De novo Transcriptome Analysis to Identify Anthocyanin Biosynthesis Genes Responsible for Tissue-Specific Pigmentation in Zoysiagrass (*Zoysia japonica* Steud.)

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ABSTRACT

Zoysiagrass (*Zoysia japonica* Steud.) is commonly found in temperate regions and widely used for lawns, in part, owing to its uniform green color. However, some zoysiagrass cultivars accumulate red to purple pigments in their spike and stolon tissues, thereby decreasing the aesthetic value. The anthocyanin contents were analyzed in two zoysiagrass cultivars ‘Anyang-jungji’ (AJ) and ‘Greenzoa’ (GZ) that produce spikes and stolons with purple and green colors, respectively, and cyanidin and petunidin were found to be primarily accumulated in the pigmented tissues. In parallel, a *de novo* transcriptome assembly was performed to identify differentially expressed genes between the two cultivars. Two anthocyanin biosynthesis genes encoding anthocyanidin synthase (ANS) and

dihydroflavonol 4-reductase (DFR) were preferentially upregulated in the purple AJ spike upon pigmentation. Both *ANS* and *DFR* genes were also highly expressed in other zoysiagrass cultivars with purple spikes and stolons, but their expression levels were significantly low in the cultivars with green tissues. Recombinant ZjDFR1 and ZjANS1 proteins were observed to successfully catalyze the conversions of dihydroflavonols into leucoanthocyanidins and leucoanthocyanidins into anthocyanidins, respectively. These findings suggest that upregulation of *ANS* and *DFR* is responsible for tissue-specific anthocyanin biosynthesis and differential pigmentation in zoysiagrass. The zoysiagrass *DFR* and *ANS* genes were also shown to complement Arabidopsis *tt3* and *tt18* mutants, restoring pigment accumulation ability in the plant stem.

Keywords: anthocyanidin synthase (ANS), anthocyanin biosynthesis, *de novo* assembly, dihydroflavonol 4-reductase (DFR), transcriptome, *Zoysia japonica*

Student number: 2009-30969

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LIST OF ABBRIVIATIONS

ABRE	Abscisic acid responsive element
AJ	*Zoysia japonica* ‘Anyang-jungji’
ANS	Anthocyanin synthase
AP2	APETALA2
BLAST	Basic local alignment search tool
BS-Seq	Bisulfite sequencing
CDS	Coding DNA sequence
CHI	Chalcone isomerase
CHS	Chalcone synthase
COP	CONSTITUTIVE PHOTOMORPHOGENIC1
DEG	Differentially expressed gene
DFR	Dihydroflavonol 4-reductase
DHK	Dihydrokaempferol
DHM	Dihydromyricetin
DHQ	Dihydroquercetin
DMR	Differentially methylated region
F3H	Flavonone 3-hydroxylase
F3’H	Flavonone 3’-hydroxylase
F3’5’H	Flavonone 3’5’-hydroxylase
FLS	Flavonol synthase
GO	Gene ontology
GZ	*Zoysia japonica* ‘Greenzoa’

HPLC	High-performance liquid chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside
LC-MS	Liquid chromatography-mass spectrometry
MBS	Myb binding site
MYB	Myeloblastosis
NGS	Next generation sequencing
NR	Non-redundant
ORF	Open reading frame
PAL	Phenylalanine ammonia-lyase
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
RACE	Rapid amplification of cDNA ends
TAIL-PCR	Thermal asymmetric interlaced polymerase chain reaction
UFGT	Uridine diphosphate-glucose flavonoid glucosyl transferase

LITERATURE REVIEW

Zoysia japonica

Zoysiagrass (*Zoysia* spp.) is a perennial creeping grass that belongs to Chloridoideae including a subfamily of genus *Zoysia*, which consists of 11 species commonly found from tropical regions of South-east Asia to temperate regions in North-east Asia (Engelke and Anderson, 2003). Among the 11 recognized species, three species *Z. japonica*, *Z. matrella*, and *Z. pacifica* have been used as turfgrass. Natural and man-made hybrids of these three turfgrass species are also used as turf throughout the world (Engelke and Anderson, 2003). Especially, *Z. japonica*, *Z. matrella*, and *Z. tenuifolia* for main creeping grasses, and *Z. sinica* and *Z. macrostachya* along the seashore are main turfgrasses of *Zoysia* genus that grow naturally in Korea (Hyun et al., 2012). The genome size of zoysiagrass estimated by flow cytometry was 421 Mbp with 2n = 4x = 40 of chromosome composition (Arumuganathan et al., 1999; Forbes, 1952). Zoysiagrass is commonly used in golf courses, sports ground, and parks in Korea because of its dense growth habit and tolerance to low mowing heights, cold, shade, salinity, and drought with little insect and disease problems (Christians, 1998). Pistils of zoysiagrass emerge and mature 7-10 days prior to the emergence of stamens (Tsuruta et al., 2011), thus outcrossing is needed for sexual reproduction.

Green appearance is one of the most commercial values of zoysiagrass. Most aerial parts of zoysiagrass are kept green under temperate climate, though creeping stolons and regenerative spike tissues appear purple colors, which could be a nuisance to scenery value of green lawns. Therefore, development of new cultivars with less purple coloration in such tissues is one of crucial goals for breeders. Cultivars Zenith and Millock are good examples with green colored spikes and stolons (Choi et al., 2006), while cultivars Meyer and Senock still display purple coloration in those tissues (Choi et al., 2004; Emmons, 1995; Sleper et al., 1989).

Current biological studies on this plant have been mainly conducted at the physiological level, including photosynthesis, stress responses (Fry et al., 1986), physiology (Bae et al., 2010), and growth regulation (Lee et al., 2010). In contrast, a few genetic studies have been done with rough molecular linkage maps constructed from interspecific hybrids of *Z. japonica* and *Z. matrella* (Ebina et al., 1998; Yaneshita et al., 1999), an AFLP-based linkage map for *Z. japonica* (Cai et al., 2004), and SSR markers in several *Zoysia* species (Cai et al., 2005; Li et al., 2009; Tsuruta et al., 2005).

The absence of a sequenced genome hampers many valuable experiments in *Z. japonica* such as gene expression studies and comparative genomics with other monocot species, and genetic manipulation to determine gene functions.

Anthocyanin Biosynthesis

Flavonoids represent a large class of secondary plant metabolites, of which anthocyanins are the most conspicuous class due to the wide range of colors resulting from their diverse structures and conjugates (Holton and Cornish, 1995). Anthocyanins play diverse functions such as attracting pollinators, aiding in seed dispersal, and protecting plants from UV irradiation damage. Diverse functions and many different types of anthocyanins facilitate to study the synthesis and regulation of these compounds. Anthocyanins, a group of phenolic compounds widely found in the plant kingdom, are responsible for the orange, red, purple and blue colors in nature (Mazza and Miniati, 1993). Anthocyanins are water-soluble pigments that occur in almost all vascular plants and a number of studies describe their chemistry, distribution, and biosynthesis (Grotewold, 2006).

After Mendel's work on flower color of peas, genetic studies on anthocyanin biosynthesis of several plant species have flourished during the past century. Following by structural analysis of flavonoids include anthocyanins, correlation of structural alteration of single genes related to specific anthocyanins or with the presence or absence of particular flavonoids were studied (Holton and Cornish, 1995). The chemistry and genetics of anthocyanin pigmentation have been elucidated mainly utilizing undifferentiated cells derived from *Petroselinum hortense* (parsley) (Hahlbrock et al., 1981) and pigmented tissues of *Zea mays* (maize), *Antirrhinum majus* (snapdragon), and *Petunia hybrid* (petunia) (Dooner et al., 1991; Holton and Cornish,

1995; Martin and Gerats, 1993). Many of flavonoid biosynthesis regulatory genes in these plant species were cloned and characterized (Mol et al., 1996). Transcriptional controls play an important role in regulating the overall activity of flavonoid biosynthesis. The pathway is also controlled in response to different developmental and environmental cues (Jaakola et al., 2002).

Anthocyanin biosynthesis pathway is largely shared among flowering plants (Forkmann, 1991; Mol et al., 1989). Regulation of anthocyanin biosynthesis has been studied in several flowering plants using anthocyanin deficient mutants (Park et al., 2011; Yang et al., 2013). There are two parts of the pathway that lead to the anthocyanin production. First, phenylalanine is converted to 4-coumaroyl-CoA through the general phenylpropanoid pathway, and the product is required for the production of lignins, coumarins, and stilbenes. Anthocyanin biosynthesis pathway is initiated by chalcone synthase, which synthesizes chalcone from a *p*-coumaroyl-CoA and three malonyl-CoAs, and chalcone is subsequently converted to flavanone by chalcone isomerase. Flavanones are hydroxylated by flavanone-3β-hydroxylase to make dihydroflavonols, which are further converted by flavonol synthase to flavonols or reduced by dihydroflavonol reductase to leucoanthocyanidins, crucial intermediates for anthocyanin synthesis. The B-ring hydroxylation at 3’ and 5’ positions proceeds by flavonoid hydroxylases. Leucoanthocyanidins are converted to anthocyanidins by anthocyanidin synthase and glycosylated via UDP-Glc: flavonoid-3-O-glycosyltransferases. Peonidin, malvidin, and petunidin are produced

by methylation of 3’ and 5’ hydroxyl groups of anthocyanin. Additionally, upstream genes related to the anthocyanin synthesis have been speculated to evolve slowly than downstream genes in both monocots and dicots (Lu and Rausher, 2003).

Next Generation Sequencing (NGS)-based RNA-Seq

Whole genome sequencing demands a long-term expensive investment. Instead, it is relatively easy to obtain useful information from the transcriptome (Crowhurst et al., 2008; Newcomb et al., 2006). Recent RNA-Sequencing based NGS allows bioinformatics studies on species without a reference; *Myrica rubra* (bayberry), *Cicer arietinum* (chickpea), and *Hevea brasiliensis* (Pará rubber tree) (Feng et al., 2012; Garg et al., 2011; Xia et al., 2011). Short read sequencing technologies such as the Solexa/Illumina (Illumina), 454 (Roche) and SOLiD (ABI) platforms have made it increasingly feasible to perform *de novo* transcriptome sequencing (Hudson, 2008; Wang et al., 2009). It has become widely applied to model and non-model organisms to obtain mass sequence data for molecular marker development, gene discovery, and transcriptional analysis (Brautigam et al., 2011; Dubey et al., 2011; Hahn et al., 2009; Parchman et al., 2010; Shi et al., 2011; Wang et al., 2010; Wu et al., 2010; Xiang et al., 2010). Compared with traditional laboratory methods, RNA-Seq is a high throughput technology, overcoming the weakness of microarrays in exploring unknown genes. Transcriptomes represent a fraction of the total genome in size, contain fewer repetitive elements, and by selecting specific tissues they can be

enriched for genes relevant to the particular aims of the research. In addition, if the RNA sample is not normalized the relative abundance of different reads has been shown to accurately reflect the expression level of transcripts in the tissue (Oshlack et al., 2010).

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CHAPTER I

De novo Transcriptome Assembly and Differential Gene Expression Profiling of Anthocyanin Biosynthesis in Zoysiagrass (*Zoysia japonica* Steud.)

ABSTRACT

Zoysiagrass (*Zoysia japonica* Steud.) is commonly found in coastal areas and widely used for lawns in temperate regions. *Z. japonica* ‘Greenzoa’ is genetically neighbor to *Z. japonica* ‘Anyang-jungji’, but ‘Greenzoa’ lacks purple coloration at stolons and spikes. HPLC analysis revealed that high levels of cyanidin chloride and petunidin chloride existed in ‘Anyang-jungji’ but not in ‘Greenzoa’, suggesting the absence of anthocyanin pigmentation in ‘Greenzoa’. To elucidate the genetic mechanisms responsible for the differential pigmentation between two cultivars, RNA-Seq analyses were performed and *de novo* transcriptomes were obtained. Through the comparison to known transcriptome data, zoysiagrass was found to be genetically related to *Setaria italica* the most and followed by *Sorghum bicolor*. Surveying the sequence information of anthocyanin biosynthesis genes resulted in a total of 24 anthocyanin biosynthesis genes, which were identical at the nucleotide level between the two cultivars but several of them were differently expressed. Specifically, *ZjDFR1* and *ZjANS1*, in the later steps of the anthocyanin biosynthesis pathway, were significantly down-regulated in ‘Greenzoa’ consistent with the lack of anthocyanins. With other collected zoysiagrass cultivars appearing green or purple colors in stolons and spikes, similar expression differences of the two genes were found. In consequence, the data strongly suggest that proper

expressions of *ZjDFR1* and *ZjANS1* are keys to purple pigmentation in stolons and spikes of zoysiagrass.

Keywords: anthocyanin, DEG, *de novo* assembly, expression level, quantitative RT-PCR, *ZjDFR1*, *ZjANS1*, *Zoysia japonica*

INTRODUCTION

Zoysiagrass (*Zoysia japonica* Steud.) is a perennial creeping grass commonly found in Southeast and East Asia and Australia (Engelke and Anderson, 2003). Zoysiagrass is widely used for lawns, mainly because of its quick spreading on the ground and fascinating green color under a broad range of environmental conditions (Inokuma et al., 1996). Several zoysiagrass cultivars such as Meyer, Anyang-jungji, and Zenith are popular choices for warm-season lawn in Korea and other Northeast Asian countries.

Green appearance is one of the most commercial values of zoysiagrass. Most aerial parts of zoysiagrass are kept green under temperate climate, though creeping stolons and spike tissues often develop purple colors in some cultivars, which may compromise the uniform aesthetic value of green lawns. Therefore, development of new cultivars with less purple coloration is one of the breeding goals. For example, zoysiagrass cultivars ‘Zenith’ and ‘Millock’ have green spikes and stolons, whereas cultivars ‘Meyer’ and ‘Senock’ develop purple colors in the same tissues (Choi and Yang, 2004, 2006; Emmons, 1995; Sleper et al., 1989). And most likely, the accumulation of anthocyanin pigments should result in purple coloration.

Despite its commercial values, only a limited number of molecular-based studies have been reported so far. Zoysiagrass is an allotetraploid (2n = 4x = 40) with a genome size of approximately 421 Mb

(Arumuganathan et al., 1999; Forbes, 1952). Even though several molecular linkage maps have been reported from *Z. japonica* and other *Zoysia* spp. (Cai et al., 2004, 2005; Li et al., 2009; Tsuruta et al., 2005; Yaneshita et al., 1999), no comprehensive genetic studies have been done in zoysiagrass.

Anthocyanins, a group of phenolic compounds commonly found in many plant species, are responsible for red to purple colors in nature (Mazza and Miniati, 1993). Anthocyanins are produced in various kinds of tissues in higher plants including leaves, stems, roots, flowers, and fruits. Anthocyanins play beneficial roles using their vivid colors, attracting pollinators and seed dispersers to flowers and fruits, and protecting cells from photooxidative damage in photosynthetic tissues by absorbing high-energy light (Neill and Gould, 2003; