant Physiol* 2007, **145(2):**450-464.
83. Chiu WL, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J: **Engineered GFP as a vital reporter in plants.** *Curr Biol* 1996, **6(3):**325-330.
84. Duarte JM, Cui LY, Wall PK, Zhang Q, Zhang XH, Leebens-Mack J, Ma H, Altman N, dePamphilis CW: **Expression pattern shifts following duplication indicative of subfunctionalization and neofunctionalization in regulatory genes of Arabidopsis.** *Mol Biol Evol* 2006, **23(2):**469-478.
85. Cao H, Bowling SA, Gordon AS, Dong XN: **Characterization of an Arabidopsis Mutant That Is Nonresponsive to Inducers of Systemic Acquired Resistance.** *Plant Cell* 1994, **6(11):**1583-1592.
86. Kesarwani M, Yoo JM, Dong XN: **Genetic interactions of TGA transcription factors in the regulation of pathogenesis-related genes and disease resistance in Arabidopsis.** *Plant Physiol* 2007, **144(1):**336-346.
87. Zhang YL, Tessaro MJ, Lassner M, Li X: **Knockout analysis of Arabidopsis transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance.** *Plant Cell* 2003, **15(11):**2647-2653.
88. Stenzel I, Hause B, Miersch O, Kurz T, Maucher H, Weichert H, Ziegler J, Feussner I, Wasternack C: **Jasmonate biosynthesis and the allene oxide cyclase family of Arabidopsis thaliana.** *Plant Mol Biol* 2003, **51(6):**895-911.
89. Alonso JM, Hirayama T, Roman G, Nourizadeh S, Ecker JR: **EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis.** *Science* 1999, **284(5423):**2148-2152.
90. Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M: **Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression.** *Plant Cell* 2000, **12(3):**393-404.
91. Li FL, Wu XZ, Tsang E, Cutler AJ: **Transcriptional profiling of imbibed Brassica napus seed.** *Genomics* 2005, **86(6):**718-730.
92. Finkelstein RR, Gampala SSL, Rock CD: **Abscisic acid signaling in seeds and seedlings.** *Plant Cell* 2002, **14:**S15-S45.
93. Finkelstein RR, Lynch TJ: **The arabidopsis abscisic acid response gene ABI5 encodes a basic leucine zipper transcription factor.** *Plant Cell* 2000, **12(4):**599-609.
94. Imamura A, Hanaki N, Umeda H, Nakamura A, Suzuki T, Ueguchi C, Mizuno T: **Response regulators implicated in His-to-Asp phosphotransfer signaling in Arabidopsis.** *Proc Natl Acad Sci USA* 1998, **95(5):**2691-2696.
95. Mahonen AP, Bishopp A, Higuchi M, Nieminen KM, Kinoshita K, Tormakangas K, Ikeda Y, Oka A, Kakimoto T, Helariutta Y: **Cytokinin signaling and its inhibitor AHP6 regulate cell fate during vascular development.** *Science* 2006, **311(5757):**94-98.
96. Dong X: **SA, JA, ethylene, and disease resistance in plants.** *Curr Opin Plant Biol* 1998, **1(4):**316-323.
97. Durrant WE, Dong X: **Systemic acquired resistance.** *Annu Rev Phytopathol* 2004, **42:**185-209.
98. Robert-Seilaniantz A, Navarro L, Bari R, Jones JD: **Pathological hormone imbalances.** *Curr Opin Plant Biol* 2007, **10(4):**372-379.
99. Duan MR, Nan J, Liang YH, Mao P, Lu L, Li LF, Wei CH, Lai LH, Li Y, Su XD: **DNA binding mechanism revealed by high resolution crystal structure of Arabidopsis thaliana WRKY1 protein.** *Nucleic Acids Res* 2007, **35(4):**1145-1154.
100. Wu KL, Guo ZJ, Wang HH, Li J: **The WRKY family of transcription factors in rice and Arabidopsis and their origins.** *DNA Res* 2005, **12(1):**9-26.
101. Guo AY, Chen X, Gao G, Zhang H, Zhu QH, Liu XC, Zhong YF, Gu XC, He K, Luo JC: **PlantTFDB: a comprehensive plant transcription factor database.** *Nucleic Acids Res* 2008, **36:**D966-D969.
102. Bowers JE, Chapman BA, Rong JK, Paterson AH: **Unravelling angiosperm genome evolution by phylogenetic analysis of chromosomal duplication events.** *Nature* 2003, **422(6930):**433-438.
103. Cannon SB, Mitra A, Baumgarten A, Young ND, May G: **The roles of segmental and tandem gene duplication in the evolution of large gene families in *Arabidopsis thaliana*.** *BMC Plant Biol* 2004, **4(10):**.
104. Thomas BC, Pedersen B, Freeling M: **Following tetraploidy in an Arabidopsis ancestor, genes were removed preferentially from one homeolog leaving clusters enriched in dose-sensitive genes.** *Genome Res* 2006, **16(7):**934-946.
105. Rossberg M, Theres K, Acarkan A, Herrero R, Schmitt T, Schumacher K, Schmitz G, Schmidt R: **Comparative sequence analysis reveals extensive microcolinearity in the Lateral suppressor regions of the tomato, Arabidopsis, and Capsella genomes.** *Plant Cell* 2001, **13(4):**979-988.
106. Lysak MA, Koch MA, Pecinka A, Schubert I: **Chromosome triplication found across the tribe Brassiceae.** *Genome Res* 2005, **15(4):**516-525.
