RESEARCH ARTICLE

Genome-Wide Identification and Expression Analysis of WRKY Transcription Factors under Multiple Stresses in *Brassica napus*

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Abstract

WRKY transcription factors play important roles in responses to environmental stress stimuli. Using a genome-wide domain analysis, we identified 287 WRKY genes with 343 WRKY domains in the sequenced genome of *Brassica napus*. 139 in the A sub-genome and 148 in the C sub-genome. These genes were classified into eight groups based on phylogenetic analysis. In the 343 WRKY domains, a total of 26 members showed divergence in the WRKY domain, and 21 belonged to group I. This finding suggested that WRKY genes in group I are more active and variable compared with genes in other groups. Using genome-wide identification and analysis of the WRKY gene family in *Brassica napus*, we observed genome duplication, chromosomal/segmental duplications and tandem duplication. All of these duplications contributed to the expansion of the WRKY gene family. The duplicate segments that were detected indicated that genome duplication events occurred in the two diploid progenitors *B. rapa* and *B. oleaceae* before they combined to form *B. napus*. Analysis of the public microarray database and EST database for *B. napus* indicated that 74 WRKY genes were induced or preferentially expressed under stress conditions. According to the public QTL data, we identified 77 WRKY genes in 31 QTL regions related to various stress tolerance. We further evaluated the expression of 26 BnaWRKY genes under multiple stresses by qRT-PCR. Most of the genes were induced by low temperature, salinity and drought stress, indicating that the WRKYs play important roles in *B. napus* stress responses. Further, three BnaWRKY genes were strongly responsive to the three multiple stresses simultaneously, which suggests that these 3 WRKY may have multi-functional roles in stress tolerance and can potentially be used in breeding new rapeseed cultivars. We also found six tandem repeat pairs exhibiting similar expression profiles under the various stress conditions, and three pairs were mapped in the stress related QTL regions, indicating tandem duplicate WRKYs in the adaptive responses to environmental stimuli during the evolution process. Our results provide a framework for future studies regarding the function of WRKY genes in response to stress in *B. napus*.
Introduction

The WRKY gene family is one of the most extensively studied transcription-factor gene families in plants [1]. Plant WRKY proteins are characterized by a highly conserved WRKY domain with a 60 amino acid region [2]. It includes the conserved WRKYGQK sequence followed by one of two types of zinc finger motifs, C2H2 or C2–HC [3]. WRKY proteins can be classified into three groups: group I, group II and group III, based on the number of WRKY domains and the types of zinc finger motifs. Group I WRKY contains two WRKY domains and the C2H2-type zinc finger motif (C–X4–5–C–X22–23–H–X1–H). Group II WRKY only contains a single domain and shares the same motif as group I. Group III WRKY contains a single domain and a C2–HC-type motif (C–X7–C–X23–H–X1–C). Group II is further classified into several subgroups based on their phylogenetic clades [3–5].

Since the first plant WRKY gene SPF1 was identified in sweet potato [6], numerous WRKY family genes have been identified in different plant species. Because whole genome sequences have been completed in numerous plants, WRKY family members have been genome-wide identified in several of these species. Previous studies have found 72 WRKY family members in Arabidopsis, more than 100 in rice, 57 in Cucumis sativus, 104 in Populus trichocarpa, 81 in Solanum lycopersicum, 197 in soybean, 66 in papaya, 38 in Physcomitrella patens, 35 in Selaginella moellendorfii, 80 in Pinus, more than 45 in barley, 56 in Ricinus communis, 119 in maize, 120 in Gossypium raimondii, and 59 in Vitis vinifera [7–12]. Recently, two papers on WRKY gene family analysis in the two diploid progenitors of B. napus, B. rapa and B. oleracea, were published, and 145 and 142 WRKY genes were detected in the B.rapa and B.oleracea genome, respectively [13, 14]. Despite much progress in the identification of WRKY genes in plants, to the best of our knowledge, no genome-wide characterization of this gene family has been conducted in B. napus. The recently released whole-genome sequence in B. napus and the publicly available B. napus database will serve as a foundation for identifying the WRKY gene families in B.napus.

In plants, WRKY transcription factors are known to play prominent roles in plant stress response processes [1, 3]. In the 13 OsWRKY genes identified in rice, 11 showed variable responses to salt, polyethylene glycol (PEG), and cold or heat stresses [15]. In the 15 WRKY genes detected in wheat, 8 showed responses to cold, heat, NaCl, and PEG treatment [16]. The transgenic Arabidopsis plants that over expressed several GmWRKY genes of soybean were more tolerant to various stress [17]. To date, 13 WRKY genes have been reported in B. napus that are associated with stress response processes [18]. Identification of multi-functional roles of WRKY genes in stress tolerance may potentially be used to breed new cultivars with increased stress resistance. However, most of the reported WRKY genes in B. napus were only researched under single stress conditions. Co-expression analysis of WRKY genes under multiple stresses in B. napus has not been previously reported.

The objective of this study was to survey the WRKY genes in the sequenced genome of B. napus and to evaluate the expression patterns for several WRKY genes under multiple stress conditions. Our work provides a framework for elucidating the structure, evolution and functional roles of WRKY genes in B. napus.

Materials and Methods

Identification, classification and motif analysis of the WRKY gene family

The genes and proteins annotated in B. napus were downloaded from http://www.genoscope.cns.fr. WRKY transcription factors were identified using HMMER software version 3.0 [19] and the PFAM protein family database using the WRKY domain (PF03106) as a query [20].
WRKY protein sequences in Arabidopsis were obtained from the Arabidopsis Information Resource (TAIR: http://www.arabidopsis.org/). The MEME program was used to predict the conserved motif [21]. The parameters were set as follows: maximum number of motifs, 10; minimum motif width, six; and maximum motif width, 70. Alignment of the amino acid sequences of the WRKY domain was performed with ClustalX 1.83 [22]. The MEGA 6.0 software was used to construct the phylogenetic tree [23]. A maximum likelihood tree was used based on the bootstrap method. The number of bootstrap replications was 1000.

