ORIGINAL PAPER

Over-expression of a WRKY transcription factor gene *BoWRKY6* enhances resistance to downy mildew in transgenic broccoli plants

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Abstract WRKY transcription factors play an important role in plant growth, development and immunity. In our study, a WRKY family member gene designated *BoWRKY6* was isolated from broccoli (*Brassica oleracea* var. *italica*), and its expression was induced by downy mildew (*Hyaloperonospora parasitica*). Five transgenic broccoli lines over-expressing *BoWRKY6* driven by the CaMV 35S promotor were obtained by *Agrobacterium tumefaciens* mediated transformation, and they demonstrated significant increased resistance to downy mildew, with resistant levels from low to very high. Real time-qualitative PCR analysis indicated that expressions of both *BoWRKY6* and the pathogenesis-related gene 1 (*BoPR1*) in transgenic plants were obviously higher than those in WT plants after *H. parasitica* treatment. Lines of *BWK14* and *BWK31* exhibited very high resistance to downy mildew, and may serve as promising candidate materials for broccoli molecular breeding in the near future.

Keywords *Brassica oleracea* var. *italica* · Downy mildew · Over-expression · WRKY transcription factor

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Introduction

Plants are constantly exposed to a wide range of biotic and abiotic environmental stresses, and extreme temperatures, drought, salinity, chemical toxicity, oxidative stress, bacteria, virus, fungi, nematodes and insect pests are regarded as serious threats to plant production, by affecting vegetative growth, biomass accumulation as well as quality properties (Gupta and Sharma 2013). Abiotic stress factors have a huge impact on crop losses, and it has been estimated that they reduce average yields by more than 50 % for most major crop plants globally (Bray et al. 2000; Wang et al. 2003). Likewise, biotic stresses cause serious impact on crop, and there are approximately 10 000 species of insect pests, 50 000 species of fungal pathogens, 1 800 species of weed plants and 15 000 species of nematodes that destroy agricultural crops (Klassen and Schwartz, 1985; Koul 2011). To cope with these unfavorable environmental conditions, plants have evolved with complex mechanisms, including changes at cellular, molecular, and physiological levels (Akpinar et al. 2012; Atkinson and Urwin 2012). At molecular level, plant stress responses are regulated by diverse signal transduction pathways that are mediated by hormones like salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA) (Ludwig et al. 2005; Sánchez-Vallet et al. 2012).

A variety of genes have been reported to be induced by environmental stresses, and the induced gene products include late embryogenesis abundant proteins, chaperones, detoxification enzymes, transcription factors, protein kinases as well as phosphoinositide-metabolizing enzymes, which function in protecting cells against oxidative damage (Xiong et al. 2002; Kaur and Gupta 2005). Among them, the cellular abundance of transcription factors are important regulatory proteins that induce or regulate the expression of a series specific genes during stress conditions by interacting with *cis*-elements in

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promoter regions, in *Arabidopsis* for example, there are more than 1500 transcription factors covering over 5 % of its genome, and AP2/EREBP, ABI3/VP1, ARF, bHLH, bZIP, HB, HSF, MYB, NAC as well as WRKY are recognized as known stress responsive transcription factor families (Riechmann et al. 2000; Shameer et al. 2009).

WRKY domain has been defined as an approximately 60 amino acid motif sequence of WRKYGQK at its N-terminus as well as a zinc finger structure of C2H2 or C2HC (C and H indicate cysteine and hisidine amino acid residues, respectively.) at C-terminus (Eulgem et al. 2000; Zhang and Wang 2005). The WRKY gene family participates in plant growth, development, and stress responses. Though discovered relatively recently, they have been regarded as one of the best characterized transcription factor families (Chen et al. 2012). However, to our knowledge, little is known about the biological functions of WRKYs in downy mildew (*Hyaloperonospora parasitica*) resistance. Downy mildew is one of the most destructive diseases of cruciferous crops, and it causes serious problems in commercial production of cabbage, broccoli, cauliflower, radish and mustard. Broccoli (*Brassica oleracea* var. *italica*) is susceptible to infection by downy mildew at all growth stages, causing damages on leaves, stems as well as flower parts, which consequently decreased flower head yield and quality (Wang et al. 2001). In our study, a *Brassica oleracea* var. *italica* WRKY gene, designated *BoWRKY6*, was isolated and introduced into broccoli driven by the CaMV 35S promoter, and five broccoli lines illustrating significantly higher resistance against downy mildew were obtained.

