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Research Article

Cloning and Sequence Analysis of an *Anthocyanidin reductase (ANR)* Gene from *Ginkgo biloba*

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ABSTRACT

Ginkgo biloba leaves mainly contain these two physiological active ingredients: flavonoids and terpene lactones. Flavonoids have an important role in regulating plant growth and development. Anthocyanidin reductase is a key enzyme involved in the flavonoids biosynthesis. Based on the EST sequence, the 1597 bp length cDNA sequence of anthocyanidin reductase gene (designated as *GbANR*) was isolated from *G. biloba* leaves by PCR technology. The open reading frame (ORF) of GbANR was 1023 bp, encoding 340 amino acids, and the molecular weight was 37.38 kDa, with the isoelectric point of 5.87. Bioinformatics analysis showed that the deduced GbANR shared more than 50% identity with ANR proteins from other plants. Phylogenetic tree analysis revealed that the GbANR was closely related to PsANR, and clustered into a single group, suggesting GbANR and ANR from other gymnosperm plants may be the same ancestor. These results indicated that GbANR belongs to ANR family and serve as a basis for functional analysis of the *ANR* gene.

Keyword: *G. biloba*; anthocyanidin reductase; flavonoids; sequence analysis

INTRODUCTION

Ginkgo biloba has existed since the late Triassic period, more than 200 million years ago. *G. biloba* is the only surviving member of the Ginkgoaceae family, and is often called "a living fossil" [1, 2]. *G. biloba* tree has strong adaptability, long life cycle, fan-shaped leaves and graceful tree performance, with a high ecological and ornamental value. Meanwhile, *G. biloba* also has some economic and scientific value. *G. biloba* leaves mainly contain these two physiological active compounds: flavonoids and terpene lactone [3]. In plant, flavonoids are a large group of polyphenolic secondary metabolites, and are associated with an array of functions, including protect against UV light damage [4], regulation of auxin transport, defense against pathogens and insects [5], modulation of flowers and fruits color [6], and environmental stress responses [7]. Flavonoid biosynthesis takes place through the phenylpropanoid pathway, and depending on the genetic constitution of the plant naringenin can have several different fates leading to the formation of flavonoid metabolites that include anthocyanins, flavones and anthocyanidins [8, 9]. Anthocyanidin reductase (ANR) is one of the key enzymes in the biosynthesis of proanthocyanidins and is responsible for catechin biosynthesis in plants [9]. Anthocyanidin reductase has been isolated in

several plant species, such as *Vitis bellula* [10], *Populus trichocarpa* [11], *Fagopyrum esculentum* [12], *Gossypium hirsutum* [13], *Rosa rugosa* [14], *Medicago truncatula* and *Arabidopsis thaliana* [15]. In *G. biloba*, a putative anthocyanidin reductase gene was isolated and characterised, but the function is still not clear [16]. For a better understanding of the role of ANR in flavonoids biosynthesis in *G. biloba*, we describe the isolation and sequence analysis of the *ANR* gene from *G. biloba* in the study; our results will be an important foundation for further understanding function of ANR in the regulation of flavonoids biosynthesis in *G. biloba*.

MATERIALS AND METHODS

Plant material

In this study, the *G. biloba* used for cloning of the *GbANR* gene was 18-year-old, growing in the Botanical Garden of Yangtze University, in China. The fresh leaves were gathered, immediately frozen in liquid nitrogen and stored at -80°C prior to RNA extraction.

cDNA Sequence amplification of *GbANR*

G. biloba total RNA was extracted from fresh leaves using the MiniBEST Plant RNA extraction kit (Dalian TaKaRa, China). The cDNA was used as the template to amplify GbANR fragment was reverse transcribed by using the PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). The obtained first-strand cDNA was used as the template to amplify *GbANR* fragment. Based on transcriptome sequence of *G. biloba*, a pair of specific primers *GbANRS* (5′-CAGGTACGCACAAGGTGTT-3′) and *GbANRA* (5′-ATACTATATTACTAAAATTCTTGGGTC-3′) were designed, and synthesised by Shanghai Sangon Biotechnology Company (In China). PCR reaction was performed with the following conditions: denaturation at 94°C for 4 min; 32 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for the 90s; and a final extension at 72°C for 10 min. The amplified product was ligated into a pMD18-T vector (Dalian TaKaRa, China) after purified according to the manufacturer's instructions. The cloning vectors were transformed into *E. coli* TOP10 competent cells; positive clones were selected and identified by PCR with M13 universal primers. The selected clones were sequenced by Shanghai Sangon Biotechnology Company.

