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Cloning, Bioinformatics and Expression Analysis of *SmWRKY53* Gene in *Solanum melongena L.*

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Abstract In this study, *SmWRKY53* gene was selected from the data of high temperture transcriptome and gene expression detection of "Tewangda" seedlings. It was then cloned, bioinformatics and preliminary expression analyzed. The results showed that the open reading frame of *SmWRKY53* gene was 1 083 bp, encoding 360 amino acids. The 138-144 amino acids contain a typical "WRKYGQK" conserved domain, and the zinc finger structure is C_2HC type, belonging to the Group III subfamily. The homology comparison and phylogenetic tree analysis showed that SmWRKY53 was closely related to StWRKY53 of potato (*Solanum tuberosum*), and the homology is 91.41%. Bioinformatics prediction showed that SmWRKY53 was a basic and unstable hydrophilic protein with no signal peptide and transmembrane domain. The ratio of random coil in the secondary structure of protein was the highest, 63.89%. There was amino acid disorder structural regions in the SmWRKY53 protein. Subcellular localization prediction indicated that the protein was most likely located in the nucleus. Quantitative Real-time PCR results showed that the relative expression levels of *SmWRKY53* was the highest in mature stem and the lowest in flesh. The expression of *SmWRKY53* in roots, stems and leaves of eggplant seedlings first increased and then decreased, and reached the peak at 3 h under high temperature stress. This study provides the reference for further exploring the function of *SmWRKY53* gene.

Keywords Eggplant; *SmWRKY53*; Gene cloning; Bioinformatics; Expression analysis

The WRKY family is one of the unique transcription factor families in plants, with diverse and complex functions and wide regulatory ranges, covering various physiological activities such as plant growth and development and stress response (Yu and Zhang, 2020). The WRKY transcription factors contain at least one WRKY domain, which is a segment of approximately 60 highly conserved amino acid sequences. At the N-terminal of this conserved domain, there is a core heptapeptide WRKYGQK with extremely high conservation, hence its name (Agarwal et al., 2011). There is a Zinc-finger motif at the C-terminal, which is usually composed of $CX_{4\text{-}5}CX_{22\text{-}23}$ HXH (C_2H_2 type) or $CX_7CX_{23}HXC$ (C_2H_2 type). According to the number of WRKY domains and the type of zinc finger, the WRKY family can be divided into three subfamilies (Zhao et al., 2020): The I subfamily contains two WRKY domains, and the zinc finger structure is C_2H_2 type; The II subfamily only contains one WRKY domain, and the zinc finger is also C_2H_2 type; Like the II subfamily, the III subfamily only contains one WRKY domain, but its zinc finger is C_2HC type. The II subfamily is further subdivided into five subgroups based on different amino acid core sequences (IIa, IIb, IIc, IId and IIe). Yang et al. (2019) identified 58 WRKY transcription factors in eggplant, including 13 members of family I and 39 members of family II (6 of IIa, 7 of IIb, 13 of IIc, 8 of IId and 5 of IIe), 6 members of the III family.

WRKY transcription factor regulates transcription by specifically binding to the target gene promoter W-box *cis*-element TTGACC/T (Cheng et al., 2019). In the *cis*-element, TGAC is the core sequence, and any nucleotide mutation can cause a decrease or even complete disappearance of binding activity, which proves that W-box plays an indispensable role in the function of WRKY transcription factors (Phukan et al., 2016). WRKY transcription factor plays a positive role in the response of plants to external environmental stress. Overexpression of

PtrWRKY73 in Arabidopsis can enhance its disease resistance (Duan et al., 2015). *VvWRKY30* is induced by salt stress and signal molecules H_2S and H_2O_2, and overexpression of *VvWRKY30* in Arabidopsis can improve its resistance to salt stress (Zhu et al., 2019). Transgenic tomatoes overexpressing *SlWRKY8* showed lighter wilting or yellowing phenotype, higher osmotic substance content and antioxidant enzyme activity than WT under drought and salt stress (Gao et al., 2019). The above reports provide a basis for studying the response mechanism of plant WRKY transcription factors under different biotic and abiotic stress.

Eggplant (*Solanum melongena* L.) is an important fruit vegetable in China, which has been cultivated for a long time and covers a wide area. Eggplant has rich nutritional value and can be supplied annually, making it an economical and affordable popular vegetable. But with the change of global environment and climate, temperature stress has brought great influence on eggplant agricultural production. Our research team conducted transcriptome analysis on seedlings of 'Tewangda' eggplant variety under high temperature stress in the early stage, and the data showed that *WRKY53* expression was significantly up-regulated (Zhang et al., 2020). In this study, the *SmWRKY53* gene was successfully isolated from the eggplant variety 'Tewangda'. With the help of bioinformatics website and software, the protein encoded by the gene was analyzed by bioinformatics such as domain prediction, multiple sequence alignment, phylogenetic tree, physical and chemical properties, signal peptide and transmembrane structure, disorder, secondary and tertiary structure prediction and subcellular localization, and the gene was preliminarily expressed by qRT-PCR test. This study is of great significance for further research on the *SmWRKY53* gene and the cultivation of new eggplant varieties that are resistant to stress, especially high temperature.

1 Results and Analysis

1.1 Cloning and protein domain analysis of *SmWRKY53*

Extract the seedling RNA of eggplant variety 'Tewangda' and reverse transcribe it into cDNA, design the full-length primer *SmWRKY53 SmWRKY53-F/R*, and use this as a template for PCR amplification to obtain gel electrophoresis image (Figure 1). The target gene (*SmWRKY53*) fragment was cloned and sequenced. The sequencing results showed that the open reading frame of *SmWRKY53* was 1 080 bp, encoding 360 amino acids. The protein was predicted through the NCBI conserved domain analysis website, and the results showed that the transcription factor *SmWRKY53* contained a WRKY conserved domain (Figure 2). The C-terminal zinc finger is C_2HC type, so it is inferred that *SmWRKY53* belongs to the WRKY transcription factor Group Ⅲ subfamily.