Materials and methods

Plant and fungal materials

Broccoli (*B. oleracea* var. *italica*) line Bo112 (highly resistant to downy mildew) was cultured in a growth chamber to two- erous c s42as01Tfcripti9-85lTc2.45420Td7.6.

employed for expression analysis. RT-PCR assays were conducted using 30 ng cDNA templates of downy mildew treated and control leaves, employing the same cycling conditions as gene cloning. PCR products were separated on a 1.0 % agarose gel, and then photographed using Gel Doc XR$^+$ System (BIO-Rad, UK). Actin gene was used as an internal control, and two primers, ACTUP (5′-TCTCGATGGAAGAGC TGGTT-3′) and ACTDN (5′-GATCCTTACCGAGGGA GGTT-3′), were applied to amplify actin gene fragments using following profile: 94 °C for 5 min; 32 cycles of 94 °C for 30 s, 55.6 °C for 45 s, and 72 °C for 60 s; followed by a final extension at 72 °C for 7 min.

Expression vector construction and transformation

Purified PCR products were digested with two restriction enzymes of *Sma* I and *Sac* I, and then cloned into binary vector pBI121 vector which was digested by the same two enzymes. The recombinant vector was then introduced into *Agrobacterium tumefaciens* strain LBA4404 competent cells. Broccoli line Bo0283 (susceptible to downy mildew) was used for transgenic study. Stems of 18-day old seedlings were harvested, and dipped in 0.1 % $HgCl_2$ for 9 min, and were then inoculated with *A. tumefaciens*. Murashige and Skoog (MS) supplemented with 0.02 mg/L of α-naphthaleneacetic acid (NAA), 4.0 mg/L of 6-benzylaminopurine (6-BA) and 5.0 mg/L of $AgNO_3$ was prepared for pre- and co-culture mediums. Shoot induction medium was made up of MS plus 0.02 mg/L of NAA, 4.0 mg/L of 6-BA, 4.0 mg/L of $AgNO_3$ and 50.0 mg/L of kanamycin (Km). Regenerated shoots were rooted on half strength MS medium containing 0.2 mg/L of NAA and 50.0 mg/L of Km (Jiang et al. 2012).

Screening of transgenic plants

Genome DNA of broccoli transgenic lines was isolated using CTAB method, and primer pair WK6UP3 (5′-TCAACA AAGGGTAATATCCGG-3′)/WK6DN3 (5′-CAAGGCTT GATTTTGGGTG-3′) was employed to amplify fragments with partial 35S promoter and *BoWRKY6* gene. Approximately 30 ng DNA of each line was used as PCR templates. The PCR conditions were as follows: 95 °C for 5 min; 32 cycles of 95 °C for 30 s, 53.5 °C for 50 s, and 72 °C for 75 s; followed by a final extension at 72 °C for 10 min. PCR products were electrophoretically separated on a 1.0 % agarose gel at 120 V for 30 min, and then photographed using the Gel Doc XR$^+$ System (BIO-Rad, UK).

Expression of BoWRKY6 and pathogenesis-related gene 1

WT and transgenic plants were inoculated with *H. parasitica*, and leaf samples were collected at 0 h and 24 h after infection. Total RNA was isolated from 100 mg of leaves using Trizol (Invitrogen, USA), and the 1st cDNA was synthesized using the SMART cDNA PCR KIT (Clontech, USA). For Real time-qualitative PCR (RT-qPCR), three primer pairs were used to determine gene expression of *BoWRKY6*, *BoPR1* (pathogenesis related gene 1), and *BoActin* (a reference gene) (Lovelock et al. 2013). The sequences of gene specific primers were as follows: BoWRKY6rtup (TATCTCGTCACAGC CGCC), BoWRKY6rtdn (AACTGGTCCCAATCTTTTCT), BoPR1up (GCGACTGCAGACTCGTACAC), BoPR1dn (TCTCGTTGACCCAAAGGT), BoActinINup (ACGTGGACATCAGGAAGGAC) and BoActindn (GAACCACCGATCCAGACACT). All the qRT-PCR runs were carried out in a LightCycler® 96 real-time PCR System (Roche, Switzerland) by using YBR FastStart Essential DNA Green Master Mix (Roche, Switzerland). The following reagents were added to 20 μl tubes: 10 μl of Master Mix; 0.2 μl of each primer (20 μM), 3 μl of diluted cDNA(0 h and 24 h), and 6.6 μl of ddH2O. The following PCR conditions were used: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s; 55 °C for 15 s, and 72 °C for 30 s. 60 °C for 1 min; 72 °C for 30 s. All qRT-PCR assays were conducted in triplicate. Mean fold changes in gene expression of *BoWRKY6* and *BoPR1* were normalized against *BoActin* gene by using the equation as described by Livak and Schmittgen (2001).

Disease resistance assessment and statistical analysis

Interaction phenotypes were determined 7 d after inoculation. Scales ranging from 0 to 9 were used for classifying leaf infection by downy mildew pathogen: 0 = no necrotic flecks, no sporulation; 1 = small necrotic flecks, no sporulation; 3 = necrotic flecks, one to few sporangiophores; 5 = necrotic lesions, sparse scattered sporulation usually confined to necrotic areas; 7 = necrotic lesions, sometimes with accompanying chlorosis, scattered, heavy to abundant sporulation in both chlorotic and necrotic areas; 9 = necrosis and some chlorosis may be evident, uniformly heavy sporulation over abaxial surface of leaf (Li et al. 2010). The data were statistically analyzed by using one-way analysis of variance (ANOVA) and Duncan's test at a significance level of $p = 0.05$.

Results

Isolation and characterization of BoWRKY6

Primer pair WK6UP1/WK6DN1 was employed to amplify *BoWRKY6* gene by using leaf genome DNA and cDNA as templates, respectively. Sequencing results indicated *BoWRKY6* was 1498 bp in length, with two introns of 467 and 179 bp (Fig. 1). The complete coding sequence of *BoWRKY6* was 852 bp in length encoding 283 amino acids,

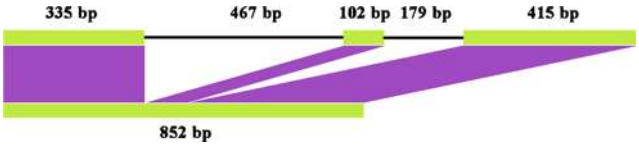

Fig. 1 Gene structure of *BoWRKY6*. *Boxes* indicate exons; *black lines* represent introns

carrying a nuclear localization sequence (NLS) of PVKGKRGCYKRKKK, and a WRKY motif of WRKYGQK, together with a zinc finger of C-X_7-C-X_{23}-H-X_1-C (X represents any amino acid) at its C-terminal (Fig. 2).

Sequence comparison and phylogenetic analysis of BoWRKY6

For sequence comparison and phylogenetic analysis, 17 homologous protein sequences were downloaded from NCBI, and were aligned with ClusterX 1.81. These protein sequences together with BoWRKY6 varied in length, ranging from 276 (*B. rapa*) to 338 (*T. halophila*), with an average size of 299. BoWRKY6 demonstrated the highest degree of homology to *B. napus* (CDY32013.1) and *B. rapa* (NP_001288847.1) by performing BLAST searches via NCBI, with identities of 99 and 94 %, respectively, and the lowest homology were observed in *B. distachyon* (XP_003566949.1) and *O. sativa* (AAU44093.1), with sequence identities less than 40 %. However, the 18 WRKY domains were relatively well conserved both in length and amino acid composition, and each domain contained a WRKYGQK motif as well as a C-X_7-C-X_{23}-H-X_1-C pattern zinc finger sequence (Fig. 3).