Bioinformatics analysis

To analyse cDNA sequence and protein of *ANS* gene, bioinformatics tools Vector NTI Suite V 11.5 and ORF Finder (NCBI, https://www.ncbi.nlm.nih.gov/orffinder/) was used to analyse the nucleic acid structure, DNAMAN 8 was used to deduce the amino acid sequence of *GbANR* gene. Molecular mass and theoretical isoelectric point of GbANR protein were predicted with compute pI/Mw tool (http://web.expasy.org/compute_pi/). The secondary structure of GbANR protein was analysed by SOPMA tool. Sequence alignment of ANR proteins from different plants was completed with Blastp (http://www.ncbi.nlm.nih.gov/BLAST/) and Align X (Vector NTI). Phylogenetic tree of anthocyanidin reductase proteins was constructed with software Clustal X 2.0 and MEGA 6.0 using the neighbor-joining (NJ) method [17].

RESULTS

cDNA sequence of *GbANR*

With one pair specificity primer, a 1597 bp length cDNA fragment of *ANR* gene (designated as *GbANR*) was amplified by PCR from the cDNA of *G. biloba*. The *GbANR* open reading frame from ATG codon to TGA stop codon was 1023 bp long encoding 340 amino acids. The 5'-untranslated region and 3'-untranslated region of *GbANR* cDNA were 181 bp and 393 bp, respectively (Fig. 1).