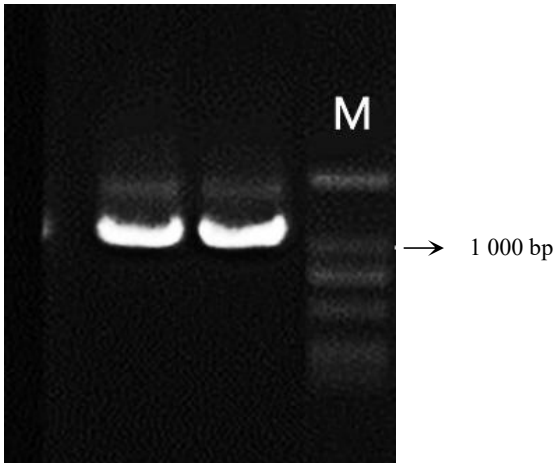

Figure 1 Full-length PCR clone electrophoresis of *SmWRKY53* gene

Note: M: DL2000 DNA Marker

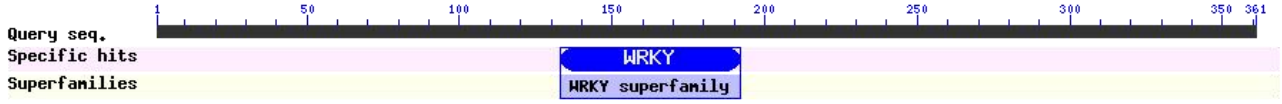

Figure 2 The conversed domain predicts of transcription factor SmWRKY53

1.2 Multiple sequence alignment of WRKY53 in different species

Based on the WRKY53 amino acid sequence of eggplant, BLAST homology search and comparison were conducted in NCBI to obtain WRKY amino acid sequences with high similarity in different plants. Multiple alignment of the homologous protein sequences of eggplant SmWRKY53 with different species such as pepper (*Capsicum annuum*) (NP_001311621.1), potato (*Solanum tuberosum*) (XP_006352253.1), tobacco (*Nicotiana attenuata*) (XP_019233122.1), sweet potato (*Lpomoea triloba*) (XP_031095353.1), and upland cotton (*Lpomoea triloba*) (XP_031095353.1) showed that their amino acid sequence consistency was 77.76%, and they all contained WRKY's typical conserved domains WRKYGQK and C_2HC type zinc finger (Figure 3).

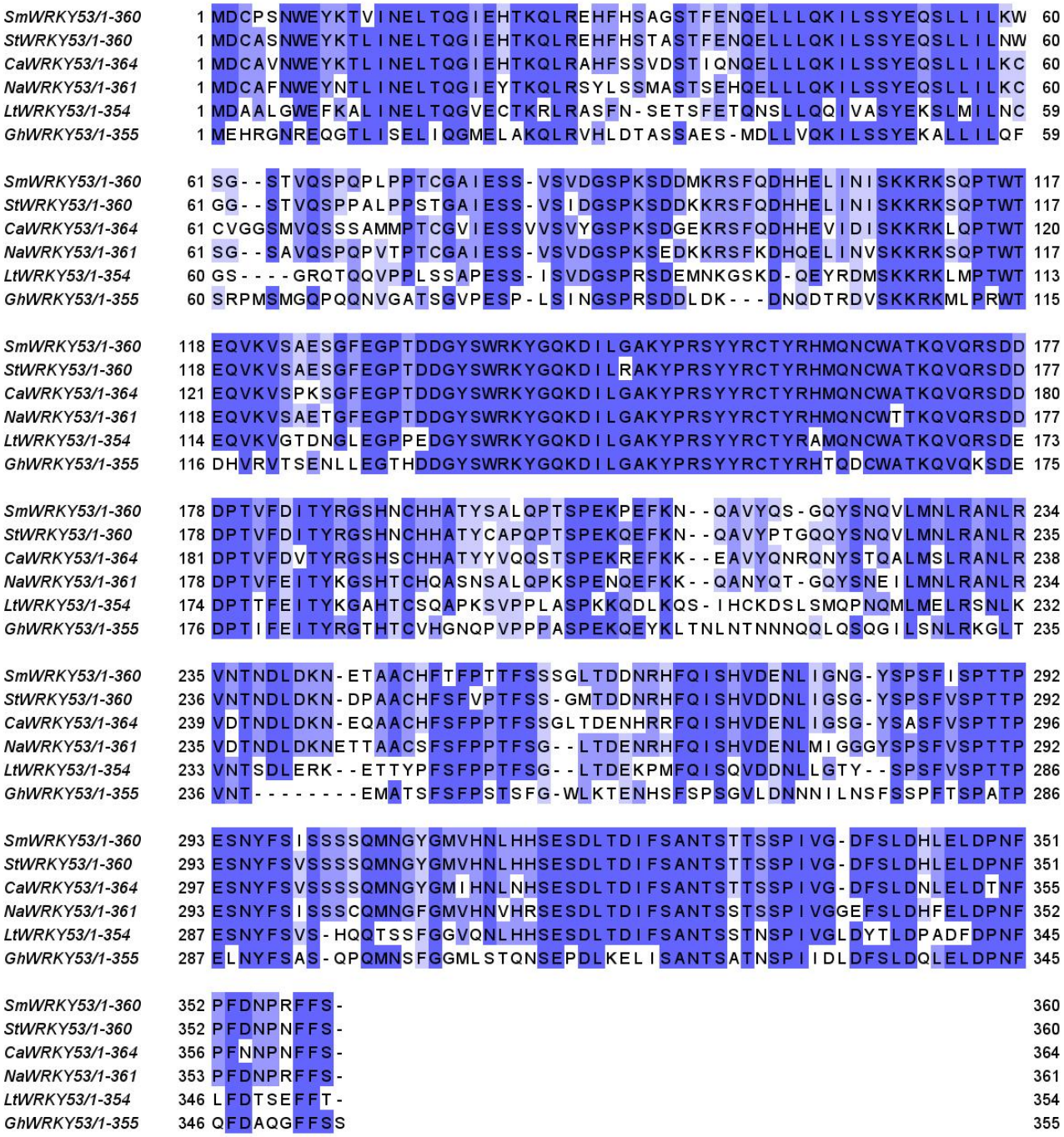

Figure 3 Multiple sequences alignment analysis of SmWRKY53 and 5 other plant homologous proteins

