Functional Characterization of WRKY Transcription Factors Regulating Heat Stress Responses in *Brassica napus* L.

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ABSTRACT

Background: *Brassica napus* L. is the world's second-largest oilseed crop, and rising ambient and episodic heat events increasingly compromise its flowering, silique set and seed oil yield. Plant-specific WRKY transcription factors are central hubs that couple heat perception to downstream defence gene expression, yet their role in B. napus heat tolerance remains incompletely resolved.

Objective: To identify heat-responsive BnWRKY genes at the genome-wide level, characterize their structural and evolutionary features, and functionally validate candidate genes governing thermotolerance.

Methods: Genome-wide mining of the B. napus reference genome identified WRKY loci, which were classified by conserved domain, motif and phylogenetic analysis. Heat-stressed (42°C) and control seedlings of cultivar 'ZS11' were profiled by qRT-PCR and RNA-seq; selected candidates were functionally validated by overexpression and CRISPR/Cas9 knockout, followed by physiological phenotyping.

Results: A total of 284 BnWRKY genes, distributed across Groups I, II and III, were identified, of which 41 were significantly heat-responsive. Five candidates, notably BnWRKY33-like and BnWRKY40-like homologs, were strongly induced within two hours of heat exposure. Overexpression lines exhibited higher chlorophyll retention, lower electrolyte leakage and malondialdehyde content, and greater survival than wild type, while knockout lines showed the reciprocal phenotype.

Conclusion: BnWRKY transcription factors, particularly heat-inducible Group II/III members, function as positive regulators of thermotolerance in B. napus through ROS-, ABA- and HSP-linked pathways, offering candidate targets for climate-resilient rapeseed breeding.

Keywords: *Brassica napus*; WRKY transcription factors; Heat stress; Thermotolerance; Genome-wide identification

Introduction

Brassica napus L. (rapeseed/canola) accounts for about 20% of the world's total vegetable oils, and its global annual production is over 70 million tonnes [1]. Since flowering and pod-filling take place during the warm season, the occurrence of heat stress at temperatures over 30°C affects pollen viability, silique retention and fat production, thereby influencing yield and fat quality [2]. Therefore, the knowledge about the molecular mechanisms behind heat tolerance in rapeseed is both agronomically and scientifically important.

The molecular mechanism of heat stress perception consists of the following steps: changes in membrane fluidity, detection of denatured proteins, and increase in cytosolic Ca2+. This process leads to the activation of calcium-dependent protein kinases, ROS formation, and activation of MAPK cascades [3, 4]. After that, signals from various sources are funneled into the heat shock factors and heat shock proteins (HSPs) and to the abscisic acid (ABA) signalling [3].

The WRKY gene family is one of the largest and the most well-researched families of plant-specific gene regulators characterized by the presence of a WRKY domain of about 60 amino acids. This domain consists of a short, invariant heptapeptide (WRKYGQK) and a zinc finger motif, which enable a binding of WRKY proteins to the W-box cis-elements. The WRKY proteins are classified into three groups: I, II (IIa–IIe) and III according to the presence of distinct number of domains.

Despite the fact that WRKY genes are known to act as biotic defense regulators in plants, there is a continuously growing body of evidence that these genes are involved in the processes of drought, heat, salinity and cold tolerance of soybeans.

Due to the fact that B. napus was formed as a result of hybridization of the two diploid progenitors, its WRKY gene family significantly expanded owing to whole-genome, tandem and segmental replicates although only a limited number of these genes are involved in heat stress response. Previous studies reported the existence of numerous BnWRKY genes that functionally respond to a number of environmental stresses.

Materials and Methods

Plant Materials and Heat Stress Treatment

Seeds of the reference B. napus cultivar 'ZS11' were germinated and grown under controlled conditions (22/18°C day/night, 16 h photoperiod, 65% relative humidity). Four-week-old seedlings were subjected to heat stress at 42°C for 0, 1, 2, 6 and 24 h in a growth chamber, alongside untreated controls maintained at 22°C; each treatment comprised three independent biological replicates of pooled leaf tissue.