To understand the relationship between BoWRKY6 and its homologous sequences, phylogenetic tree was constructed using Mega software. Based on the results, 18 WRKY proteins were divided into 4 distinct groups, namely I, II, III and IV (Fig. 4). It could be observed that BoWRKY6 shared a close lineage with other Cruciferae plants of *B. napus*, *B. rapa*, *E. salsugineum*, *T. halophila,* and *C. rubella*, with 100 % bootstrap confidence value. WRKY proteins of three Leguminosae plants, *C. arietinum*, *P. vulgaris* and *G. max*, were closely related in our phylogenetic tree, with confidence of 100 %. WRKY sequences of *J. curcas*, *C. clementina*, *R. communis*, *P. trichocarpa* and *T. cacao* group together, however, the confidence value was lower (83 %) when compared to group I, II, and IV. Group IV was consisted of two Gramineae species, *B. distachyon* and *O. sativa*, with 100 % bootstrap confidence value.

Expression analysis

RT-PCR was performed to determine the expression patterns of *BoWRKY6* in both control and downy mildew challenged leaves. Results indicated that *BoWRKY6* expressed constitutively in control leaves at a relatively lower level, however, increasing levels of expression were detected 6–24 h after inoculation, and then decreased at 36 h (Fig. 5).

Screening of transgenic plants

BoWRKY6 gene driven by a cauliflower mosaic virus (CaMV) 35S promoter was transformed into broccoli, and T_0 plants with abnormal morphological characteristics were discarded. Totally five transgenic lines, namely *BWK05*, *BWK14*, *BWK16, BWK31* and *BWK54*, which exhibited normal growth and developmental traits, were screened out of 75 kanamycin-resistant plants, and they were later confirmed by PCR using

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1    ATGGATATTGCTAGTAATAACAAAGCAATAAAGCTAAAAGTTAGGGACCAACTTCTTCAATGCCACGAGATGACCACTAAG
1     M  D  I  A  S  N  N  K  A  I  K  L  K  V  R  D  Q  L  L  Q  C  H  E  M  T  T  K
82   GTTCAGCAACTCCTCTCTCAAGACGGGTCGGATTTGGGTCCAGCGAAGGATCTCGTGGAGAAAATATTGGGGTCTATCAGT
28    V  Q  Q  L  L  S  Q  D  G  S  D  L  G  P  A  K  D  L  V  E  K  I  L  G  S  I  S
163  GACACAATCTCTGCTCTTGATTCCTTCGAACCCATCTCCCCCTCTTATCTCGTCACAGCCGCCGAAGGCTCTCAAAATGCT
55    D  T  I  S  A  L  D  S  F  E  P  I  S  P  S  Y  L  V  T  A  A  E  G  S  Q  N  A
244  TCCTGCGACAACGACGGCAAGCTTGAGGATTCTGGCGATAGTCAGAAAAGATTGGGACCAGTTAAGGGTAAAAGAGGATGC
82    S  C  D  N  D  G  K  L  E  D  S  G  D  S  Q  K  R  L  G  P  V  K  G  K  R  G  C
325  TACAAGAGAAAGAAGAAATCAGAGACGTGGACTGTAGAGTCTACCGTACTTGAGGACACATTTTCTTGGAGGAAATATGGA
109   Y  K  R  K  K  K  S  E  T  W  T  V  E  S  T  V  L  E  D  T  F  S  W  R  K  Y  G
406  CAAAAACAGATTCTTAATGCCAAATTCCCAAGAAGTTACTTTAGGTGCACACACAAATACACTCAAGGGTGCAAGGCAACA
136   Q  K  Q  I  L  N  A  K  F  P  R  S  Y  F  R  C  T  H  K  Y  T  Q  G  C  K  A  T
487  AAGCAAGTGCAGAAGCTAGAGTCTGAACCCAGGATGTTCAGCATCACATACATCGGAAACCACACGTGTAATACCAACGAA
163   K  Q  V  Q  K  L  E  S  E  P  R  M  F  S  I  T  Y  I  G  N  H  T  C  N  T  N  E
568  GTAACACCCAAAATCAAGCCTTGTATTCATCATGATGAGATCATCACGGATTCTGAAGAGATCCAGAGTCCTAGTTTGATG
190   V  T  P  K  I  K  P  C  I  H  H  D  E  I  I  T  D  S  E  E  I  Q  S  P  S  L  M
649  ACCTCGATGAAGGAAGAGGAAGAAAATCACCATCATGGTTCGTCAACGGAGAGTGACTTGCAATTGGTGTGGCAGGAAATG
217   T  S  M  K  E  E  E  E  N  H  H  H  G  S  S  T  E  S  D  L  Q  L  V  W  Q  E  M
730  TTGGTCTTTGCAGAGGAACACCATCATCATCATGAGGCTGTTTACGGTTGTGGGGAAACTAGTACATCTATCAATGGTTTG
244   L  V  F  A  E  E  H  H  H  H  H  E  A  V  Y  G  C  G  E  T  S  T  S  I  N  G  L
811  GATTCCGCGGATCTGTGGAGTTGCCAGCAGTTTTCGGTTTAG
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Fig. 2 Complete coding sequence of *BoWRKY6* and its deduced amino acids. *Dashed line* indicates a nuclear localization sequence; *Line* represents the WRKY motif; *Boxed sequence* indicates a zinc finger domain