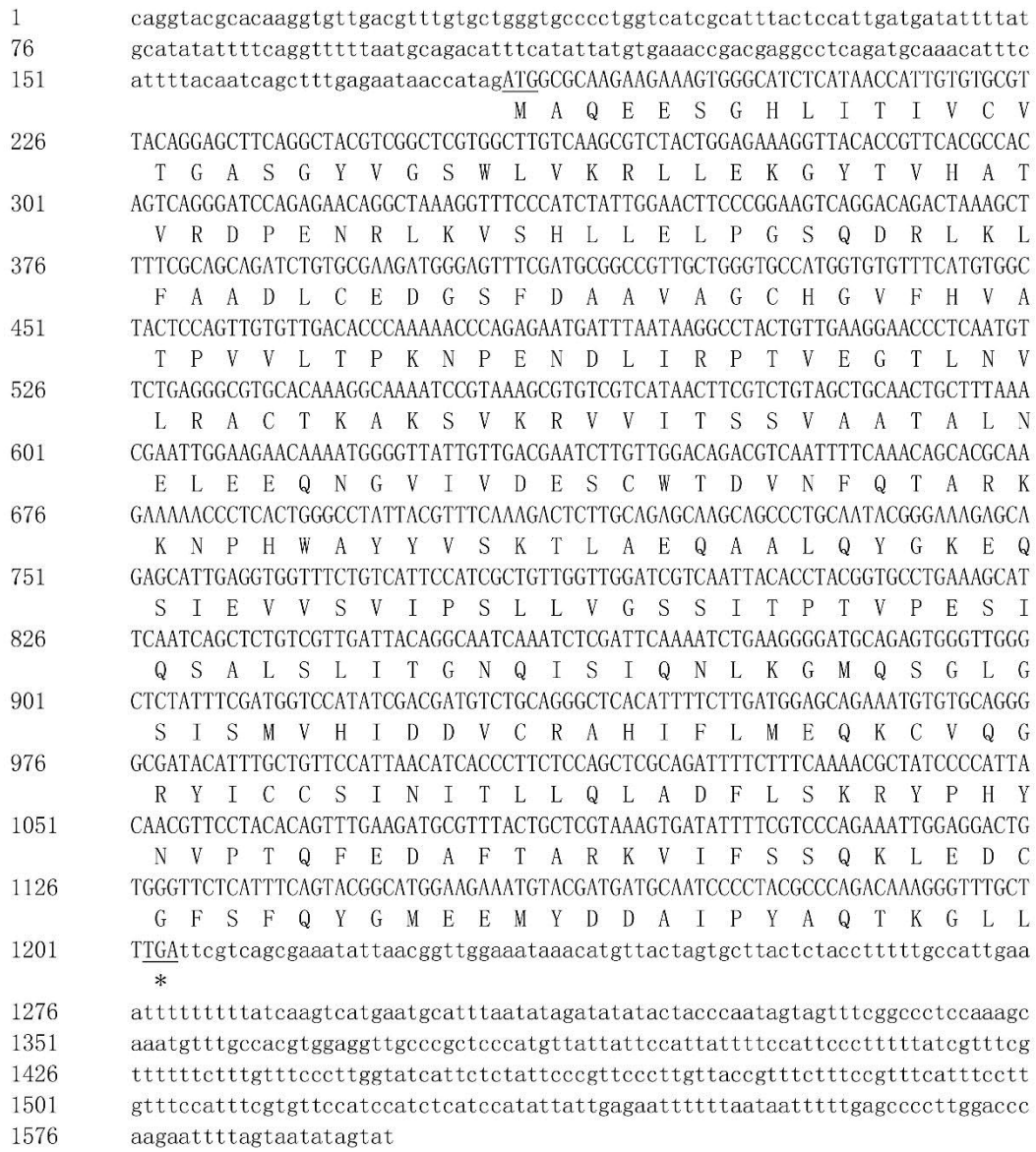

Fig. 1: The nucleotide acid sequence and deduced the amino acid sequence of GbANR: *The start codon (ATG) and the stop codon (TGA) are underlined, non-coding regions are indicated in lowercase.*

Characterization of the predicted GbANR protein

The length of putative GbANR protein sequence was 340 amino acids. The calculated molecular mass and theoretical isoelectric point (pI) of GbANR was 37.38 kDa and 5.87, respectively. Secondary structure prediction discovered that alpha helix, extended strand, beta turn and random coil in the secondary structure were predicted to be 41.18%, 20.59%, 11.47% and 26.76% respectively.

Sequence comparison via Blastp (NCBI) search and Align X (Vector NTI) showed that GbANR had a certain homology with other ANR proteins (Fig.2), the amino acid sequences alignment showed that GbANR had some identity with *Picea sitchensis* (66% identity, ABR18365.1); *Nelumbo nucifera* (56% identit, XP_010241022.1); *Vitis bellula* (55% identity, AFG28175.1); *Vitis vinifera* (54% identity, NP_001267885.1); *Camellia sinensis* (54% identity, ADF43751.1); *Populus euphratica* (54% identity, XP_011003389.1); *Camellia sinensis* (53% identity, AAT68773.1); *Gossypium hirsutum* (54% identity, ABM64802.1); *Prunus cerasifera* (55% identity, AKV89239.1); *Gossypium arboretum* (54% identity, NP_001316937.1); *Theobroma cacao* (53%

identity, ADD51353.1); *Rubus idaeus* (54% identity, AMP19723.1). Conserved protein domain NADB_Rossmann that distributed over numerous dehydrogenases of metabolic pathways was found in the GbANR protein, FR_SDR_e (flavonoid reductase FR, extended SDRs), Epimerase, NAD_binding_4 and other domains also exist. These results indicated that cloned GbANR was a member of the ANR family.