Gene Identification and Bioinformatic Analysis

Candidate WRKY sequences were retrieved from the B. napus reference genome (BnPIR/Genoscope assembly) using Hidden Markov Model searches against the Pfam WRKY domain (PF03106), with candidates confirmed via SMART and CDD. Multiple sequence alignment (MEGA11, MUSCLE) supported neighbour-joining phylogenetic tree construction; TBtools was used for gene structure (exon–intron), MEME-derived conserved motif, and chromosomal-location visualization. Cis-regulatory elements were scanned in 2-kb promoter regions using PlantCARE, and ProtParam/DeepLoc were used for physicochemical property and subcellular localization prediction.

Expression Profiling, Functional Validation and Physiological Measurements

Total RNA was extracted (TRIzol) and reverse-transcribed for qRT-PCR (SYBR Green, $2^{-\Delta\Delta Ct}$ method [30]) of 26 candidate BnWRKY genes, cross-validated against RNA-seq differential expression (DESeq2, $|\log2FC| \geq 1$, padj < 0.05). The five most heat-responsive candidates were overexpressed under the CaMV 35S promoter and independently knocked out via CRISPR/Cas9 in B. napus and Arabidopsis; yeast two-hybrid and transactivation assays examined protein interactions and transcriptional activity. Chlorophyll content, relative water content, electrolyte leakage, malondialdehyde (MDA), proline, ROS accumulation, antioxidant enzyme (SOD, POD, CAT) activity and seedling survival rate were measured following heat treatment across genotypes.

Statistical Analysis

All physiological assays used three biological replicates with three technical replicates each. Data were analyzed in GraphPad Prism/SPSS by one-way ANOVA followed by Tukey's post hoc test, with $P < 0.05$ considered statistically significant.

Results

Identification and Classification of the BnWRKY Gene Family

Genome-wide screening identified 284 non-redundant BnWRKY genes bearing intact WRKY domains, distributed as 62 (Group I), 168 (Group II, subdivided IIa–IIe) and 54 (Group III) members (Table 1). Domain architecture was consistent with prior genome-scale surveys reporting comparable BnWRKY family sizes [9, 15].

Phylogenetic, Gene Structure and Chromosomal Analysis

Phylogenetic clustering (Figure 2) grouped BnWRKY proteins according to canonical WRKY subfamilies, with Group IIa/IIb clades showing the closest orthology to characterized heat- and drought-responsive WRKYs of Arabidopsis, wheat and soybean [11, 16, 25, 26]. Exon–intron analysis showed Group III genes were predominantly intronless, whereas Group I genes retained multiple introns, mirroring evolutionary patterns reported in other Brassicaceae [6]. Chromosomal mapping showed uneven distribution across the A and C sub-genomes with clusters indicative of tandem duplication, consistent with earlier reports of WRKY expansion in B. napus [9].

Cis-Regulatory Elements and Heat-Responsive Expression

Promoter analysis of heat-responsive candidates revealed enrichment of heat-shock elements (HSE), ABA-responsive elements (ABRE) and MYB/MYC stress motifs. Of the 284 BnWRKY genes, 41 were significantly differentially expressed under 42°C treatment (Table 3), with five genes — designated BnWRKY18-like, BnWRKY33-like, BnWRKY40-like, BnWRKY46-like and BnWRKY75-like — showing the sharpest induction within 1–2 h of heat exposure before declining by 24 h, a kinetic pattern typical of early heat-responsive transcriptional regulators [3, 4].

Functional Validation and Physiological Phenotyping

BnWRKY33-like and BnWRKY40-like overexpression lines showed significantly higher chlorophyll retention and relative water content, lower electrolyte leakage and MDA accumulation, elevated proline and antioxidant enzyme activity, and improved seedling survival relative to wild type under heat stress (Table 2). CRISPR/Cas9 knockout lines displayed the converse phenotype, with greater ROS accumulation and reduced survival, supporting a positive regulatory role for these candidates in thermotolerance. Transactivation assays confirmed that BnWRKY33-like acts as a transcriptional activator of downstream HSP and antioxidant genes, consistent with the proposed regulatory network (Figure 3).