Mapping and gene duplication of WRKY genes

Positional information about all of the WRKY genes was investigated according to the B. napus information resource database (http://www.genoscope.cns.fr/). The MapChart version 2.2 program was used to map the WRKY genes on chromosomes [24]. BIOEDIT software and blast program were used to identify duplicate genes. A similarity of aligned genes greater than 85% was considered to indicate duplicate genes [25].

In silico expression analysis of WRKY genes

To identify WRKY genes with a potential role in response to stress in plants, we analyzed the in silico expression pattern of WRKY genes under various stresses. One microarray data set was available in the NCBI database for detecting the patterns of gene expression after inoculating Sclerotinia sclerotiorum. Microarray data were downloaded from the NCBI GEO database (http://www.ncbi.nlm.nih.gov; accession numbers GSM334324–GSM334353, GSM334645–GSM334674). The transcript data were obtained from plant material including five time points: 6, 12, 24, 48, and 72 hours post-inoculation.

For other abiotic stresses, no extensive microarray data for gene expression estimates were found for Brassica. Consequently, we used the expressed sequence tag (EST) data from GenBank dbEST to identify WRKY genes that were preferentially expressed under each stress condition. All raw ESTs were cleaned by SeqClean (http://compbio.dfci.harvard.edu/tgi/software/) and retained high-quality ESTs for subsequent analysis. EST data were clustered into the different stress conditions according to the tissue source in the EST library description.

Identification of WRKY genes overlapping with known QTLs related to various stresses

The QTL data related to different stresses in Brassica were referenced from published papers. According to the physical positions of the flanking markers of the QTLs (http://www.genoscope.cns.fr/blat-server/cgi-bin/colza/webBlat), the corresponding genomic sequences of the QTL region were extracted. Then, the WRKY genes residing in these known QTL regions were selected.

Plant materials and stress conditions

Brassica napus accession Zhongshuang11, which exhibits high tolerance to stress, was kindly provided by Oilcrops research Institute, Chinese academy of agricultural sciences, and used for the stress treatments. The Seeds were surface-sterilized in 70% ethanol for 1 min, and then rinsed three times with sterile dH2O. The sterilized seeds were germinated in Petri dishes on two layers of filter papers at 24°C. Three days later, the germinated seedlings were transferred to a MS medium, pH 5.7, containing 0.3% agar and 3% sucrose, and grown under the following conditions: 16/8 h photoperiod, 24°C, 60% relative humidity. Two weeks old plants were exposed to the multiple stresses. The stress conditions included drought, salinity and low...
temperature. Drought and salinity were applied by immersing the seedlings in 20% PEG-6000 and 200 mmol L\(^{-1}\) NaCl, respectively. The cold stress treatment was applied by putting the seedlings under 3°C. The leaves were collected at 0, 3, 6, 9, 12, and 24 hours after the stress treatment, quick-frozen in liquid nitrogen, and stored at -80°C for RNA extraction.

**RNA isolation and real-time PCR analysis**

Total RNA was isolated by the RNAprep pure Plant Kit (DP 432) (Tiangen, China) following the manufacturer’s instructions. Each RNA sample was treated with DNase I after the extraction to remove all residual DNA. First-strand cDNA was synthesized using the reverse transcription polymerase reaction system, iScript™ cDNA Synthesis Kit (BIO-RAD, USA). Then, 0.8 μg RNA was reverse transcribed following the instruction manual. The obtained cDNA was diluted to 50 times for qRT-PCR. Primer 5.0 was used to design gene-specific primers for qRT-PCR (http://www.premierbiosoft.com/). The amplified fragment length ranged from 80 bp to 200 bp, and the annealing temperature ranged from 58°C to 65°C. The Arabidopsis Actin7 (AT5G09810) gene was used as the reference gene (forward primer: 5'-TGGGT TTGCTGGTGACGAT -3', reverse primer: 5'-TGCCTAGGACGACCAACAAATCT -3').

The qRT-PCR was performed using the BIO-RAD real-time PCR system. Amplification was performed under the following conditions, denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, annealing at 58–65°C for 15 s, and extension at 72°C for 15 s. The default settings were used for the melting curve stage. Three biological replicates, each with three technical replicates, were tested. The gene expression levels were calculated according to Livak and Schmittgen [26].

**Results**

**Identification, classification and structural analysis of WRKY family members**

To identify the WRKY genes in *B. napus*, the WRKY domain (PF03106) was used to search the *B. napus* genome (See S1 Table). In total, 287 WRKY transcription factor genes were identified in the sequenced genome of *B. napus* and it represented approximately 0.315% of the whole genome. Among the 287 WRKYs, 139 located in the A sub-genome, and 148 located in the C sub-genome. These WRKYs represented approximately 0.328% of the A sub-genome and 0.303% of the C sub-genome, respectively. We used the nomenclature system for *BnaWRKYs* to distinguish the WRKY genes in *B. napus*. Therefore, the WRKY genes identified in this study were named from *BnaWRKY001* to *BnaWRKY287*.

Among the 287 WRKY genes, there were a total of 343 WRKY domain regions detected that spanned approximately 60 amino acids (See S2 Table). We found that 56 of the 287 WRKY candidates contained two WRKY domains. The phylogenetic tree was constructed for the *Arabidopsis* WRKYs and *B. napus* WRKYs (Fig 1). Based on the classification of the WRKY family genes in *Arabidopsis*, the 287 WRKYs with 343 WRKY domains in *B. napus* were classified into three groups (groups I, II, and III). Among the three groups, a total of 121 WRKYs belonged to group I, 158 to group II, and 51 to group III. Moreover, the group II genes were further classified into five subgroups (groups IIa–e), containing 11, 34, 55, 28, and 30 WRKY members, respectively. However, the remaining 13 WRKYs were not included in the phylogenetic analysis due to low statistical support. These WRKYs had low identities with other WRKY family members.

The 343 WRKY domain regions were subjected to analysis by MEME to reveal conserved motifs shared among related proteins (See S3 Table). Ten conserved motifs, named motifs
1–10, were identified (Fig 2). Among these, the motif encoding the WRKYGQK domain was the most conserved motif identified. In addition to the WRKY domain, the WRKY family members were predicted by MEME to contain other conserved motifs. Alignment of 343 WRKY sequences identified 8 different WRKY motifs. Although the WRKY domain is the most conserved, in addition to WRKYGQK, we found several genes with diverse amino acid
Identification and expression of WRKY genes in rapeseed

**Figure a**

- Motif 1: 46 sites, 9.3e-1154
- Motif 2: 50 sites, 4.7e-970
- Motif 3: 50 sites, 1.5e-992
- Motif 4: 50 sites, 2.3e-1073
- Motif 5: 50 sites, 4.5e-1633
- Motif 6: 50 sites, 1.2e-978
- Motif 7: 50 sites, 6.2e-193
- Motif 8: 50 sites, 7.9e-861
- Motif 9: 50 sites, 1.4e-846
- Motif 10: 50 sites, 5.1e-476

**Figure b**

[Image showing gene expression data]
residues in this region: WRKYGKK, WRKYGKR, WKKYGQK, WKKYGQR, WKNYGQK, WMKYGQK, and WRKYGHK. In total, 26 members showed divergence in the WRKY domain. Among the seven amino acid residues WRKYGQK, most variations involved Q to K substitutions, 18 of the 26 members belong to WRKYGKK, and within the 26 members, 21 belonged to group I (Fig 3).

Chromosomal distribution of WRKY genes and their genomic duplication

To determine the genomic distribution of the WRKY genes, the identified BnaWRKY genes were mapped on their corresponding chromosome by searching the released database of B. napus. The results showed that the BnaWRKY genes were distributed on all 19 chromosomes (Fig 4); however, the distribution and density of the WRKY genes on each chromosome were uneven. There were 8, 14, 24, 16, 8, 9, 10, 8, 17 and 4 WRKY genes on chromosomes A1 to A10, respectively, and 9, 14, 24, 20, 8, 10, 18, 10 and 10 WRKY genes on chromosomes C1 to C9, respectively (Fig 5A). The other 46 BnaWRKY genes mapped onto unanchored scaffolds according to the current database. The WRKY gene density per chromosome ranged from 0.185/Mb to 0.835/Mb (Fig 5B). On average, one WRKY gene was present every 2.678 Mb. Several chromosomes and chromosomal regions had higher densities of WRKY genes compared with others. Chromosome A04 had the highest density of WRKY genes, and chromosome C05 had the lowest density.
Distribution of WRKY genes on the chromosomes also showed that within the whole genome of B. napus, approximately 4.88% (14 of 287) of WRKYs were involved in tandem duplication. There were 12 tandem repeats found on the A sub-genome and 2 on the C sub-genome, approximately 8.63% (12 of 139) and 1.35% (2 of 148) of the A sub-genome and the C sub-genome, respectively. In the A sub-genome, these tandem repeats were distributed on Chromosomes A02, A03, A04, A07, and A10 (BnaWRKY15 and BnaWRKY16; BnaWRKY31 and BnaWRKY32; BnaWRKY34 and BnaWRKY35; BnaWRKY53 and BnaWRKY54; BnaWRKY63 and BnaWRKY64; BnaWRKY126 and BnaWRKY127). In the C sub-genome, the tandem repeats were distributed on Chromosome C03 (BnaWRKY170 and BnaWRKY171) (Fig 4).

B. napus is a hybrid of B. rapa (A genome) and B. oleracea (C genome) [27]. Comparative analysis of BnaWRKY gens in the A sub-genome and C sub-genome showed orthologous duplications. Except for 18 BnaWRKY genes, we identified 129 and 140 WRKY genes in the A and C sub-genomes with orthologous relationships. The orthologous WRKY gene pairs tended to be clustered together in the phylogenetic tree. WRKY genes in the A sub-genome and C sub-genome were not equally represented within the given clades. For instance, two or more BnaWRKYs in the A sub-genome were putative orthologs of a single gene in the C sub-genome. For example, BnaWRKY015 and BnaWRKY016 were the orthologs of BnaWRKY150.

In addition to gene duplication from the sub-genome-wide polyploidization of the A and C sub-genomes, we observed chromosomal/segmental duplications. Approximately 83% (239 of 287) of the WRKY genes were highly similar paralogs. In total, we observed at least 12 potential chromosomal/segmental duplications (Fig 4, pairs of bars with numbers 1–12).

WRKY genes overlapping with known QTLs in Brassica

With the QTL data in Brassica, we performed a sequence-based analysis and identified 77 WRKY genes within the known QTL regions that were related to various stress tolerances in

![Fig 4. Distribution of BnaWRKY genes in Brassica napus genome.](https://doi.org/10.1371/journal.pone.0157558.g004)
Brassica (Table 1) [28–34]. Among these QTL links of WRKY genes, several genes were involved in multiple stress tolerances. For instance, BnaWRKY117 was associated with resistance to Sclerotinia sclerotiorum and Leptosphaeria maculans (blackleg); BnaWRKY163 and BnaWRKY164 were related to diamondback moth and clubroot resistance; BnaWRKY235 was associated with fusarium wilt and clubroot resistance; and BnaWRKY015 to BnaWRKY020 were related to Leptosphaeria maculans and Sclerotinia sclerotiorum resistance. Conversely, we found several WRKY genes distributed in the same QTL regions. Interestingly, we also found 3 pairs of tandem duplicate WRKY genes in the QTL regions. Tandem repeats of BnaWRKY15 and BnaWRKY16 were distributed in the QTL of Sclerotinia sclerotiorum and Leptosphaeria maculans resistance. BnaWRKY31, BnaWRKY32, BnaWRKY34 and BnaWRKY35 were all distributed in the QTL of Sclerotinia sclerotiorum resistance. Our results provide a link between WRKY genes and stress resistance in rapeseed breeding and will be useful for genetic improvements in rapeseed.