1.3 Protein similarity analysis and phylogenetic tree construction of WRKY53 in different species

In the GenBank database, BLAST searches for amino acid sequences of the *WRKY53* gene in other species. Compare the amino acid sequence of eggplant SmWRKY53 with various species such as pepper, upland cotton, clover potato, tobacco, tomato, potato, etc (Table 1). On this basis, MAGA software was used to construct phylogenetic tree of homologous proteins of the above species (Figure 4). The results showed that among these species, SmWRKY53 and StWRKY53 had the shortest evolutionary distance, with a high homology of 91.41%, indicating that the Solanaceae plant potato WRKY53 had the closest genetic relationship with SmWRKY53, followed by tomato and walnut.

Table 1 Information of WRKY53 protein of other species aligned to SmWRKY53

Number	Species	Protein sequence ID	Identities (%)
1	*Solanum tuberosum*	XP_006352253.1	91.4
2	*Solanum lycopersicum*	XP_004244630.1	89.2
3	*Nicotiana attenuata*	XP_019233122.1	84.3
4	*Capsicum annuum*	NP_001311621.1	83
5	*Lpomoea triloba*	XP_031095353.1	60.6
6	*Olea europaea var. sylvestris*	XP_022886946.1	56.5
7	*Gossypium hirsutum*	NP_001314458.1	53.1
8	*Camellia sinensis*	XP_028115466.1	53.1
9	*Pistacia vera*	XP_031270893.1	52.6
10	*Durio zibethinus*	XP_022761163.1	52.3
11	*Sesamum indicum*	XP_011101023.1	51.9
12	*Juglans regia*	XP_018815563.1	50.4

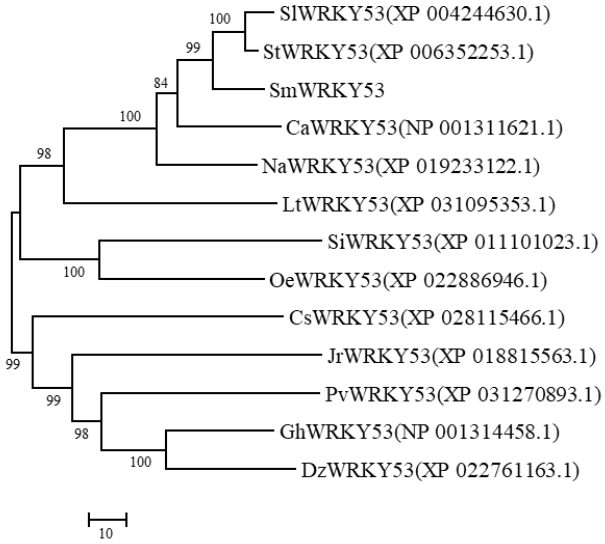

Figure 4 Phylogenetic tree of SmWRKY53

Note: The Neighbor-Joining method was applied to construct the phylogenetic tree

1.4 Physicochemical properties and hydrophobicity analysis of SmWRKY53 protein

Use the Protparam website to predict the physicochemical properties of SmWRKY53 protein. The molecular formula of the protein was found to be $C_{1789}H_{2698}N_{496}O_{578}S_{12}$, with a theoretical molecular weight of 40 786.80 kD and an instability coefficient (II) of 49.31, greater than 40, therefore, it may be an unstable protein. The total number of positively charged residues (Arg+Lys) is 30, and the total number of negatively charged residues (Asp+Glu) is 42, with an isoelectric point of 5.7. Therefore, it is speculated that the protein is an alkaline protein.

Hydrophilic analysis showed that the 46th lysine (Lys) of SmWRKY53 had the strongest hydrophobicity, while the 112th serine (Ser) had the strongest hydrophobicity, with hydrophobicity scores of 1.189 and -2.633, respectively

(Figure 5). The average hydrophobicity (GRAVY) was -0.777, indicating that the number of hydrophilic amino acids encoded by this gene is higher than that of hydrophobic amino acids, indicating that it should be a hydrophilic protein. It is speculated that SmWRKY53 may be a hydrophilic unstable alkaline protein.