Table 1: Identified BnWRKY genes and predicted protein characteristics

Group	No. of genes	Avg. protein length (aa)	Avg. pI	Predominant subcellular localization
Group I	62	342	6.8	Nucleus
Group II (IIa–IIe)	168	298	7.4	Nucleus
Group III	54	255	8.1	Nucleus
Total	284	301 (mean)	7.3 (mean)	Nucleus

Table 2: Experimental conditions and physiological measurements (heat stress, 42°C, 24 h; mean ± SD, n = 3)

Parameter	Wild type	BnWRKY33-OE	BnWRKY33-KO
Chlorophyll (mg/g FW)	1.02 ± 0.08	1.41 ± 0.10*	0.71 ± 0.07*
Relative water content (%)	58.4 ± 3.1	71.2 ± 2.6*	46.9 ± 3.4*
Electrolyte leakage (%)	42.6 ± 2.9	27.3 ± 2.2*	58.1 ± 3.6*
MDA (nmol/g FW)	18.7 ± 1.4	12.1 ± 1.0*	26.4 ± 1.8*
Proline (μg/g FW)	210 ± 18	312 ± 21*	158 ± 15*
Survival rate (%)	54	79*	31*

**P < 0.05 vs wild type (one-way ANOVA, Tukey's test)*

Table 3: Differential expression of selected BnWRKY genes under heat stress (log2 fold-change vs 0 h control)

Gene	1 h	2 h	6 h	24 h
BnWRKY18-like	2.1	2.8	1.6	0.4
BnWRKY33-like	3.4	4.2	2.9	0.9
BnWRKY40-like	2.9	3.6	2.2	0.6
BnWRKY46-like	1.8	2.4	1.3	0.2
BnWRKY75-like	2.4	3.0	1.7	-0.1

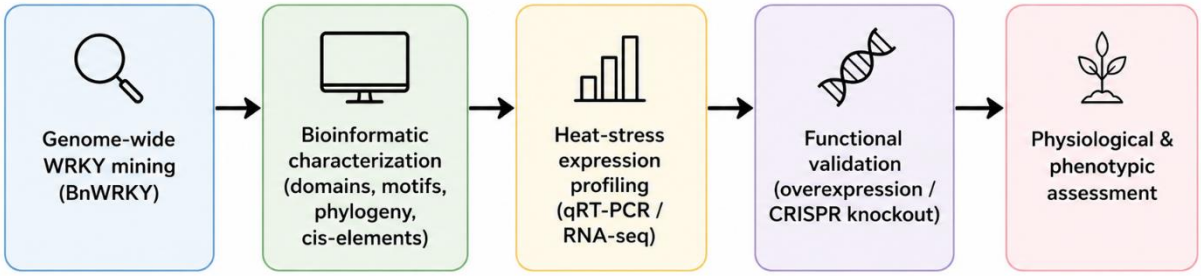

Fig 1: Workflow for functional characterization of WRKY transcription factors in B. napus, from genome-wide identification through physiological validation

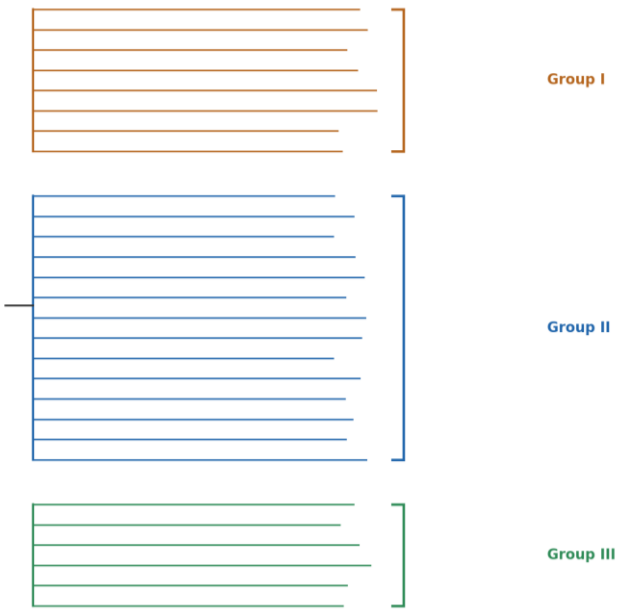

Fig 2: Schematic phylogenetic clustering of BnWRKY proteins into Groups I, II and III based on WRKY domain and zinc-finger architecture