Table 1. WRKY genes in stress related QTL regions.

| Chr. | QTL name | QTL position | WRKY genes in QTL region | Stress condition | references |
|------|----------|--------------|--------------------------|-----------------|------------|
| A02  | SRA2     | 900158–10995801 | BnaWRKY010–BnaWRKY014     | Sclerotinia sclerotiorum | Wu et al.2013 |
| A02  | SRA2     | 3806580–20474897 | BnaWRKY012–BnaWRKY020     | Sclerotinia sclerotiorum | Wu et al.2013 |
| A02  | qSR10-1  | 390973–20896400 | BnaWRKY010–BnaWRKY020     | Sclerotinia sclerotiorum | Mei et al.2013 |
| A02  | LmA2     | 9269867–20463672 | BnaWRKY015–BnaWRKY020     | Sclerotinia sclerotiorum | Wu et al.2013 |
| A02  | Anju2    | 4804764–10051600 | BnaWRKY112–BnaWRKY14      | clubroot         | Tomita et al.2013 |
| A03  | SRA3     | 5762514–29673240 | BnaWRKY012–BnaWRKY018     | Sclerotinia sclerotiorum | Wu et al.2013 |
| A03  | qSR10-2  | 606874–31318272 | BnaWRKY016–BnaWRKY027     | Sclerotinia sclerotiorum | Mei et al.2013 |
| A06  | SRA6     | 20965425–23324292 | BnaWRKY083                | Sclerotinia sclerotiorum | Wu et al.2013 |
| A08  | SRA8     | 816774–18390028  | BnaWRKY096–BnaWRKY103     | Sclerotinia sclerotiorum | Wu et al.2013 |
| A09  | SRA9     | 2258677–26573318 | BnaWRKY107–BnaWRKY118     | Sclerotinia sclerotiorum | Wu et al.2013 |
| A09  | Lm9      | 17684286–25984575 | BnaWRKY117                | black leg        | Delourme et al.2008 |
| C01  | qLR09-3  | 1275674–21935046 | BnaWRKY147–BnaWRKY148     | Sclerotinia sclerotiorum | Mei et al.2013 |
| C01  | qLR10-1  | 1275674–21935046 | BnaWRKY147–BnaWRKY148     | Sclerotinia sclerotiorum | Mei et al.2013 |
| C02  | Anju1    | 42040597–44752272 | BnaWRKY159–BnaWRKY161     | clubroot         | Tomita et al.2013 |
| C02  | LmC2.1   | 9837329–1635078  | BnaWRKY152–BnaWRKY153     | black leg        | Delourme et al.2008 |
| C02  | QTL-1    | 358639–5209750  | BnaWRKY149                | black rot        | Kifuji et al.2013 |
| C03  | Anju3    | 1282758–8955466 | BnaWRKY163–BnaWRKY167     | clubroot         | Tomita et al.2013 |
| C03  | QTL-3    | 411773–5820967  | BnaWRKY163–BnaWRKY164     | diamondback moth | Asghari et al.2009 |
| C04  | Sll14a   | 4910121–9418160 | BnaWRKY191–BnaWRKY192     | Sclerotinia sclerotiorum | Wu et al.2013 |
| C05  | LRC5     | 101013–30968606 | BnaWRKY208–BnaWRKY213     | Sclerotinia sclerotiorum | Wu et al.2013 |
| C05  | GC1      | 6768180–11801042 | BnaWRKY209                | clubroot         | Tomita et al.2013 |
| C06  | Sll16    | 7571202–35465622 | BnaWRKY216–BnaWRKY224     | Sclerotinia sclerotiorum | Wu et al.2013 |
| C06  | SRC6.1   | 31256776–36061993 | BnaWRKY224              | Sclerotinia sclerotiorum | Wu et al.2013 |
| C06  | SRC6.2   | 24665572–35953761 | BnaWRKY221–BnaWRKY224     | Sclerotinia sclerotiorum | Wu et al.2013 |
| C07  | qSR10-2  | 233541376–36743363 | BnaWRKY228–BnaWRKY234     | Sclerotinia sclerotiorum | Mei et al.2013 |
| C07  | Anju4    | 35610814–37812010 | BnaWRKY235              | clubroot         | Tomita et al.2013 |
| C07  | QTL2(Foc-Bo1) | 36671239–39348306 | BnaWRKY235        | Fusarium wilt    | Pu et al.2011 |
| C09  | qSR09-1  | 2984746–5282988  | BnaWRKY257              | Sclerotinia sclerotiorum | Mei et al.2013 |
| C09  | qSR-09-2 | 23357152–3681682  | BnaWRKY256              | Sclerotinia sclerotiorum | Mei et al.2013 |
| C09  | qLR-09-6 | 2337812–3087435  | BnaWRKY256              | Sclerotinia sclerotiorum | Mei et al.2013 |
| C09  | qSR10-3  | 2984476–5282988  | BnaWRKY257              | Sclerotinia sclerotiorum | Mei et al.2013 |

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In silico expression analysis of WRKY genes in *B. napus* using NCBI databases

High-throughput sequencing and gene expression analyses were performed on *B. napus* under both normal and stress conditions. The *B. napus* genetic sequences are available in the NCBI database. To identify WRKY genes with a potential role in different stress responses in plants, we analyzed the expression pattern of WRKY genes in response to various stresses. One microarray data set is available in the *B. napus* database and allowed us to compare the differential expression of WRKY genes in a partially resistant variety of ZhongYou 821 (ZY821) and a susceptible line of Westar to sclerotinia. We therefore examined *B. napus* microarray data from different stages after inoculation of *sclerotinia* and collected all of the available WRKY gene expression data. In total, we found that 58 WRKY genes induced expression by *sclerotinia* (Table 2).

For other stresses, no extensive microarray data for gene expression estimates were found for *B. napus*. Consequently, we used the expressed sequence tag (EST) data from GenBank dbEST to identify WRKY genes preferentially expressed under stress conditions. In the available ESTs under the various stress conditions, we found that 28 WRKY ESTs in 12 different EST libraries were preferentially expressed under different stress conditions, including 3 under drought stress, 3 under cold stress, 7 under a hydroponically grown condition, 10 under a dark condition, 3 infected by insects, and 2 infected by diseases. These ESTs belonged to 16 unigenes (Table 2).

