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Molecular Cloning and Sequence Analysis of *GbWRKY31*, A Novel Transcription Factor Gene from *Ginkgo biloba*

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Abstract

WRKY proteins are a class of plant-specific transcription factors involved in stress response signaling pathways. In this work, a novel WRKY transcription factor gene, named after *GbWRKY31*, was isolated by RT-PCR method from the *Ginkgo biloba*. The full-length cDNA of *GbWRKY31* was 1738 bp and contained a 1281bp open reading frame (ORF) encoding 427 amino acids. The estimated isoelectric point (pI) and molecular weight of the putative *GbWRKY31* protein were 9.10 and 47.05 kDa, respectively. Homology analysis indicated that the deduced *GbWRKY31* protein was highly homologous to other WRKY Proteins from different species and had a typical WRKY conserved domain database. Phylogenetic analysis showed that *GbWRKY31* belongs to the WRKY IIb group. The results suggest that *GbWRKY31* belongs to WRKY families and may be involved in the modulation of senescence and defense in *G. biloba*.

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Introduction

Ginkgo biloba, the oldest species in the world, has been growing for nearly 200 million years on the earth (He et al., 2009). It has many good characters, such as disease-resistance, drought-resistance (Cheng et al., 2013). Recent studies have shown that WRKY transcription factors were play an important role in plant growth involved in physiological metabolism process of biological signal stimulation and defense responses against pathogens.

It has been reported that WRKY transcription factors are crucial regulators in the transcription and disease resistance (Eulgem and Somssich, 2007). For example, *AtWRKY70* in *Arabidopsis thaliana*, appears to affect the balance between signaling branches promoting SA-dependent and suppressing JA-dependent responses (Li et al., 2006); The rice carrying *OsWRKY71* gene had a stronger resistance to *Xanthomonas oryzae*, in addition, the expression of disease resistance related gene was also significantly increased (Liu et al., 2007).

The WRKY protein includes one or more WRKY domains, which were composed of conserved WRKYGQK peptide and typical zinc finger motif, $CX_{4-5}CX_{22-23}HXH$ (C_2H_2) or $CX_7CX_{23}HXC$ (C_2HC), at the N-terminal and the C-terminal separately (Eulgem et al., 2000). According to the quantity of WRKY domain and the zinc finger motif, WRKY gene families can be divided into three groups: Group I included two WRKY domains with a C_2H_2 zinc-finger structure; Group II and III consist of only one WRKY domain with a C_2H_2 and a C_2HC zinc-finger structure, respectively. Meanwhile, the group IIWRKY genes were further divided into IIa, IIb, IIc, IId, and IIe based on their primary amino acid sequence (Rushton et al., 2012).

In terms of molecular biology, WRKY proteins perform themselves function through specifically combining with the W-box (5′-TTGACC/T-3′), which is the cis-element and the minimal consensus required for specific DNA binding (Rushton et al., 1996; Ciolkowski et al., 2008). W-box sequences generally exist in promoter region involving in stress response. Moreover, WRKY genes promoter regions also often have W-box (Maleck et al., 2000). In recent years, a lot of WRKY transcription factors had been identified, and then discovered the association with secondary metabolism. The function of *AtWRKY75* has been confirmed in regulating Pi starvation responses (Devaiah et al., 2007). *TcWRKY1* participates in regulation of taxol biosynthesis in *Taxus chinensis* cells (Li et al., 2013). Previously, there are few WRKY genes that had been isolated and characterized from *G. biloba*. At present, transcriptome analysis of *G. biloba* was performed by our laboratory research group designed through using Illumina HiSeq™2500 sequencing platform. Based on the transcriptome data, we cloned and characterized *GbWRKY31* from *G. biloba*.

Materials and methods

Plant material and reagents

The leaves of ginkgo were collected from Botanical Garden of Yangtze University, China, and stored at -80 °C refrigerator immediately. Both the primers synthesis and DNA sequencing were performed by Shanghai Sangon Biotechnology Company, in China. Agarose Gel DNA purification Kit Ver.4.0, pMD19-T vector kit, AMV Reverse Transcriptase, RNase, PrimeScript™ 1st Strand cDNA Synthesis kit PrimeScript™ RT-PCR kit and Taq DNA polymerase were purchased from Takara Company, Dalian, China.

RNA extraction and reverse transcription

Total RNA was isolated from plant frozen plant tissues using the MiniBEST Plant RNA Extraction kit (TaKaRa, Dalian, China). According to the instructions of PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China), first-strand cDNA was synthesized.