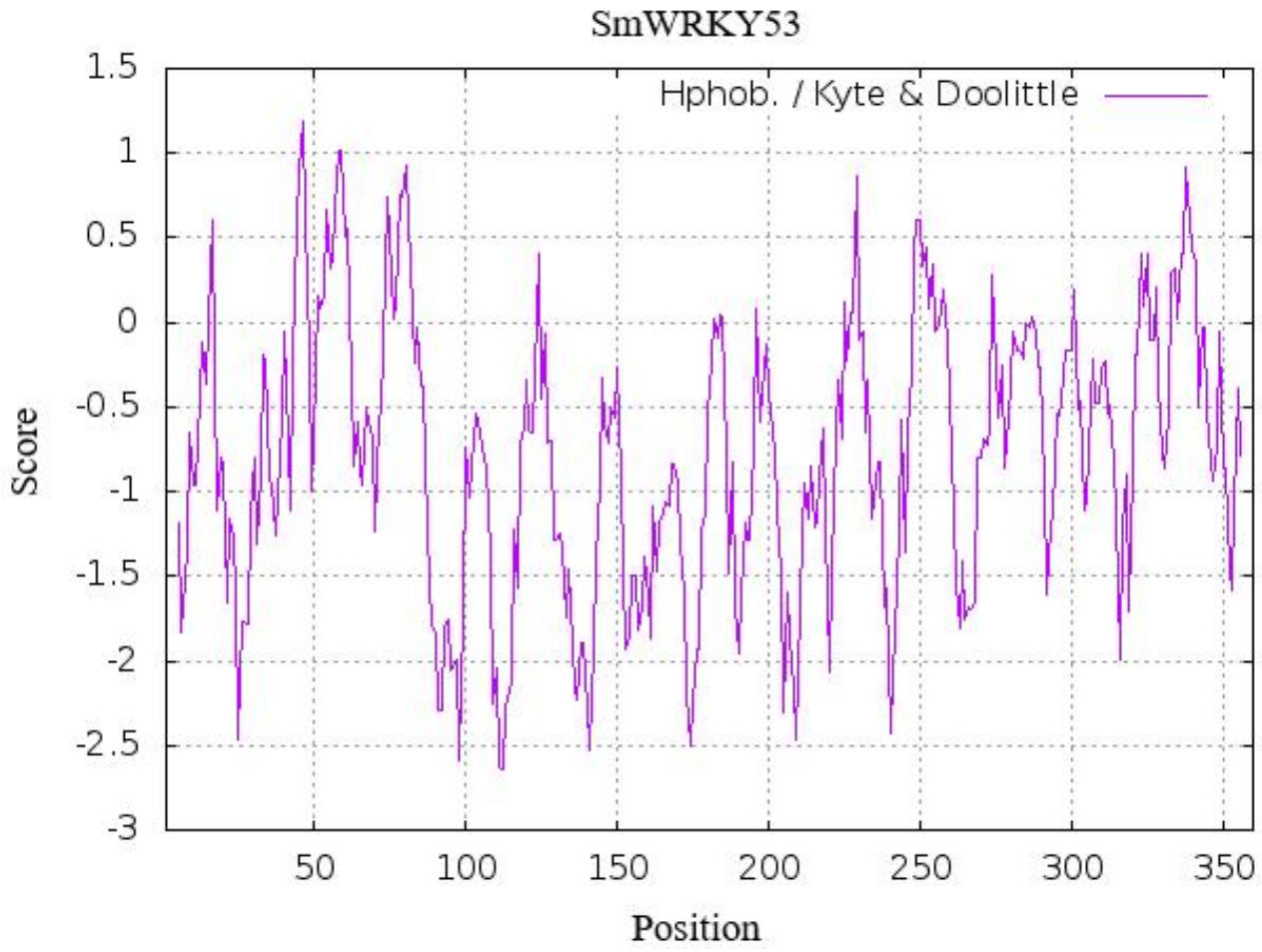

Figure 5 Predicted hydrophobicity and hydrophilicity of deduced amino acid sequence of SmWRKY53

1.5 Prediction of SmWRKY53 protein signal peptide and transmembrane structure

The online analysis tools SignalP and TMHMM were used to predict the signal peptide and transmembrane structure of *SmWRKY53* gene. The results showed that there was no signal peptide at the N-terminal of SmWRKY53 protein, suggesting that it may be a non-secretory protein and has no transmembrane domain.

1.6 SmWRKY53 protein phosphorylation analysis

The online analysis tool Netphos 2.0 server predicts the phosphorylation sites of SmWRKY53 protein, showing that serine is the most phosphorylated site in this protein, followed by threonine and tyrosine (Figure 6).

1.7 SmWRKY53 secondary structure prediction and subcellular localization analysis

The SOPMA website predicts the secondary structure of SmWRKY53 protein online, and the results showed (Figure 7) that the secondary structure of the protein contains α-helix, random coil, extended strand, and β-turn, with random coil accounting for the highest proportion, reaching 63.89%. Random coil can connect other secondary structural elements; However, there are relatively few other secondary structures, α-helix accounts for 22.78%, extended strand accounts for 10.28%, β-turn accounts for 3.06%. Using the online Prediction tool of PSORT website to predict the subcellular localization of this protein, the result showed that SmWRKY53 had the highest probability of localization in the nucleus (Table 2).