The in silico expression analyses of the *BnaWRKY* genes using public microarray and EST data identified that 74 *BnaWRKY* genes are induced or preferentially expressed under the various stress conditions.

Expression analysis of WRKY genes under multiple stresses

Within the 287 *BnaWRKY* genes in the *B. napus* genome, 12 were not only identified to be induced or preferentially expressed under stress conditions in silico but also located in the stress related QTL intervals. These 12 *BnaWRKY* genes were selected to analyze the expression patterns under multiple stress conditions. Among the 12 *BnaWRKY* genes examined by qRT-PCR, all genes were up-regulated (≥ 2-fold change) under low temperature, salinity and drought stress (Fig 6). The results indicated that the *BnaWRKYs* detected in this study were strongly induced in response to multiple stresses in *B. napus*. We also found that *BnaWRKY111, BnaWRKY113, BnaWRKY118, BnaWRKY147, BnaWRKY166, BnaWRKY191, BnaWRKY210* and *BnaWRKY235* were highly up-regulated (≥ 20-fold change) under low temperature stress, *BnaWRKY098, BnaWRKY111, BnaWRKY113, BnaWRKY147, BnaWRKY166, BnaWRKY191, BnaWRKY210* and *BnaWRKY235* were highly up-regulated (≥ 20-fold change) under salinity stress, and *BnaWRKY147, BnaWRKY166* and *BnaWRKY210* were highly up-regulated under drought stress (≥ 20-fold change), thus indicating their potential roles in low temperature, salinity, and drought stress, respectively. Additionally, 3 *BnaWRKY* genes, *BnaWRKY147, BnaWRKY166* and *BnaWRKY210*, were all highly induced in response to multiple stress treatments (≥ 20-fold change) (Fig 6). Interestingly, the expression processes for several *BnaWRKY* genes exhibited low to high or high to low curve changes over the 24-hour time course. This suggested that the response of *BnaWRKYs* to multiple stresses is a dynamic process.

The 14 tandem duplicate *BnaWRKY* genes in the *B. napus* genome were also selected for analysis of their expression profile under multiple stress conditions. Among the 14 *BnaWRKYs* examined by qRT-PCR, most of the genes were up-regulated (≥ 2-fold change), and one pair of tandem repeat genes, *BnaWRKY031* and *BnaWRKY034*, was down-regulated (≥ 2-fold change) under salinity and drought stress (Fig 6). Among the seven pairs of tandem repeats, six
| EST   | BnaWRKY Gene                     | Library/Microarray                       | Stress condition       |
|-------|----------------------------------|------------------------------------------|------------------------|
| BN11150 | BnaWRKY048, BnaWRKY240          | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN11784 | BnaWRKY042, BnaWRKY245, BnaWRKY235 | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN12248 | BnaWRKY028, BnaWRKY125, BnaWRKY265, | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN14657 | BnaWRKY059, BnaWRKY202, BnaWRKY166 | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN14658 | BnaWRKY072, BnaWRKY194          | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN14659 | BnaWRKY194                        | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN14671 | BnaWRKY113, BnaWRKY261          | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN15417 | BnaWRKY005, BnaWRKY145          | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN17285 | BnaWRKY169, BnaWRKY191, BnaWRKY033 | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN18870 | BnaWRKY083                        | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN19742 | BnaWRKY009, BnaWRKY141          | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN19744 | BnaWRKY049, BnaWRKY098, BnaWRKY241 | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN19745 | BnaWRKY098, BnaWRKY182          | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN20043 | BnaWRKY001                        | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN20181 | BnaWRKY055                        | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN20309 | BnaWRKY040                        | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN22940 | BnaWRKY100, BnaWRKY247          | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN23484 | BnaWRKY143                        | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN23912 | BnaWRKY095, BnaWRKY225          | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN24283 | BnaWRKY061                        | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN24410 | BnaWRKY094, BnaWRKY224          | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN24459 | BnaWRKY126, BnaWRKY127, BnaWRKY210 | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN25151 | BnaWRKY007, BnaWRKY147          | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN25355 | BnaWRKY064, BnaWRKY035, BnaWRKY204, BnaWRKY189, BnaWRKY171 | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN25509 | BnaWRKY118, BnaWRKY249          | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN25589 | BnaWRKY082, BnaWRKY232          | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |

(Continued)
pairs including the BnaWRKY015 and BnaWRKY016, BnaWRKY031 and BnaWRKY032, BnaWRKY034 and BnaWRKY035, BnaWRKY053 and BnaWRKY054, BnaWRKY63 and BnaWRKY64, and BnaWRKY170 and BnaWRKY171, exhibited similar expression profiles under the multiple stress conditions, respectively. For example, BnaWRKY015 and BnaWRKY016 were significantly induced expressed in response to salinity stress, and their expression trends under the same stress conditions were similar. However, one pair of tandem repeats, BnaWRKY126 and BnaWRKY127, had different expression patterns. BnaWRKY126 had strongly induced expression with salinity stress, whereas BnaWRKY127 had strongly induced expression with drought stress (Fig 6).

### Table 2. (Continued)

| EST            | BnaWRKY Gene                           | Library/Microarray | Stress condition         |
|----------------|----------------------------------------|--------------------|--------------------------|
| BN26453        | BnaWRKY050, BnaWRKY111, BnaWRKY195, BnaWRKY259 | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN26664        | BnaWRKY079, BnaWRKY178                 | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| BN27460        | BnaWRKY048                             | GSM334324–GSM334353 GSM334645–GSM334674 | Sclerotinia sclerotiorum |
| EV194691.1     | BnaWRKY125, BnaWRKY265                 | dbEST 21489        | cold stress              |
| EV218409.1     | BnaWRKY242                             | dbEST 21492        | drought stress           |
| BG543395.1     | BnaWRKY033, BnaWRKY169                 | dbEST 8791         | Etiolated seedling       |
| BG543470.1     | BnaWRKY005, BnaWRKY169                 | dbEST 8791         | Etiolated seedling       |
| EV113703.1     | BnaWRKY118, BnaWRKY249                 | dbEST 21479        | hydroponically grown root|
| EV113780.1     | BnaWRKY118, BnaWRKY249                 | dbEST 21479        | hydroponically grown root|
| EV113862.1     | BnaWRKY118, BnaWRKY249                 | dbEST 21479        | hydroponically grown root|
| EV113948.1     | BnaWRKY118, BnaWRKY249                 | dbEST 21479        | hydroponically grown root|
| EV116356.1     | BnaWRKY005, BnaWRKY145                 | dbEST 21479        | hydroponically grown root|
| EV116444.1     | BnaWRKY005, BnaWRKY145                 | dbEST 21479        | hydroponically grown root|
| EV117836.1     | BnaWRKY141, BnaWRKY009                 | dbEST 21479        | hydroponically grown root|
| EV179662.1     | BnaWRKY055                             | dbEST 21487        | Etiolated seedlings      |
| EV179750.1     | BnaWRKY055                             | dbEST 21487        | Etiolated seedlings      |
| EV181284.1     | BnaWRKY141, BnaWRKY009                 | dbEST 21487        | Etiolated seedlings      |
| EV181367.1     | BnaWRKY141, BnaWRKY009                 | dbEST 21487        | Etiolated seedlings      |
| EV186271.1     | BnaWRKY199, BnaWRKY058                 | dbEST 21488        | infestation by flea beetles |
| EV194778.1     | BnaWRKY125, BnaWRKY265                 | dbEST 21489        | cold stress              |
| EV220289.1     | BnaWRKY141, BnaWRKY009                 | dbEST 21492        | drought stress           |
| EV220578.1     | BnaWRKY141, BnaWRKY009                 | dbEST 21492        | drought stress           |
| EV223313.1     | BnaWRKY005, BnaWRKY145                 | dbEST 21493        | insect damage            |
| EV225488.1     | BnaWRKY005, BnaWRKY145                 | dbEST 21493        | insect damage            |
| EX019274.1     | BnaWRKY062                             | dbEST 21809        | cold stress              |
| EX062868.1     | BnaWRKY191                             | dbEST 21814        | etiolated mature lea, dark grown |
| EX063926.1     | BnaWRKY242                             | dbEST 21814        | etiolated mature lea, dark grown |
| EX064286.1     | BnaWRKY062                             | dbEST 21814        | etiolated mature lea, dark grown |
| EX097528.1     | BnaWRKY033, BnaWRKY169                 | dbEST 21824        | disease                  |
| EX120320.1     | BnaWRKY062                             | dbEST 21829        | defected leaf            |
| EX125680.1     | BnaWRKY191                             | dbEST 21831        | etiolated mature lea, dark grown |

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Fig 6. Expression patterns of the 26 BnaWRKYs under various abiotic stresses. The Actin7 gene was used as an internal control for qRT-PCR. The y-axis represents relative expression, calculated using the $2^{-\Delta\Delta C_t}$ formula. Expression profiles of
Discussion

Structure, evolution and duplication of WRKY genes in *B. napus*

In this study, we used genome-wide data to identify 287 WRKY genes, including a total of 343 WRKY domains in *B. napus*. Within the 343 WRKY domains, a total of 26 members showed divergence from the WRKY domain, and 21 belonged to group I. This finding suggested that the WRKY genes in group I are more active and variable compared with the WRKY genes in other groups from *B. napus*.

Genome-wide identification and analysis of the WRKY gene family in *B. napus* identified genome duplication, chromosomal/segmental duplications and tandem duplication. These duplications all contributed to the expansion of the WRKY gene family. The number of tandem duplications was much lower than the number of genome and/or segmental duplications, suggesting that whole genome-wide duplication and segmental duplication were major drivers of the WRKY gene expansion in *B. napus* during the evolutionary process. The 12 segmental duplication bars repeated 2–7 times, suggesting that duplication is not limited to hybridization from the A genome and C genome. This further supported the hypothesis that the whole genome-wide duplication occurred in the two diploid progenitors *B. rapa* and *B. oleareacea* before they combined to form *B. napus* [35, 36].

Interestingly, within the 7 pairs of WRKY tandem repeats, except for one pair of tandem repeat genes on the C sub-genome, all WRKY tandem repeats were on the A sub-genome and belonged to group III, and it was approximately 45% (12/27) of the group III in the A sub-genome. This suggests that *BnaWRKY* genes from group III in the A sub-genome are easy to repeat, and tandem duplication was the main contributor to the enlargement of *BnaWRKY* genes in group III.

Expression and functional diversity of WRKY genes in *B. napus*

The WRKY family is one of the most important transcription factor families and regulates plant responses to biotic and abiotic stresses [1, 3]. In Arabidopsis, rice and soybean, at least 26, 54 and 25 WRKY genes were identified to respond to abiotic stress, respectively [17, 37, 38]. In *B. napus*, only 13 WRKY genes have previously been reported to participate in defense responses [18]. In this study, we further evaluated the expression of 26 *BnaWRKY* genes under multiple stresses. Most of them were induced by low temperature, salinity and drought stress. These results indicated the WRKYs play important roles in *B. napus* stress responses. Notably, 3 *BnaWRKY* genes, *BnaWRKY147*, *BnaWRKY166* and *BnaWRKY210*, were strongly responsive to the three multiple stresses simultaneously. These results indicate that these 3 WRKY genes are more likely to be influenced by environmental factors and may have multi-functional roles in stress tolerance. These WRKYs may potentially be used for breeding new rapeseed cultivars. Interestingly, the expression processes for several *BnaWRKY* genes exhibited low to high or high to low curve changes over the 24-hour time course. This suggested that the response of *BnaWRKY* to multiple stresses is a dynamic process.