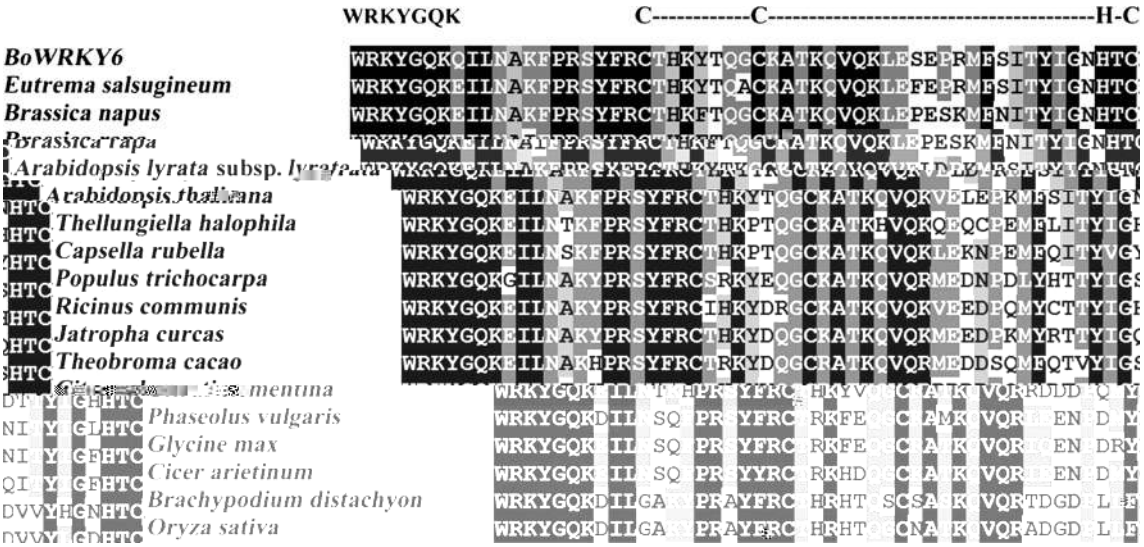

Fig. 3 Comparisons of WRKY domains between BoWRKY6 and its homologous sequences

WK6UP3/WK6DN3 primer pair. Fragments containing partial 35S promoter and *BoWRKY6* were amplified only in transgenic lines, and no band was observed in WT line (Fig. 6).

Expression analysis of BoWRKY6 and BoPR1

There was no obvious difference in the basal expression levels of *BoWRKY6* between the WT and transgenic leaves. However, *BoWRKY6* transcripts increased greatly after infiltration with *H. parasitica*, and higher expression levels at 24 h after infection were observed in all transgenic lines when compared to WT. The expression levels increased 2.11 times in WT, while in transgenic plants, 3.44 to 9.85 times of expression levels were detected when infected by *H. parasitica* in 24 h (Fig. 7).

PR1 is regarded as a marker gene of the SA signaling pathway, and in our present study, qRT-PCR was carried out to analyze its expression level in both WT and transgenic broccoli lines. The results indicated no significant difference before *H. parasitica* infection (0 h), however, after 24 h of *H. parasitica* inoculation, *BoPR1* expression levels increased 2.15 times in WT plants, and it increased 6.62 to 13.90 times in those of transgenic lines (Fig. 7).