Cloning of *GbWRKY31*

The specific primer GbWRKY31-F (5′-AAGGTTTCC AATGCGGATAAG-3′) and GbWRKY31-R (5′-AGTG CCGCAGTGAAGTTGGGA-3′) were designed using the software of DNAMAN6. The PCR was performed using the one-step RT-PCR kit (TaKaRa, Dalian, China) under the following conditions: per-denaturation at 94°C for 3 min; denaturation at 94°C for 30s, anneal at 58°C for 30s, and extension at 72°C for 40s, by 35 cycles; extend at 72°C for 10 min. The PCR product was purified and cloned into pMD19-T vector kit (TaKaRa, Dalian, China), followed by sequencing for confirmation.

Bioinformatic analysis

The sequence was analyzed by the bioinformatics software on websites (http://www.xpasy.org and http://www.ncbi.nlm.nih.gov), protein sequences were retrieved from NCBI GenBank. Sequence alignment was performed using ClustalX 2.0 and phylogenetic tree was constructed by neighbor-joining method using MEGA 6.0. The bootstrap statistical analysis was carried out with 1000 replicates.

Results

Cloning and sequence analysis of *GbWRKY31*

The cDNA sequence of *GbWRKY31* gene was obtained according to a pair of specific primers and total RNA reverse transcription product. The length of *GbWRKY31* was 1738 bp, and the gene contained 1281 bp-length open reading frame and encoding 427 amino acids (Fig. 1). We compared the nucleotide sequence of *GbWRKY31* with the nucleotide sequences of other plants acquired from the NCBI database and found the nucleotide sequence of *GbWRKY31* had high similarity with WRKY genes of other plants (Table 1).

Characterization of the deduced *GbWRKY31* protein

The *GbWRKY31* protein encodes 552 amino acids (Fig. 1). Using the online website (http://web.expasy.org/ compute_pi/), the molecular weight could be known and theoretical isoelectric point of the *GbWRKY31* protein were 47.05 kDa and 9.10, respectively. Using BLAST search of GeneBank and Vector NTI, the Fig. 2 shows that the similarity of the *GbWRKY31* protein with other WRKY proteins was made. Sequencing analysis indicated that the predicted *GbWRKY31* protein contains WRKY-motif and Zinc-finger motif. *GbWRKY31* protein was 43-44% similarity to Group IIb WRKY proteins including *EgWRKY31* of *Eucalyptus grandis*, *PeWRKY31* of *Populus euphratica*, *PkWRKY* of *Picrorhiza kurrooa*, *MaWRKY31* of *Musa acuminata*, *CsWRKY31* of *Cucumis sativus*, *VrWRKY31* of *Vigna radiata*, *GmWRKY23* of *Glycine max* and *GsWRKY6* of *Glycine soja*.

Table 1. Nucleotide sequence of *GbWRKY31* similarity to the WRKY genes of other plant species.

Species	GenBank no.	Identity	E-value
Ziziphus jujuba	XM_016039435	97%	5e-04
Gossypium hirsutum	KF669851	93%	1e-05
Gossypium hirsutum	KF031106.1	93%	1e-05
Eucalyptus grandis	XM_010030989.1	88%	5e-04
Brassica rapa	NM_001301983.1	81%	1e-04
Tarenaya hassleriana	XM_010531148.1	76%	2e-12
Arabidopsis lyrata	XM_002872646.1	76%	9e-12
Solanum tuberosum	KF280263.1	74%	1e-15

Note: The nucleotide sequence of *GbWRKY31* was 97%, 93%, 93%, 88%, 81% similarity to *ZjWRKY72* in *Ziziphus jujuba*, *GhWRKY90* in *Gossypium hirsutum*, *GhWRKY80* in *Gossypium hirsutum*, *EgWRKY72* in *Eucalyptus grandis*, *BrWRKY47* in *Brassica rapa*, respectively.