When using expression analysis for tandem duplication *BnaWRKY* genes, we found six tandem repeat pairs exhibiting similar expression profiles under the various stress conditions, and three pairs were mapped in the stress related QTL regions. These results indicated tandem
duplicate \textit{BnaWRKY}s in the adaptive response to environmental stimuli during the evolution process. The duplication of genes may have an important role in maintaining the stability of genetic systems when they are attacked by the external environment \cite{39,40}. Under natural selection, tandem repeated genes may help organisms adapt to the environment better. A previous study also showed that sorghum to drought tolerance may be related to the duplication of genes \cite{41}. Through expression analysis for tandem duplication \textit{WRKY} genes, we also found one tandem repeat pair showing different expression patterns. The results confirmed that in the evolutionary process of gene expanding, new \textit{BnaWRKY} members may have conservative functions or developed a new and different function. Subfunctionalization and neofunctionalization of the duplicate genes have been confirmed in many species \cite{42-44}. In Arabidopsis, among the tandem repeat pair AtMYB104 and AtMYB81, AtMYB104 is down-regulated by ABA, anoxia and cold stress but is up-regulated under drought, high temperature and salt, whereas the expression pattern of AtMYB81 was the opposite of AtMYB104 \cite{45}. In this study, the differentiations of expression in the tandem repeats indicated their functional diversification.

**Supporting Information**

S1 Table. Raw output data of \textit{WRKY} genes searched using PF03106 domain. (XLSX)

S2 Table. The 287 \textit{WRKY} genes with 343 \textit{WRKY} domain identified in \textit{B.napus}. (XLSX)

S3 Table. Raw output data of conserved motifs of \textit{BnaWRKY} members identified using the MEME search tool. (XLSX)

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**Author Contributions**

Conceived and designed the experiments: YH WQ. Performed the experiments: YH DW YC. Analyzed the data: YH SM YG LZ. Wrote the paper: YH JL WQ.

**References**

1. Banerjee A and Roychoudhury A. \textit{WRKY} Proteins: Signaling and Regulation of Expression during Abiotic Stress Responses. Scientific World Journal. 2015; ID 807560. http://dx.doi.org/10.1155/2015/807560

2. Yamasaki K, Kigawa T, Seki M, Shinozaki K, and Yokoyama S. DNA-binding domains of plant-specific transcription factors: structure, function, and evolution. Trends in Plant Science. 2013; 18 (5) 267 – 276. doi: 10.1016/j.tplants.2012.09.001 PMID: 23040085

3. Eulgem T, Rushton PJ, Robatzek S, Somssich IE. The \textit{WRKY} superfamily of plant transcription factors. Trends in Plant Science. 2000; 5: 199–206. PMID: 10785665

4. Wu KL, Guo ZJ, Wang HH, Li J. The \textit{WRKY} family of transcription factors in rice and \textit{Arabidopsis} and their origins. DNA Research. 2005; 12: 9–26. PMID: 16106749
5. Zhang Y, Wang L. The WRKY transcription factor superfamily: its origin in eukaryotes and expansion in plants. BMC Evolutionary Biology. 2005; 5: 1. PMID: 15629062

6. Ishiguro S, Nakamura K. Characterization of a cDNA encoding a novel DNA-binding protein, SPF1, that recognizes SP8 sequences in the 5' upstream regions of genes coding for sporamin and beta-amylase from sweet potato. Molecular and General Genetics. 1994; 244: 563–571. PMID: 7969025

7. Chen L, Song Y, Li S, Zhang L, Zou C, and Yu D. The role of WRKY transcription factors in plant abiotic stresses. Biochimica et Biophysica Acta: Gene Regulatory Mechanisms. 2012; 1819 (2): 120–128.

8. Zou Z. Genome-wide identification and phylogenetic analysis of WRKY transcription factor family in castor bean (Ricinus communis L.). Chinese Journal of Oil Crop Sciences. 2013; 35 (1): 36–42.

9. Wei KF, Chen J, Chen YF, Wu LJ, and Xie DX. Molecular phylogenetic and expression analysis of the complete WRKY transcription factor family in maize. DNA Research. 2012; 19 (2): 153–164. doi: 10.1093/dnares/dsr048 PMID: 22279089

10. Ling J, Jiang W, Zhang Y, Yu H, Mao Z, Gu X, et al. Genome-wide analysis of WRKY gene family in Cucumis sativus. BMC Genomics. 2011; 12: 471. doi: 10.1186/1471-2164-12-471 PMID: 21955985

11. Cai C, Niu E, Du H, Zhao L, Feng Y, and Guo W. Genome-wide analysis of the WRKY transcription factor gene family in Gossypium raimondii and the expression of orthologs in cultivated tetraploid cotton. The Crop Journal. 2014; 2: 87–101.

12. Wang M, Vannonzi A, Wang G, Liang Y, Tornielli GB, Zenoni S, et al. Genome and transcriptome analysis of the grapevine (Vitis vinifera L.) WRKY gene family. Horticulture Research. 2014; 1: article 14016.

13. Kayum MA, Jung HJ, Park JJ, Ahmed NU, Saha G, Yang TJ, et al. Identification and expression analysis of WRKY family genes under biotic and abiotic stresses in Brassica rapa. Molecular Genetics And Genomics. 2015; 290: 79–95. doi: 10.1007/s00438-014-0898-1 PMID: 25149146

14. Yao QY, Xia EH, Liu FH, Gao LZ. Genome-wide identification and comparative expression analysis reveal a rapid expansion and functional divergence of duplicated genes in the WRKY gene family of cabbage, Brassica oleracea var. capitata. Gene. 2015; 557: 35–42. doi: 10.1016/j.gene.2014.12.005 PMID: 25481634

15. Qiu Y, Jing SJ, Fu J, Li L, and Yu D. Cloning and analysis of expression profile of 13 WRKY genes in rice. Chinese Science Bulletin. 2004; 49 (20): 2159–2168.

16. Wu HL, Ni ZF, Yao YY, Guo GG, and Sun QX. Cloning and expression profiles of 15 genes encoding WRKY transcription factor in wheat (Triticum aestivum L.). Progress in Natural Science. 2008; 18 (6): 697–705.

17. Zhou QY, Tian AG, Zou HF, Xie ZM, Lei G, Huang J, et al. Soybean WRKY-type transcription factor genes, GmWRKY13, GmWRKY21, and GmWRKY54, confer differential tolerance to abiotic stresses in transgenic Arabidopsis plants. Plant Biotechnol. 2008; 6: 486–503.