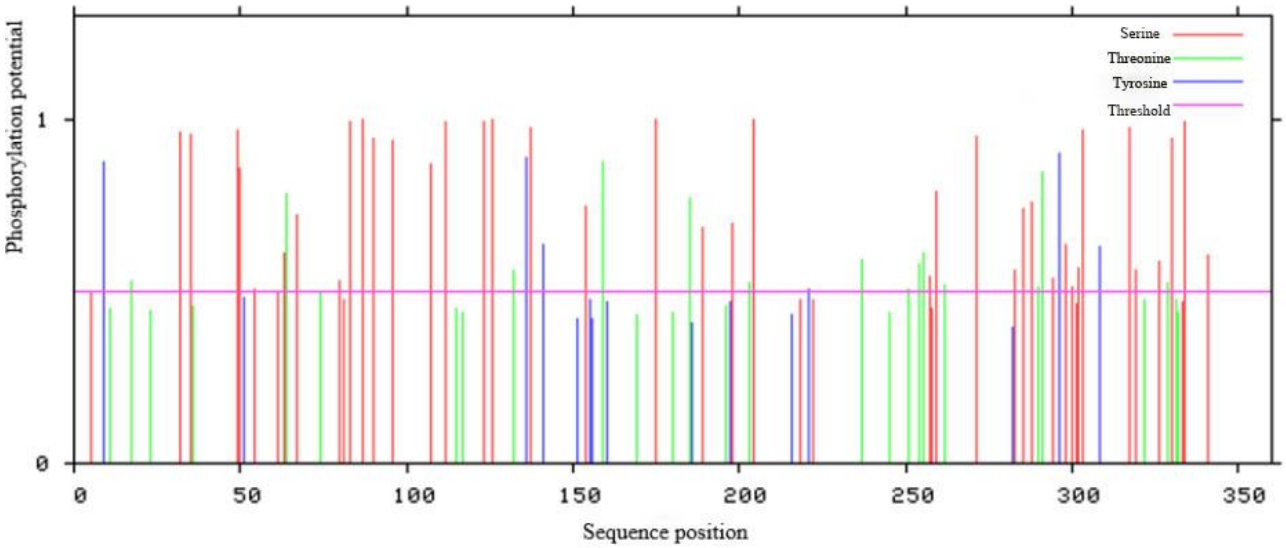

Figure 6 Prediction of phosphorylation sites of SmWRKY53

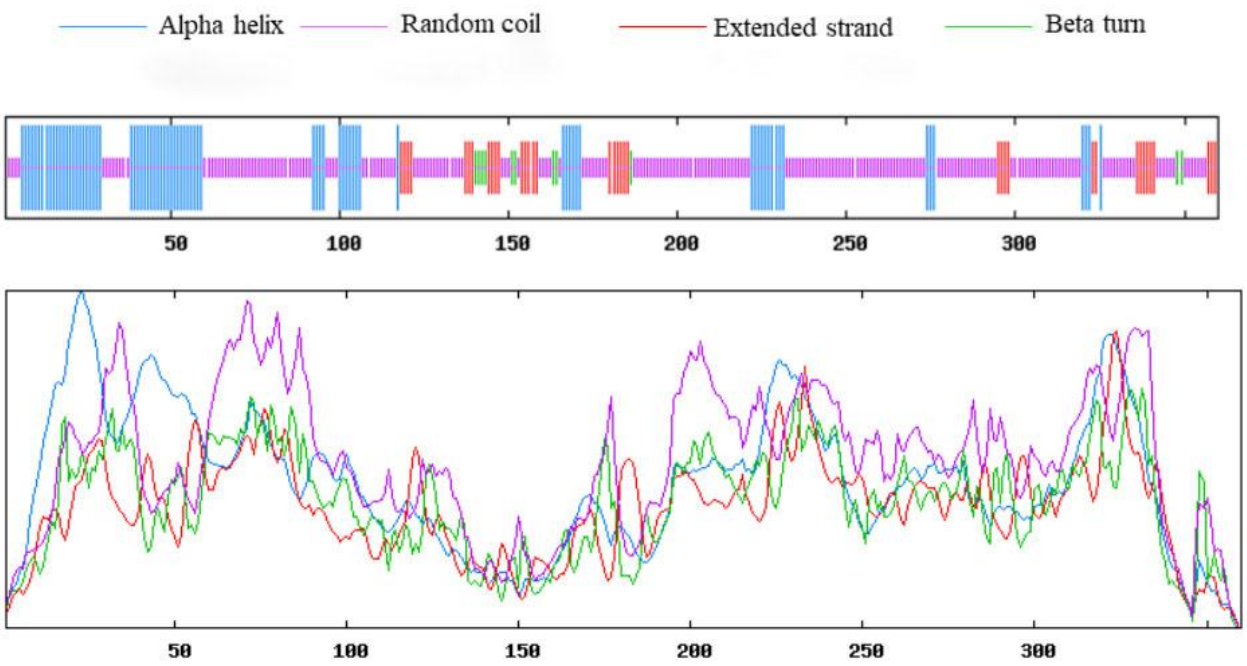

Figure 7 Secondary structure prediction of SmWRKY53

Table 2 Subcellar location prediction of SmWRKY53

Gene expression position in cells	Rate (%)
Nucleus	0.88
Microbody (peroxisome)	0.323
Mitochondrial matrix space	0.1

1.8 SmWRKY53 transcription factor folding disorder analysis and tertiary structure prediction

Disordered proteins do not have a stable three-dimensional structure, but still possess biological activity, which makes them play an important role in various life activities such as cell signal transduction and molecular recognition (Zhuang et al., 2008). Using FoldIndex to perform disordered folding analysis on the amino acid sequence of SmWRKY53 in eggplant (Figure 8), it was found that there are disordered regions in the transcription factor sequence of SmWRKY53 in eggplant.

Online modeling of the tertiary structure of the SmWRKY53 transcription factor was conducted through the SWISS-MODEL website (Figure 9). Proteins need to form a tertiary or higher spatial structure in order to function. Therefore, the prediction and modeling of protein tertiary structure is more conducive to understanding the function of protein.

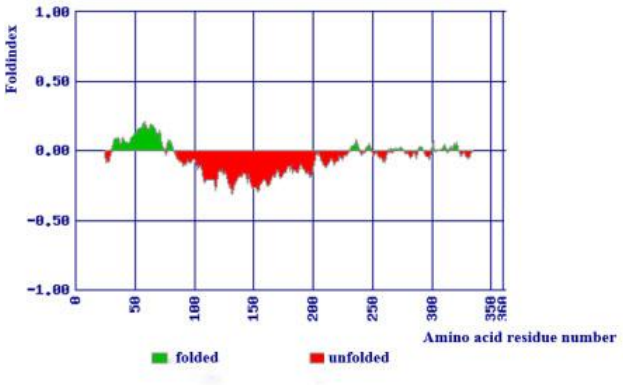

Figure 8 Analysis of the folding state of SmWRKY53 in eggplant

Figure 9 Three tertiary structure prediction of SmWRKY53

1.9 Tissue specificity and expression analysis of SmWRKY53 under high temperature stress

Analyze the expression level of *SmWRKY53* gene in different tissues of eggplant using qRT-PCR method. The results showed (Figure 10) that *SmWRKY53* was expressed in all tissues of eggplant, with the highest expression level in mature stems, significantly higher than other tissues. The expression level is the lowest in fruit flesh. It suggests that this gene may play an important role in mature stems.

In order to further explore the function of *SmWRKY53* in eggplant under high temperature, high-temperature treatment was carried out during the eggplant seedling stage. The expression levels of *SmWRKY53* gene in different stages of high temperature stress and different parts of eggplant were analyzed using qRT-PCR method. The results showed (Figure 11) that the expression of *SmWRKY53* in the roots, stems, and leaves of eggplants showed a trend of first increasing and then decreasing with the prolongation of stress time, and all reached their peak at 3 h. When subjected to high temperature stress for 3 h, the expression level of *SmWRKY53* in the stem was 12.08 times that of 0 h, while the expression level of *SmWRKY53* in the roots and leaves was 7.66 and 4.86 times that of 0 h, respectively.

2 Discussion

According to the estimation of the Food and Agriculture Organization of the United Nations (FAO) and the National Bulk Vegetable Industry Technology System in 2017, the current cultivation area of eggplant in China is about 8.0×10^5 hm^2 (Ma et al., 2017, Chinese Vegetables, (9): 1-6). Although the planting area of eggplant in China is large, there are still some problems in its development, such as the lack of new high-quality and high resistance varieties of eggplant (Liu et al., 2019). In recent years, with the continuous expansion of protected cultivation area, eggplant verticillium wilt, bacterial wilt and cotton blight have a tendency to expand and spread. In addition, with

the extreme weather occurs frequently, eggplant needs to cope with various biotic and abiotic stress in the process of growth and development, ultimately affecting its quality and yield. Therefore, accelerating the breeding of new high-quality eggplant varieties is particularly important.

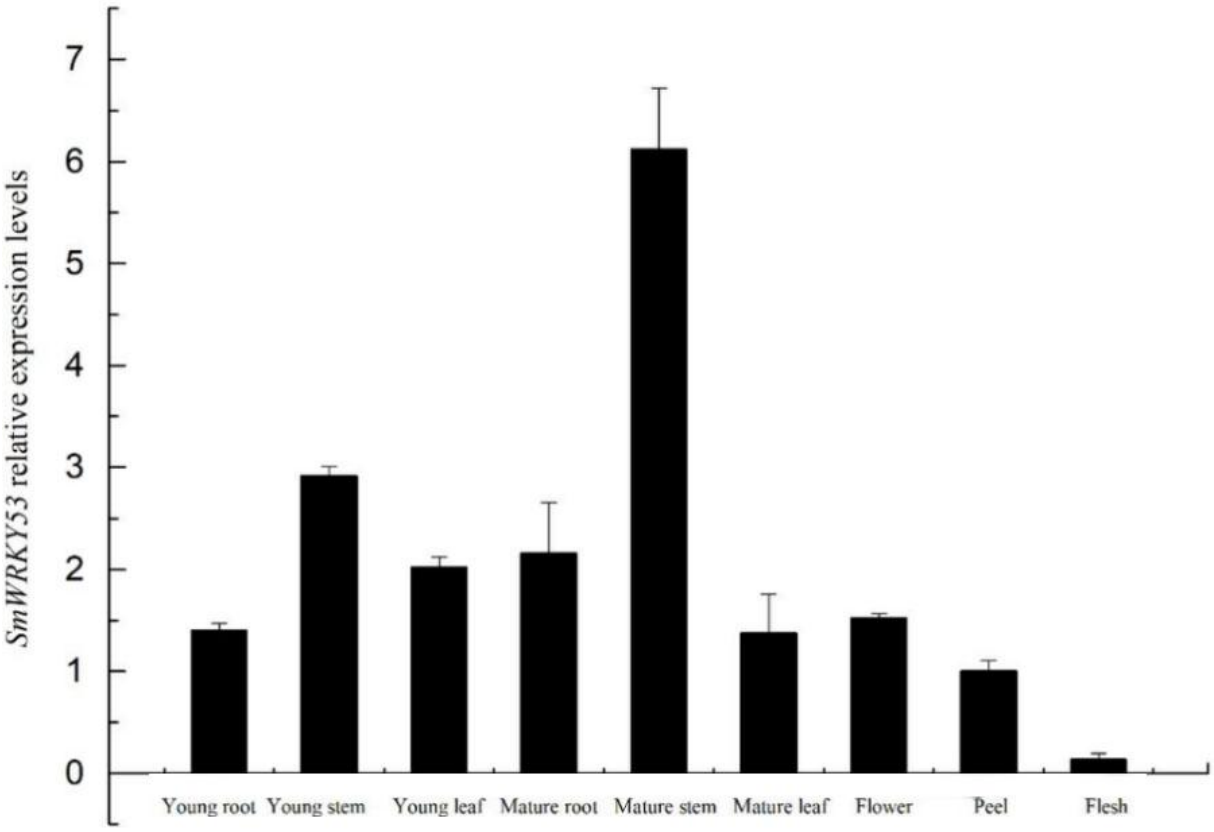

Figure 10 Relative expression levels of *SmWRKY53* gene in different tissues of eggplant

WRKY transcription factor family is involved in a variety of plant development and physiological processes, such as plant disease resistance, response to abiotic stress, aging, seed development and other development and hormone regulation processes (Xu et al., 2020). The role of WRKY transcription factors in the process of plant stress resistance (resistance to biotic and abiotic stress) has attracted much attention. At present, there is relatively little research on the function of the WRKY gene and its encoded protein in eggplants. From the transcriptome and gene expression data of eggplant 'Tewangda' seedlings under high temperature, we have screened the *SmWRKY53* gene that has obvious response to high temperature. Therefore, cloning, bioinformatics and preliminary expression analysis were performed to further explore the function of the gene.