Disease resistance evaluation

To evaluate the resistance of five transgenic lines to downy mildew, enough T_0 broccoli plants were produced using tissue culture method, and 30 plants per line were assessed, with three replicates. Disease reaction phenotypes were made at 7 d post-inoculation, and disease indices were calculated (Table 1). Significant differences in disease index were observed between WT and transgenic lines, and the WT plants demonstrated a susceptible reaction with disease index of

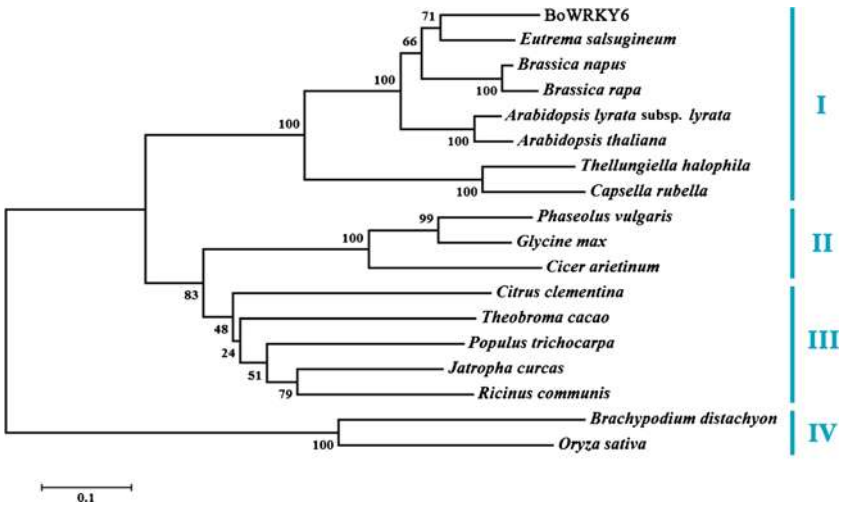

Fig. 4 Phylogenetic tree of BoWRKY6 and its homologous sequences using Neighbor-joining method

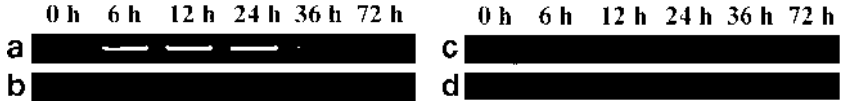

Fig. 5 Expression patterns of *BoWRKY6* gene in response to downy mildew inoculation. **a**: Leaves challenged by downy mildew; **c**: control leaves; **b** and **d**: internal control of actin gene

6.97, while the *BoWRKY6* over-expressing lines showed different levels of downy mildew resistance, with resistance classifications ranging from LR to VR. Necrotic lesions, chlorosis, and heavy sporulation were observed on WT leaves, whereas lines *BWK16* and *BWK54* exhibited fewer necrotic lesions, less chlorosis and light sporulation. *BWK31* and *BWK14*, which were identified as very resistant (VR), exhibited only small necrotic flecks and few sporangiophores on infected leaves.

Discussion

WRKY transcription factors are regarded as one of the largest families of transcriptional regulators in higher plants, and they form sophisticated signaling networks that modulate multiple plant processes (Rushton et al. 2010). Owing to the availability of increasing numbers of sequenced genomes, WRKY gene families were identified and characterized within many plants. In *Klebsormidium flaccidum*, a charophyte plant, only two WRKY genes are present in its genome, and they belong to Group I and IIb, respectively (Rinerson et al. 2015). However, the gene numbers expanded due to plant evolution, which is likely to be associated with the generation of highly complex defence mechanisms (Rushton et al. 2010). In the model plant of *Arabidopsis*, there are 72 WRKY genes, and 49 of them are differentially regulated when challenged by an avirulent strain of *Pseudomonas syringae* or treated by salicylic acid (SA) (Dong et al. 2003). Whereas in *Brachypodium distachyon*, 86 WRKY genes were identified, and dozens of *BdWRKY*s were rapidly and significantly up-regulated after inoculation of *Pseudomonas syringae* or *Boea hygrometrica* (Wen et al. 2014). There are more WRKY genes in *Oryza sativa* than in *Arabidopsis thaliana* and *B. distachyon*, respectively 98 and 102 WRKY genes were identified in *japonica* as well as in *indica* rice (Ross et al. 2007).