107. Parkin IAP, Gulden SM, Sharpe AG, Lukens L, Trick M, Osborn TC, Lydiate DJ: **Segmental structure of the Brassica napus genome based on comparative analysis with Arabidopsis thaliana.** *Genetics* 2005, **171(2):**765-781.
108. Town CD, Cheung F, Maiti R, Crabtree J, Haas BJ, Wortman JR, Hine EE, Althoff R, Arbogast TS, Tallon LJ, *et al.*: **Comparative genomics of Brassica oleracea and Arabidopsis thaliana reveal gene loss, fragmentation, and dispersal after polyploidy.** *Plant Cell* 2006, **18(6):**1348-1359.

109. Eulgem T, Somssich IE: **Networks of WRKY transcription factors in defense signaling.** *Curr Opin Plant Biol* 2007, **10(4):**366-371.
110. Penninckx I, Eggermont K, Terras FRG, Thomma B, DeSamblanx GW, Buchala A, Metraux JP, Manners JM, Broekaert WF: **Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway.** *Plant Cell* 1996, **8(12):**2309-2323.
111. Penninckx I, Thomma B, Buchala A, Metraux JP, Broekaert WF: **Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis.** *Plant Cell* 1998, **10(12):**2103-2113.
112. Xu XP, Chen CH, Fan BF, Chen ZX: **Physical and functional interactions between pathogen-induced Arabidopsis WRKY18, WRKY40, and WRKY60 transcription factors.** *Plant Cell* 2006, **18(5):**1310-1326.
113. Vijayan P, Shockey J, Levesque CA, Cook RJ, Browse J: **A role for jasmonate in pathogen defense of Arabidopsis.** *Proc Natl Acad Sci USA* 1998, **95(12):**7209-7214.
114. Robatzek S, Somssich IE: **A new member of the Arabidopsis WRKY transcription factor family, AtWRKY6, is associated with both senescence- and defence-related processes.** *Plant J* 2001, **28(2):**123-133.
115. Eckey C, Korell M, Leib K, Biedenkopf D, Jansen C, Langen G, Kogel K-H: **Identification of powdery midew-induced barley genes by cDNA-AFLP: functional assessment of an early expressed MAP kinase.** *Plant Mol Biol* 2004, **55:**1-15.
116. Shen QH, Saijo Y, Mauch S, Biskup C, Bieri S, Keller B, Seki H, Ulker B, Somssich I, Schulze-Lefert P: **Nuclear activity of MLA immune receptors links isolate-specific and basal diseaseresistance responses.** *Science* 2006, **113:**6372.
117. Wang Y, Lin JS, Wang GX: **Calcium-mediated mitochondrial permeability transition involved in hydrogen peroxide-induced apoptosis in tobacco protoplasts.** *J Integr Plant Biol* 2006, **48(4):**433-439.
118. Quirino BF, Normanly J, Amasino RM: **Diverse range of gene activity during Arabidopsis thaliana leaf senescence includes pathogen-independent induction of defense-related genes.** *Plant Mol Biol* 1999, **40(2):**267-278.
119. Goda H, Sasaki E, Akiyama K, Maruyama-Nakashita A, Nakabayashi K, Li WQ, Ogawa M, Yamauchi Y, Preston J, Aoki K, *et al.*: **The AtGenExpress hormone and chemical treatment data set: experimental design, data evaluation, model data analysis and data access.** *Plant J* 2008, **55(3):**526-542.
120. Journot-Catalino N, Somssich IE, Roby D, Kroj T: **The transcription factors WRKY11 and WRKY17 act as negative regulators of basal resistance in Arabidopsis thaliana.** *Plant Cell* 2006, **18(11):**3289-3302.
121. Pauwels L, Morreel K, De Witte E, Lammertyn F, Van Montagu M, Boerjan W, Inze D, Goossens A: **Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured Arabidopsis cells.** *Proc Natl Acad Sci USA* 2008, **105(4):**1380-1385.
122. De Vos M, Van Oosten VR, Van Poecke RM, Van Pelt JA, Pozo MJ, Mueller MJ, Buchala AJ, Metraux JP, Van Loon LC, Dicke M, *et al.*: **Signal signature and transcriptome changes of Arabidopsis during pathogen and insect attack.** *Mol Plant Microbe Interact* 2005, **18(9):**923-937.
123. Hare PD, Cress WA, van Staden J: **The involvement of cytokinins in plant responses to environmental stress.** *J Plant Growth Regul* 1997, **23(1–2):**79-103.
124. Lee B, Hong T, Byun SJ, Woo T, Choi YJ: **ESTpass: a web-based server for processing and annotating expressed sequence tag (EST) sequences.** *Nucleic Acids Res* 2007, **35:**W159-W162.
125. Frohman MA, Dush MK, Martin GR: **Rapid Production of Full-Length cDNAs from Rare Transcripts – Amplification Using a Single Gene-Specific Oligonucleotide Primer.** *Proc Natl Acad Sci USA* 1988, **85(23):**8998-9002.
126. Tamura K, Dudley J, Nei M, Kumar S: **MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0.** *Mol Biol Evol* 2007, **24(8):**1596-1599.
127. Hall B: **Phylogenetic trees made easy: A how-to manual.** 3rd edition. Sinauer Associates Inc, Sunderland, Massachusetts; 2007.
128. Baldauf SL: **Phylogeny for the faint of heart: a tutorial.** *Trends in Genetics* 2003, **19(6):**345-351.

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