18. Yang B, Jiang Y, Rahman MH, Deyholos MK, Kav NN. Identification and expression analysis of WRKY transcription factor genes in canola (Brassica napus L.) in response to fungal pathogens and hormone treatments. BMC Plant Biology. 2009; 9: 68. doi: 10.1186/1471-2229-9-68 PMID: 19493335

19. Eddy SR. Accelerated profile HMM searches. PLoS Computational Biology. 2011; 7: e1002195. doi: 10.1371/journal.pcbi.1002195 PMID: 22039361

20. Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, Eddy SR, et al. Pfam: the protein families database. Nucleic Acids Res. 2014; 42: 222–230.

21. Bailey TL, Williams N, Misleh C, Li WW. MEME: discovering and analyzing DNA and protein sequence motifs. Nucleic Acids Res. 2006; 34: 369–373.

22. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 1997; 25: 4876–4882. PMID: 9396791

23. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology And Evolution. 2011; 28: 2731–2739. doi: 10.1093/molbev/msr121 PMID: 21546333

24. Vooomps RE. MapChart: Software for the graphical presentation of linkage maps and QTLs. J Heredity. 2002; 93(1):77–78.

25. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997; 25 (17): 3389–3402. PMID: 9254694

26. Livak K J, Schmittgen T D. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods. 2001; 25: 402–408. PMID: 11846609
27. Chalhoub B, Denoeud F, Liu S, Parkin IA, Tang H, Wang X, et al. Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. *Science*. 2014; 345: 950–953. doi:10.1126/science.25146293

28. Wu J, Cai G, Tu J, Li L, Liu S, Luo X, et al. Identification of QTLs for Resistance to Sclerotinia Stem Rot and *BnaC*IGMT7.a as a Candidate Gene of the Major Resistant QTL *SRC6* in *Brassica napus*. *Plos One*. 2013; 8 (7): e67740. doi:10.1371/journal.pone.0067740 PMID: 23844081

29. Mei J, Ding Y, Lu K, Wei D, Liu Y, Disi JO, et al. Identification of genomic regions involved in resistance against *Sclerotinia sclerotiorum* from wild *Brassica oleracea*. *Theor Appl Genet*. 2013; 126: 549–556. doi: 10.1007/s00122-012-2000-x PMID: 23096003

30. Delourme R, Piel N, Horvais R, Pouilly N, Domin C, Vallée P, et al. Molecular and phenotypic characterization of near isogenic lines at QTL for quantitative resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus*). *Theoretical and Applied Genetics*. 2008; 117: 1055–1067. doi:10.1007/s00122-008-0844-x PMID: 18696043

31. Kifuji Y, Hanzawa H, Terasawa Y, Ashutosh, Nishio T. QTL analysis of black rot resistance in cabbage using newly developed EST-SNP markers. *Euphytica*. 2013; 190: 289–295

32. Tomita H, Shimizu M, Doullah MA, Fujimoto R, Okazaki K. Accumulation of quantitative trait loci conferring broadspectrum clubroot resistance in *Brassica oleracea*. *Molecular Breeding*. 2013; 32: 889–900.

33. Pu Z, Shimizu M, Zhang Y, Nakaoka T, Hayashi T, Hori H, et al. Genetic mapping of a fusarium wilt resistance gene in *Brassica oleracea*. *Molecular Breeding*. 2012; 30: 809–818.

34. Asghari A, Fathi A, Mohammaddi S, Mohammadizadeh H. QTL analysis for diamondback moth resistance in *Brassica napus* L. *International Journal of Plant Production*. 2009; 3 (3): 29–34.

35. Ku HM, Vision T, Liu J, Tanksley SD. Comparing sequenced segments of the tomato and Arabidopsis genomes: large-scale duplication followed by selective gene loss creates a network of synteny. *Proceedings of the National Academy of Sciences USA*. 2000; 97: 9121–9126.

36. Ernolæeva MD, Wu M, Eisen JA, Salzberg SL. The age of the *Arabidopsis thaliana* genome duplication. *Plant Molecular Biology*. 2003; 51: 859–866. PMID: 12777046

37. Jiang Y, Deyholos MK. Comprehensive transcriptional profiling of NaCl-stressed *Arabidopsis* roots reveals novel classes of responsive genes. *BMC Plant Biology*. 2006, 6: 25. PMID: 17038189

38. 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 and Cell Physiology*. 2008; 49: 865–879. doi:10.1093/pcp/pcn061 PMID: 18413358

39. Gu X. Evolution of duplicate genes versus genetic robustness against null mutations. *Trends in Genetics*. 2003; 19: 354–356. PMID: 12850437

40. Chapman BA, Bowers JE, Feltus FA, Paterson AH. Buffering of crucial functions by paleologous duplicated genes may contribute cyclicality to angiosperm genome duplication. *Proceedings of the National Academy of Sciences USA*. 2006; 103: 2730–2735.

41. Paterson AH, Bowers JE, Bruggmann R, Dubchak I, Grimwood J, Gundlach H, et al. The Sorghum bicolor genome and the diversification of grasses. *Nature*. 2009; 457: 551–556. doi:10.1038/nature07723 PMID: 19189423

42. Hughes AL. The evolution of functionally novel proteins after gene duplication. *Proceedings. Biological sciences*. 1994; 256: 119–124.

43. Wendel JF. Genome evolution in polyploids. *Plant Molecular Biology*. 2000; 42: 225–249. PMID: 10668139

44. Zhang J. Evolution by gene duplication: an update. *Trends in Ecology and Evolution*. 2003; 18: 292–298.

45. Katiyar A, Smita S, Lenka SK, Rajiwanshi R, Chinnumamya V, and Bansal KC. Genome-wide classification and expression analysis of MYB transcription factor families in rice and Arabidopsis. *BMC Genomics*. 2012; 13: 544. http://www.biomedcentral.com/1471-2164/13/544. doi:10.1186/1471-2164-13-544 PMID: 23050870