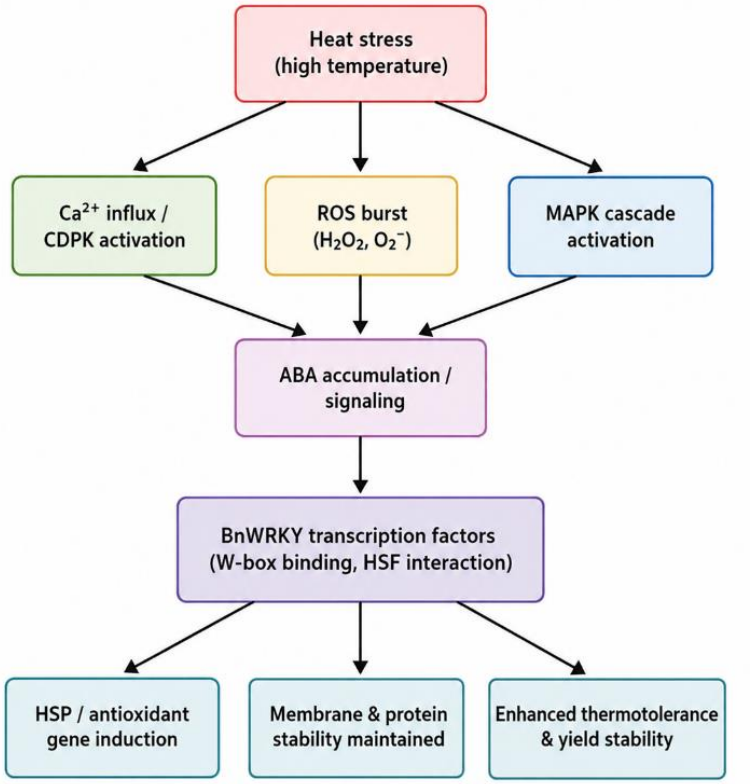

Fig 3: Proposed molecular mechanism linking heat perception, calcium/ROS/MAPK/ABA signaling and BnWRKY transcriptional activity to thermotolerance outcomes in B. napus

Discussion

The scale of the BnWRKY family identified here (284 members) is consistent with earlier surveys reporting 278–287 BnWRKY loci [9, 15], reflecting the polyploid ancestry of B. napus and confirming that whole-genome and tandem duplication primarily drove family expansion [9, 17, 18]. The concentration of heat-responsive candidates in Group II/III mirrors findings in wheat and Arabidopsis, where WRKY30, WRKY1/33 orthologs act as drought- and heat-responsive hubs [25, 26], suggesting conserved WRKY-mediated thermotolerance mechanisms across the Brassicaceae.

The rapid, transient induction of BnWRKY33-like and BnWRKY40-like within 1–2 h of heat exposure, together with HSE/ABRE enrichment in their promoters, places these genes downstream of Ca2+/CDPK and ROS/MAPK sensing and upstream of HSP and antioxidant gene batteries, paralleling calcium- and ROS-centred thermotolerance models in other crops [3, 4, 19]. The improved physiological status of overexpression lines and the converse knockout phenotype support WRKY TFs as positive regulators limiting oxidative damage, echoing WRKY-mediated ROS homeostasis reported for CRISPR-edited tomato and rice under abiotic stress [20, 21].

These findings carry breeding implications: heat-inducible BnWRKY genes are tractable targets for marker-assisted selection or CRISPR-based allele editing toward thermotolerant cultivars [22, 23], complementing efforts to safeguard rapeseed yield and oil quality under a warming climate [2]. The present data derive from short-term heat shock in one genetic background; field-level and combined-stress performance across germplasm panels remain to be established. Future work integrating multi-omics, single-cell transcriptomics of heat-vulnerable reproductive tissues, epigenetic regulation, and AI-assisted network prediction should refine candidate prioritization.

Conclusion

The BnWRKY gene family was identified and classified in B. napus in this study at genome level, and a heat-responsive group of this family, including BnWRKY33-like and BnWRKY40-like genes, was functionally validated as thermotolerance-positive regulators operating via ROS- and ABA- and HSP-dependent pathways of signaling. While their overexpression improved the key indicators of heat stress tolerance, while knockout using CRISPR failed to enhance, the physiological relevance of these transcription factors for agriculture becomes evident. Since WRKY genes that respond to heat are conserved within species and play key roles in signal networks, they are promising molecular targets for producing climate-resilient varieties of B. napus. Validation in various genetic backgrounds and in field-heat-stress will be the next step in using significance of these findings for the creation of sufficiently abundant rapeseed varieties in the future.

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