Almost all Class III WRKY transcription factors are involved in the biotic stress response of plants, which indicates that Class III WRKY transcription factors may be generated in the process of plants adapting to external environmental stresses (Wen et al., 2017). Analysis revealed that SmWRKY53 belongs to the Group III subfamily, indicating that the *SmWRKY53* gene may have evolved from eggplants during long-term adaptation to environmental changes and is related to their stress resistance. Among other species, *WRKY53* plays an important role in resisting drought stress, low temperature stress and salt stress in biotic stress and abiotic stress. The transcription factor OsWRKY53 can establish a defense system in rice by regulating MPK kinase activity, thereby resisting the invasion of herbivorous insects such as the striped stem borer (*Chilo suppressalis*) (Hu et al., 2015). In maize, low temperature (8 ℃), NaCl, H_2O_2, and PEG can significantly increase the expression level of *ZmWRKY53* (Guo and Zhang, 2020). This study indicates that *SmWRKY53* has a significant response to high temperature stress. The WRKY53 protein from multiple species exhibits high similarity in the WRKY conserved domain, indicating that their function may be related to the conserved domain. SmWRKY53 and StWRKY53 have the highest similarity, indicating a closer genetic relationship between their WRKY53 proteins. Predicting

the physical and chemical properties, hydrophobicity/hydrophilicity, phosphorylation sites, secondary and tertiary structures and disorder characteristics of eggplant SmWRKY53 protein has important reference significance for protein functional analysis. The prediction results showed that SmWRKY53 may be an alkaline unstable hydrophilic protein without signal peptide and transmembrane structure, so it is excluded as a secreted protein. Among them, the serine phosphorylation sites were the most, and 63.89% of the secondary structure was predicted to be random coil, accounting for the highest proportion, and there were disordered regions. The subcellular localization predicted that SmWRKY53 protein was most likely located in the nucleus, which was consistent with the prediction results of signal peptide and transmembrane structure.

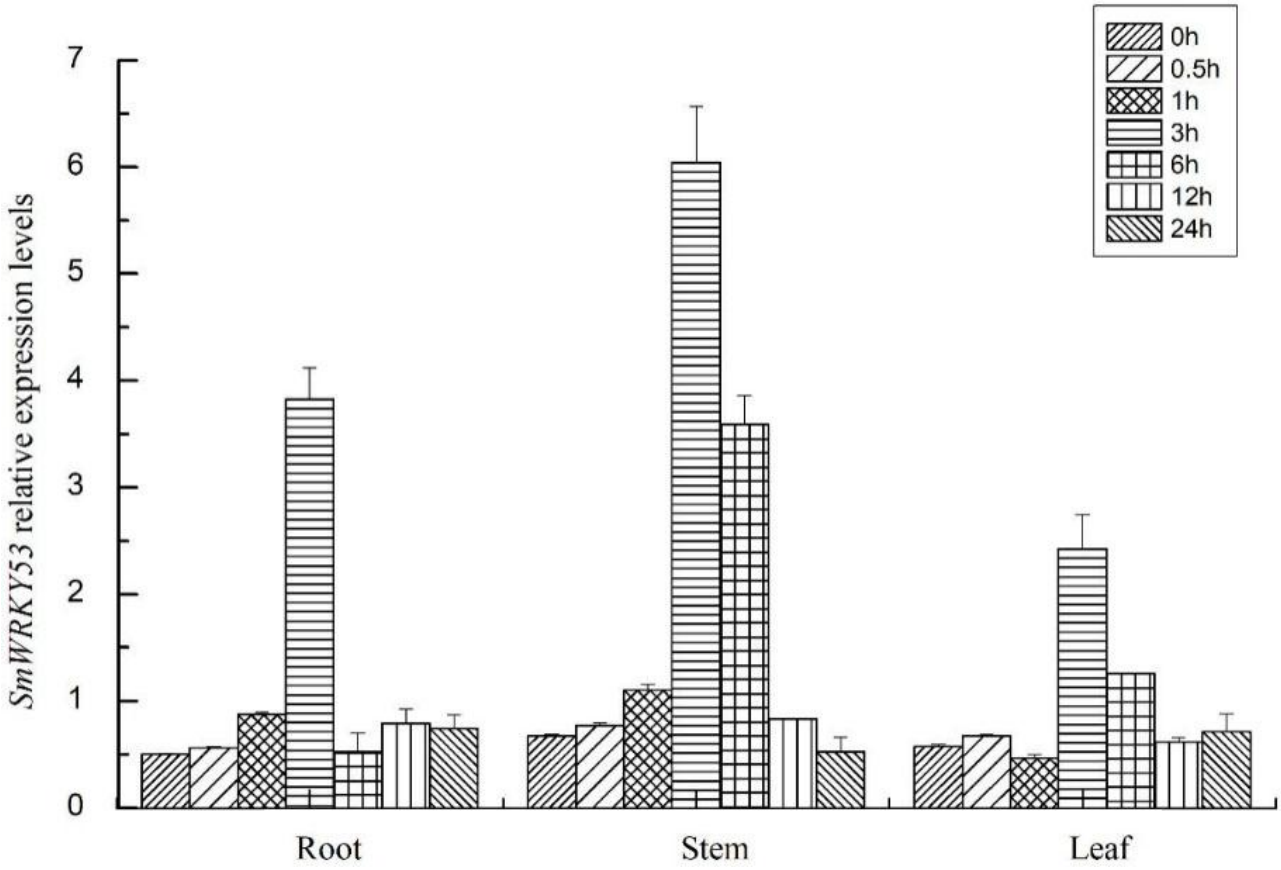

Figure 11 Relative expression levels of *SmWRKY53* gene under high temperature stress in eggplant seedling