WRKYGQK is a common signature motif present in WRKY transcription factors, and is directly involved in DNA binding, with W-box element (C/T)TGAC(C/T) in the promoter region as its target (Eulgem and Somssich 2007).

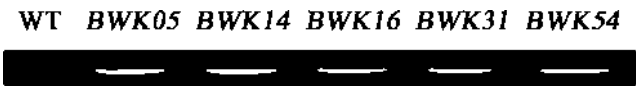

Fig. 6 PCR confirmation of *BoWRKY6* over-expressing broccoli plants

Though WRKYGQK is highly conserved in most WRKY members, variant proteins such as WRKYGKK, WRKYGEK, WRKYEDK, WKKYGQK, CRKYGQK, WHQYGLK, WRKYGMK, WSKYGQK, WQKYGQK as well as WIKYGEN were identified in *Solanum lycopersicum*, *Glycine max* and *Vitis vinifera* (Huang et al. 2012; Bencke-Malato et al. 2014; Guo et al. 2014). WRKY motif sequence is followed by a C2H2- or C2HC-type zinc finger motif, with sequences of C-$X_{4–5}$-C-$X_{22–23}$-H-X-H and C-$X_{5–8}$-C-$X_{25–28}$-H-$X_{1–2}$-C, however, there are some exceptions, e.g. in *Salvia miltiorrhiza*, six out of 61 WRKY members contain a C2HC zinc finger motif of C-X_7-C_{23}-H-X_1-C. In our study, a WRKY transcription factor gene was isolated from broccoli, with a WRKY motif of WRKYGQK and a zinc finger of C-X_7-C-X_{23}-H-X_1-C.

Plants are often subjected to abiotic and biotic stresses in their natural habitat, and adapt to such changes requires some degree of phenotypic plasticity that is mainly determined by its genome (Pandey and Somssich 2009). WRKY transcription factors play important roles not only in plant growth and development, but also in adaptive plasticity of the highly variable environments. WRKY transcription factors participate in plant immunity by regulating genes with W box elements within their promoters or by interacting with other transcription factors (Pandey and Somssich 2009). Expression of *GhWRKY15* was significantly induced in *Gossypium hirsutum* seedlings when treated with fungal, salicylic acid (SA), methyl jasmonate or methyl viologen, and tobacco over-expressing *GhWRKY15* demonstrated more resistance to *Colletotrichum*

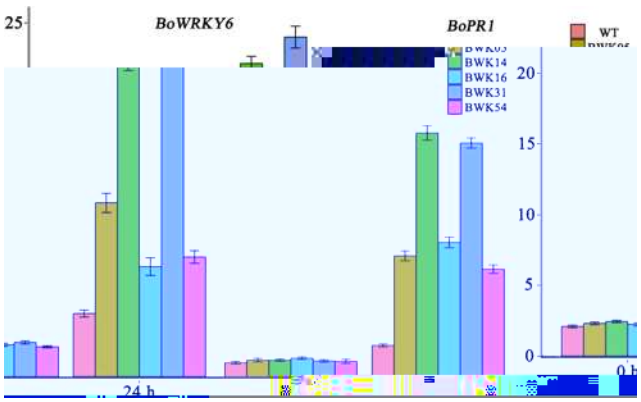

Fig. 7 Expression of *BoWRKY6* and *BoPR1* genes in WT and transgenic broccoli leaves in response to downy mildew by using qRT-PCR. *Each*

gossypii and *Phytophthora parasitica* (Yu et al. 2012). *S. lycopersicum* defense-related WRKY1 gene *SlDRW1* was significantly induced by *Botrytis cinerea*, and silencing of this gene resulted in increased severity of disease caused by *B. cinerea* (Liu *et al*. 2014). A *Populus trichocarpa* WRKY gene named *PtrWRKY73* was induced by exogenous SA, its over-expression in *Arabidopsis thaliana* resulted in increased resistance to *Pseudomonas syringae* (PstDC3000) (Duan et al. 2015). In present study, a WRKY gene designated *BoWRKY6* was isolated from broccoli, and its expression was induced by downy mildew. Five transgenic lines over-expressing *BoWRKY6* demonstrated increased resistance to downy mildew, with resistance classification from low to very high resistant.