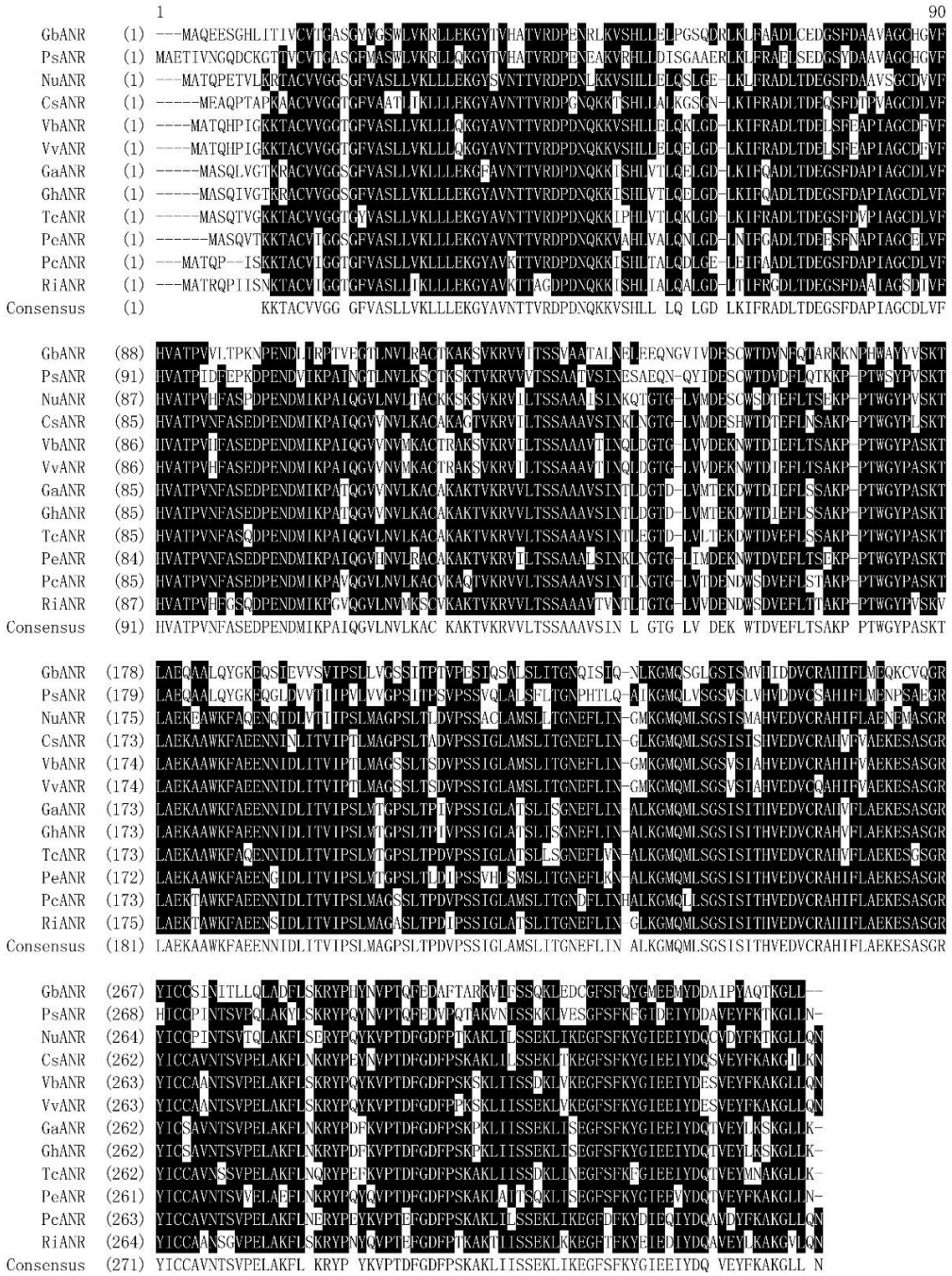

Fig.2: Sequence multi-alignment of the ANR proteins from different plants

The completely identical and conserved amino acids are indicated with a white foreground and black background. Non-similar amino acids are indicated with black foreground and white background.
The accession numbers of ANR proteins and their names are shown as follows. PsANR, Picea sitchensis (ABR18365.1); NuANR, Nelumbo nucifera (XP_010241022.1); VbANR, Vitis bellula (AFG28175.1); VvANR, Vitis vinifera (NP_001267885.1); CsANR, Camellia sinensis (ADF43751.1); PeANR, Populus euphratica (XP_011003389.1); CsANR, Camellia sinensis (AAT68773.1); GhANR, Gossypium hirsutum (ABM64802.1); PcANR, Prunus cerasifera (AKV89239.1); GaANR, Gossypium arboretum (NP_001316937.1); TcANR, Theobroma cacao (ADD51353.1); RiANR, Rubus idaeus (AMP19723.1)

Phylogenetic Analysis of ANR proteins

To investigate the evolutionary relationships of ANR proteins among the various species, a phylogenetic tree was constructed using the neighbor-joining method. As shown in Figure 3, it was clearly observed that ANR proteins from different plants were clustered into two groups, gymnosperm and angiosperm. The putative *G. biloba* ANR protein together with *Picea sitchensis* ANR was clustered into the branch of gymnosperm plants, indicated that GbANR shared a common evolutionary origin with the gymnosperm species ANR proteins. Phylogenetic analysis of ANR also showed that ANR proteins from closely related species clustered into the same subclades. For example, *Gossypium arboretum* and *Gossypium hirsutum* are Malvaceae plants, the ANR proteins GaANR and GhANR shared the same subclade; ANR proteins from *Prunus cerasifera* and *Rubus idaeus* that belongs to Rosaceae were classified into the same subclades. The phylogenetic analysis indicated that ANR proteins shared a common evolutionary origin, and GbANR may have a similar functionality with ANR proteins from other plant species.

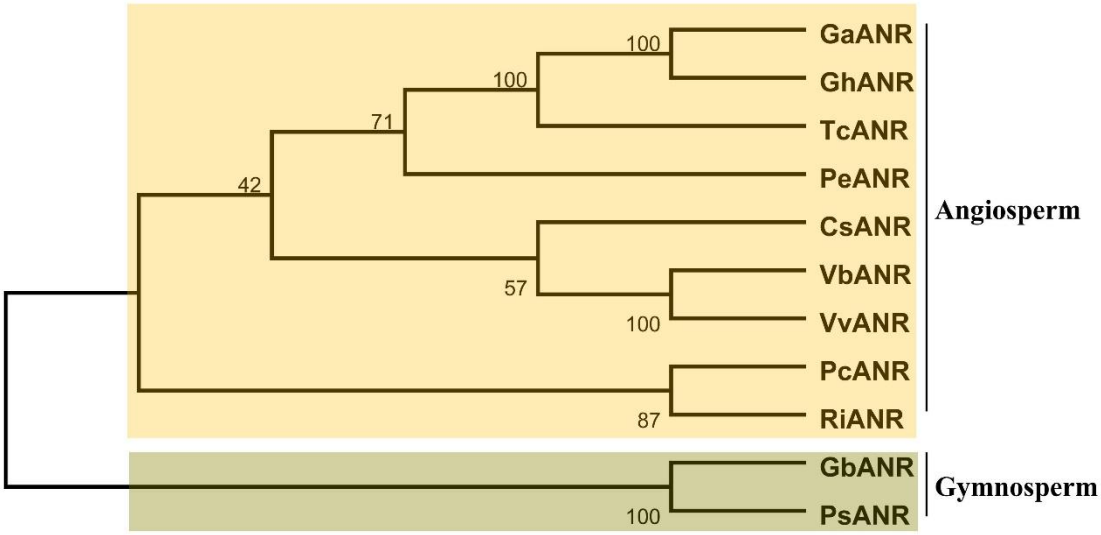

Fig. 3: Phylogenetic tree analysis of anthocyanidin reductase in different species

The numbers at each node represent the bootstrap values (with 1,000 replicates). The accession numbers of ANR proteins and translation of their names are shown as in Figure 2.

DISCUSSION

Anthocyanidin reductase (ANR, EC1.3.1.77) is an NADPH- and/or NADH-dependent enzyme [15, 18]. This reductase has been demonstrated to catalyse two hydrides transferring from two molecules of NADPH or NADH to anthocyanidins to form the corresponding flavan-3-ols [10, 18-20]. Anthocyanidin reductase was involved in the proanthocyanidins or catechin biosynthesis. Zhu *et al*. [13] found that the anthocyanidin reductase (ANR) pathway is involved in the biosynthesis of proanthocyanidins in upland cotton. In *Fragaria* × *ananassa*, experimental down-regulation of ANR, epicatechin was consequently down-regulated as a chain extension unit in the polymeric proanthocyanidins [21]. Similar results have been found in *Camellia sinensis*, variation in accumulation pattern of catechins and its fractions was found to be correlated with the expression pattern of ANR [9]. In the leaves of *V. bellula*, VbANR forms the ANR pathway, leading to the formation of three types of isomeric flavan-3-ols and Pas [10]. However, overexpression of *PtrANR1* in *poplar* resulted in a significant increase in PA levels but no impact on catechin levels. Ectopic expression of *PtrLAR1* in poplar positively regulated the biosynthesis of PAs, whereas the accumulation of anthocyanin and flavonol was significantly reduced in all transgenic plants compared to the control plants [11]. A putative anthocyanidin

reductase gene has been isolated from *G. biloba*, but functional analysis of *ANR* gene in *G. biloba* has not been published in the literature.

In the present study, an *ANR* gene was isolated from *G. biloba* leaves by PCR method. Sequence analysis indicated that the cDNA of *GbANR* was 1597 bp containing a 1023 bp open reading frame (ORF) encoding 340 amino acids. Homology analysis showed that the deduced GbANR had a certain homology with ANR proteins from other plants, and they all have conserved NADB_Rossmann domain. Phylogenetic tree analysis suggested that the GbANR was closely related to anthocyanidin reductase from other gymnosperms plants. In general, the anthocyanidin reductase cDNA sequence structure and the encoding amino acid sequence in different species had certain levels of similarity.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interests.

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