The analysis of qRT-PCR results showed that *SmWRKY53* had the highest relative expression level in the mature stem of eggplant, which is consistent with the specific expression results of SlWRKY53 in different tissues of tomato of Liu et al. (2013). However, the expression level of *SmWRKY53* is the lowest in fruit pulp, and the expression level of *SmWRKY53* in mature roots and stems is significantly higher than that in seedling roots and stems, while the opposite is true in leaves. Under high temperature stress, the expression level of *SmWRKY53* in the roots, stems, and leaves of eggplant seedlings showed a trend of first increasing and then decreasing, and reached its peak at 3 h. The above results provide a reference basis for studying the function of *SmWRKY53*.

3 Materials and Methods

3.1 Test materials

The *SmWRKY53* gene cloned in the experiment came from the heat-resistant variety 'Tewangda' of eggplant, and the expression analysis of *SmWRKY53* was conducted in the heat-sensitive eggplant variety 'Fu 2-2'. Both varieties were selected by the Eggplant Research Group of the Facility Horticulture Institute of Shanghai Academy of Agricultural Sciences.

3.2 Cloning of *SmWRKY53* gene

RNA of eggplant 'Tewangda' seedlings was extracted, and its bands were detected by agarose gel electrophoresis. Take 500 ng RNA for reverse transcription to obtain cDNA. Using this cDNA as a template, primers were designed based on the *SmWRKY53* gene sequence in the Eggplant Genome DataBase of Japan (Table 3) to clone the *SmWRKY53* gene.

Table 3 Primer information of *SmWRKY53*

Primer name	Sequence
WRKY53-F	GGGGTACCATGGATTGTCCATCCAACTG
WRKY53-R	CGGGATCCTCATGAGAAAAATCTGGG

3.3 Bioinformatics analysis of SmWRKY53 and its homologous proteins

Based on the existing *SmWRKY53* gene sequence, the amino acid sequence of the transcription factor was obtained through translation. Collect and save sequence information of its homologous proteins, and use bioinformatics analysis websites and software to predict and analyze the structure and function of the SmWRKY53 transcription factor. The specific website or software can be found in the table (Table 4).

Table 4 Websites or softwares used in this study

Analyzed items	Tools	Websides
Gene structure prediction	NCBI	https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi
Multiple sequence alignment of amino acids / Protein similarity analysis	ClustalW	https://www.genome.jp/tools-bin/clustalw
	Jalview	-
Construction of phylogenetic tree	MEGA 6.0	-
Physicochemical properties of protein	ProtParam	https://web.expasy.org/cgi-bin/protparam/protparam
Analysis of protein hydrophobicity	ProtScale	https://web.expasy.org/protscale/
Prediction of signal peptide	SignalP	http://www.cbs.dtu.dk/services/SignalP/
Prediction of transmembrane structure	TMHMM Server v. 2.0	http://www.cbs.dtu.dk/services/TMHMM/
Prediction of protein phosphorylation sites	Netphos 2.0 server	http://www.cbs.dtu.dk/services/NetPhos/
Prediction of protein secondary structure	SOPMA	https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html
Prediction of protein subcellular localization	PSORT Prediction	http://psort1.hgc.jp/form.html
Prediction of protein tertiary structure	SWISS-MODEL	https://swissmodel.expasy.org/interactive

3.4 Expression analysis and data processing of *SmWRKY53*

From May to July 2020, the young roots, stems, and leaves of 'Fu 2-2' eggplant were taken during the seedling stage (when two leaves and one heart). After the first bud appeared, the mature roots, stems, and leaves of the eggplant were taken. The fully blooming mature flowers and the skin and flesh of the eggplant 40 d after pollination were also taken. Each sample was repeated three times, frozen in liquid nitrogen and stored in a refrigerator at -80 ℃.

One hundred evenly sized and full 'Fu 2-2' seeds were soaked overnight and planted in germination boxes. After the cotyledon was fully extended, the seeds were planted in hole trays with a substrate of 1:1:1 peat, vermiculite and perlite. When the seedlings grew to two leaves, they were subjected to 42 ℃ high temperature stress, and the roots, stems and leaves of the seedlings were taken at 0, 0.5, 1, 3, 6, 12 and 24 h after sustained stress, and frozen in liquid nitrogen after sampling, and stored in the refrigerator at -80 ℃.

The total RNA of the above samples was extracted, and its bands were detected by gel electrophoresis. 1 000 ng RNA was taken for reverse transcription to obtain cDNA. Using *SmEF1a* as the internal reference gene, primers SmEF1a-F (ACCAGCATCACATTCTCTTCCAAAA) and SmEF1a-R (CCACACTTCTCATATTGCTGCTGTCA) were designed. Based on the cloned *SmWRKY53* gene sequence, primers SmWRKY53-F (ATCGAATACATCGGATC) and SmWRKY53-R (AAAGTACGACAGAGGA) were designed for detection. The

ABI QuantStudio 5 instrument was used for qRT-PCR reaction to detect the relative expression level of *SmWRKY53* in different eggplant samples, with 3 replicates per treatment. The experimental results were performed Duncan's new multiple range test using SPSS software, and plotted using Origin software.

Authors' contributions

SJ is the experimental designer and executor of this study; LXH and SJ completed data analysis and wrote the first draft of their paper; ZAD and ZZW participated in the experimental design; ZDS and KLJ participated in the analysis of the experimental results; WXX is the conceptualizer and leader of the project, guiding experimental design, data analysis, paper writing, and revision. All authors read and approved the final manuscript.

Acknowledgments

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