In plants, disease resistance is regulated by multiple signal transduction pathways, and phytohormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) act as key signaling molecules which induce different transcriptional regulation of pathogenesis-related (PR) genes (Maleck et al. 2002; Lee et al. 2014). SA pathway play an important role in defense response to biotrophic pathogens, and PR1, a reliable molecular marker for systemic acquired resistance, is dependent on SA perception and associated with induced resistance against the biotrophic fungus *H. parasitica* in *A. thaliana* (Thomma et al. 1998; Maleck et al. 2000). Over-expression of an *Arabidopsis* cysteine-rich receptor-like kinase gene *CRK13* exhibited resistance to *Pseudomonas syringae*, and high level transcript accumulations of *PR1* and other PR genes were observed (Acharya et al. 2007). In our current study, transgenic broccoli plants over-expressing the *BoWRKY6* gene demonstrated increased expression of *PR1*, which is correlated positively with enhancement of downy mildew resistance in those lines.

In conclusion, a WRKY gene namely *BoWRKY6* was isolated from broccoli, with complete coding sequence of 852 bp in length encoding 283 amino acids, carrying a WRKY motif of WRKYGQK and a zinc finger of C-X_7-C-X_{23}-H-X_1-C. Expression of *BoWRKY6* was induced by downy mildew,

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Duan Y, Jiang Y, Ye S, Karim A, Ling Z, He Y, Yang S, Luo K (2015) PtrWRKY73, a salicylic acid-inducible poplar WRKY transcription factor, is involved in disease resistance in *Arabidopsis thaliana*. Plant Cell Rep 34:831–841

Eulgem T, Rushton PJ, Robatzek S, Somssich IE (2000) The WRKY superfamily of plant transcription factors. Trends Plant Sci 5:199–206

Eulgem T, Somssich IE (2007) Networks of WRKY transcription factors in defense signaling. Curr Opin Plant Biol 10:366–371

Guo C, Guo R, Xu X, Gao M, Li X, Song J, Zheng Y, Wang X (2014) Evolution and expression analysis of the grape (*Vitis vinifera* L.) WRKY gene family. J Exp Bot 65:1513–1528

Gupta OP, Sharma P (2013) Potential role of small RNAs during stress in plants. In: Gaur RK, Sharma P (eds) Molecular approaches in plant abiotic stress. CRC Press, Boca Raton, pp 67–89

Huang S, Gao Y, Liu J, Peng X, Niu X, Fei Z, Cao S, Liu Y (2012) Genome-wide analysis of WRKY transcription factors in *Solanum lycopersicum*. Mol Genet Genomics 287:495–513

Jiang M, He CM, Miao LX, Zhang YC (2012) Overexpression of a broccoli defensin gene BoDFN enhances downy mildew resistance. J Integr Agric 11:1137–1144

Kaur N, Gupta AK (2005) Signal transduction pathways under abiotic stresses in plants. Curr Sci 88:1771–1780

Klassen W, Schwartz PH (1985) ARS research program in chemical insect control. In: Hilton JL (ed) Agricultural chemicals of future. Rowman and Allanheld, New Jersey, pp 267–291

Koul O (2011) Microbial biopesticides: opportunities and challenges. CAB Rev Perspect Agric Vet Sci Nutr Nat Resour 6:1–26

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163

Lee YH, Kim SH, Yun BW, Hong JK (2014) Altered cultivar resistance of kimchi cabbage seedlings mediated by salicylic acid, jasmonic acid and ethylene. Plant Pathol J 2014(30):323–329

Li X, Xia B, Jiang Y, Wu Q, Wang C, He L, Peng F, Wang R (2010) A new pathogenesis-related protein, LrPR4, from *Lycoris radiata*, and its antifungal activity against *Magnaporthe grisea*. Mol Biol Rep 37: 995–1001