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1       CTCAAGGTTATTTCCACACAGAAGAAAAAAAAAAAGATAGACAGACTTCTAGATGTACAA
61      ATATCATTTGTAGAATCGAAGAACCAAAAACGCATTAATGGACTCAAGAAGGTTTGATGA
21                                           M  D  S  R  R  F  D  D
121     TGGAAGGGGTATTGGCCTGGATCTGTCTATGAAGATTTCACAGGCAGATTGTACACCAGT
41       G  R  G  I  G  L  D  L  S  M  K  I  S  Q  A  D  C  T  P  V
181     GGCCAGTATTTTTCCTGTTAAGGTTTCCAATGCGGATAAGACGAACCAGATGACAGAGCT
61       A  S  I  F  P  V  K  V  S  N  A  D  K  T  N  Q  M  T  E  L
241     GGAAGCTGAGTTGAAACGTATGAATGCTGAGAACCAGAAGCTAAACTTCATGCTTAAGGA
81       E  A  E  L  K  R  M  N  A  E  N  Q  K  L  N  F  M  L  K  D
301     CATGACCAAGAACTATAATTCTCTGCAAATGCATCTTATGTCTCTGATTCAGAAAAGACA
101      M  T  K  N  Y  N  S  L  Q  M  H  L  M  S  L  I  Q  K  R  Q
361     ATTAGCACACGGTGTTGCCCAAAATCAGGTTTCAGATCGAGTTGAAGAGAATATGAGATT
121      L  A  H  G  V  A  Q  N  Q  V  S  D  R  V  E  E  N  M  R  L
421     GCCTGCAAGCAAGGATGGAAGGCAATTTATTGGTGCTTTGTCTCTGTCGAGTTTGGAGTC
141      P  A  S  K  D  G  R  Q  F  I  G  A  L  S  L  S  S  L  E  S
481     TGAACCTGCGGTTTCTCAACAGTCTCAGTCTTCAGAAGGCCATGGCCTTCGGGAAGACCG
161      E  P  A  V  S  Q  Q  S  Q  S  S  E  G  H  G  L  R  E  D  R
541     TGTGAAGGATTCCAAGTTTCTGGACAATCAACTGCCCTCTAAGAAGAGGAAGATAAATAA
181      V  K  D  S  K  F  L  D  N  Q  L  P  S  K  K  R  K  I  N  K
601     GTTGTATCAGACGGAGAATGCCATTTCTTCTAAAGATGACAGCCATCATGATGACGAGCA
201      L  Y  Q  T  E  N  A  I  S  S  K  D  D  S  H  H  D  D  E  H
661     TCAGAGCAAAGGAAGGCATCAGATAGGGCACAAGGAGTCTGATCAGACAGAGGCAACCAA
221      Q  S  K  G  R  H  Q  I  G  H  K  E  S  D  Q  T  E  A  T  K
721     ATTACGAAAGCTATCCCGGGTATCAGTGCGAACAAGATCAGATTCATCCATGATATACGA
241      L  R  K  L  S  R  V  S  V  R  T  R  S  D  S  S  M  I  Y  D
781     TGGATGTCAATGGAGAAAATACGGGCAGAAGATGACGAGAGACAATTCGAATCCGAGGGC
261      G  C  Q  W  R  K  Y  G  Q  K  M  T  R  D  N  S  N  P  R  A
841     TTATTACAGATGCGGCATGGCACCTGGCTGCCCAGTTAGAAAGCAGGTGCAAAGGTGTGC
281      Y  Y  R  C  G  M  A  P  G  C  P  V  R  K  Q  V  Q  R  C  A
901     CGAGGACAAATCGATTCTGACAACAACATATGAAGGCGAACACAATCACATTCTGAATCC
301      E  D  K  S  I  L  T  T  T  Y  E  G  E  H  N  H  I  L  N  P
961     TGTGGCAATGGCCATGGCAACAACAACCTCTGCAGCGGCTCGTATGTCCCTGTCGAATTC
321      V  A  M  A  M  A  T  T  T  S  A  A  A  R  M  S  L  S  N  S
1021    TACAACGAGCGAAGGAATGAATGAATTTGGCTTAAGGGCAGCAGGCACCCAAATGGCAGG
341      T  T  S  E  G  M  N  E  F  G  L  R  A  A  G  T  Q  M  A  G
1081    AACCCAAATGCCCTTTTTCCCAGGGTTGCCCACACTCTCGGCCTTCTCTACATTTCCCGC
361      T  Q  M  P  F  F  P  G  L  P  T  L  S  A  F  S  T  F  P  A
1141    AGTCACACTTGACCTCACCAACGGCCCCATTAACCCAGTACAGGGCTTAATGAATCCGGT
381      V  T  L  D  L  T  N  G  P  I  N  P  V  Q  G  L  M  N  P  V
1201    TTCCGGTAACATGCAGATGCAGGGCCAAGCACCGTTCTGCAACAACCAAATTGATGTGCA
401      S  G  N  M  Q  M  Q  G  Q  A  P  F  C  N  N  Q  I  D  V  Q
1261    GTCTGGATCAATGATGGGCGGTTTAAGGGTGGATCCCAACTTCACTGCGGCACTGGCAGC
421      S  G  S  M  M  G  G  L  R  V  D  P  N  F  T  A  A  L  A  A
1321    TGCCATTGCAAACTCCATGCTAACCGCGGGATCTCAGCCTGCAATGCAGTCAAAGAAGTA
441      A  I  A  N  S  M  L  T  A  G  S  Q  P  A  M  Q  S  K  K  *
1381    ATTATGATGCCTTGTCTTATGAATGCCATTGTAATTGTAACGGTGTCCTCTGCTTCAATA
1441    ATGCAGTTGGGCGATCCCGTAAGCATTACGGAAGCAAATGTTGTGTTATACAAGTCTTGT
1501    ATTGAATACCATACTTAGAGATATCCTCTTGGTCGCATTTATGCAACCGGATAAAGCTAC
1561    TGATGGAACTCGTCGACGTTTTTGGCCTTCCGGTCAGGAATTGGAAAATCAAATTGATTG
1621    TTAGGGAGCGAAGGGCCTCATGCCATCTCAAATGGAGAGTGGCAAGTCATTTCACATTAA
1681    ATTGTATATGCTCCTCCATTCATGCCCATGTATTCATAAATCGAATAAGATTTCACAA
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Fig. 1: Nucleotide sequence and deduced amino acid sequence of *GbWRKY31*.

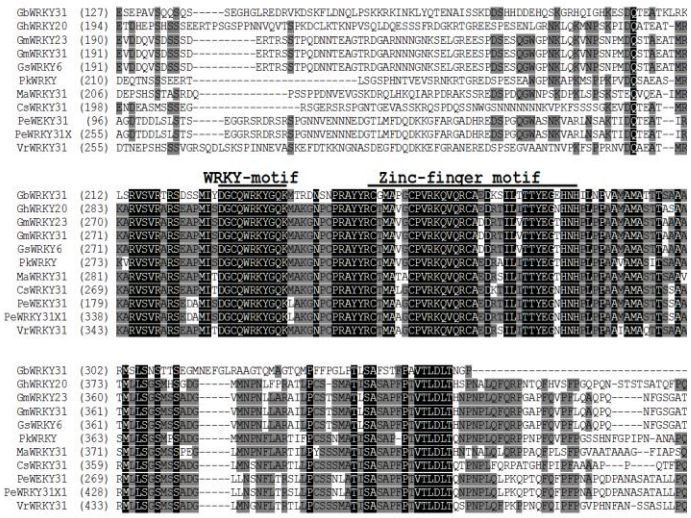

Fig. 2: Sequence multi-alignment of the deduced *GbWRKY31* protein with other WRKY proteins. The accession numbers of WRKY proteins and translation of their names are shown as follows, *GbWRKY31*: *Ginkgo biloba*; *GhWRKY20*: *Gossypium hirsutum* AJT43303.1; *GmWRKY23*: *Glycine max* ABS18425.1; *GmWRKY31*: *Glycin emax* ABS18431.1; *GsWRKY6*: *Glycine soja* KHN40870.1; *PkWRKY*: *Picrorhiza kurrooa* ACI90292.1; *MaWRKY31*: *Musa acuminata* XP_009388735.1; *CsWRKY31*: *Cucumis sativus* XP_011658698.1; *PeWRKY31*: *Populus euphratica* XP_011033773.1; *PeWRKY31x1*: *Populus euphratica* XP_011047237.1; *VrWRKY31*: *Vigna radiate* XP_014498648.1. The completely identical amino acids are indicated with white foreground and black background. Shaded in gray are conservative sequences. Conserved motifs WRKY-motif and Zinc-finger motif are marked.

Phylogenetic tree analysis of *GbWRKY31*

The phylogenetic tree showing the evolutionary relationships among *GbWRKY31* and other WRKY proteins was constructed with 17 WRKY proteins (Fig. 3). The evolutionary tree was divided into seven distinct categories. It is clearly to know kinship of the 17 species

from (Fig. 3). The result indicated that *GbWRKY31* was closely related to *AtWRKY6* of *Arabidopsis thaliana* and *TwWRKY* of *Taxus wallichiana*. From the above, *GbWRKY31* belongs to the group IIb of the WRKY families.

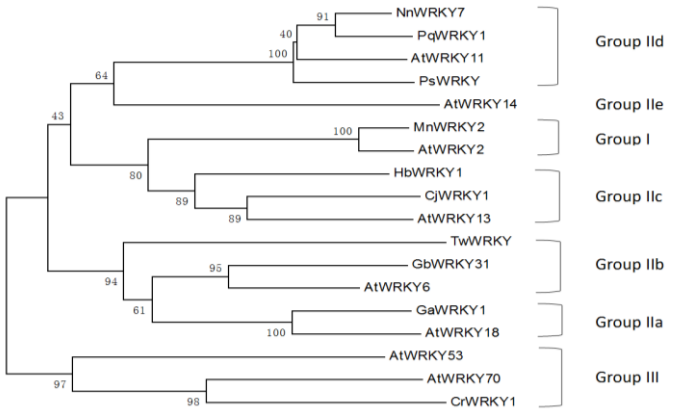

Fig. 3: Phylogenetic tree of the sequences of *GbWRKY31* and other plants WRKY protein. Bootstrap value are expressed in percentages and placed at the nodes in the tree. The GenBank accession numbers of the WRKY proteins and translation of their names are shown, *GbWRKY31*: *Ginkgo biloba*; *CjWRKY1*: *Coptis japonica* BAF41990.1; *AtWRKY13*: *Arabidopsis thaliana* AEE87071.1; *HbWRKY1*: *Hevea brasiliensis* ADF45433.1; *MnWRKY2*: *Morus notabilis* XP_010092241.1; *AtWRKY2*: *Arabidopsis thaliana* AED96743.1; *TwWRKY*: *Taxus wallichiana* AEW91476.1; *AtWRKY6*: *Arabidopsis thaliana* AEE33948.1; *GaWRKY1*: *Gossypium arboreum* AAR98818.1; *AtWRKY18*: *Arabidopsis thaliana* AEE85961.1; *AtWRKY14*: *Arabidopsis thaliana* AEE31256.1; *PsWRKY*: *Picea sitchensis* ADE77495.1; *AtWRKY11*: *Arabidopsis thaliana* AEE85928.1; *NnWRKY7*: *Nelumbo nucifera* XP_010270802.1; *PqWRKY1*: *Panax quinquefolius* AEQ29014.1; *AtWRKY53*: *Arabidopsis thaliana* AEE84809.1; *AtWRKY70*: *Arabidopsis thaliana* AEE79517.1; *CrWRKY1*: *Catharanthus roseus* ADT82685.1.

Discussion

The *GbWRKY31* gene was isolated from ginkgo in this study. The multiple sequence alignment by using bioinformatics analysis software indicated that *GbWRKY31* had high identity with other WRKY genes cloned from other plants. The homologous sequence of WRKY gene among different plants implied that the WRKY gene belongs to the group IIb of the WRKY families and keep a strong conservation during the molecular evolution. The conserved domain motif function further indicated *GbWRKY31* might involve in immune response in plants and might be the key of disease-resistant defense reaction (Peng et al., 2008; Ramiro et al., 2010).

WRKY transcription factors are involved in biological stress and non-biological stress, growth processes in plant. The functional diversity within WRKY genes determined the plant defense responses and regulation of vegetative growth. There was a research results show that *AtWRKY6*, also belonging to the WRKY IIb group, positively influenced the senescence and pathogen defense-associated PR1 promoter activity, most likely involving NPR1 function (Robatzek and Somssich, 2002). In this paper, the over expression of ginkgo confirmed that WRKY genes has a certain relationship with the regulation mechanism of disease-resistant defense reaction of PR1 promoter.

Conclusion

GbWRKY31 with an ORF about 1281bp, encoding 427 amino acids was isolated by RT-PCR from *Ginkgo biloba*. The amino acids of *GbWRKY31* contain a conserved WRKY-motif and a Zinc-finger motif displays extensive homology to WRKY transcription factors from other plants that will enable us to conclude the disease-resistant defense reaction of *G. biloba*. The phylogenetic tree analysis demonstrated the *GbWRKY31* protein belongs to the WRKY IIb group. The *GbWRKY31* was likely to participate in regulating thePR1 promoter activity of disease-resistant defense reaction in ginkgo.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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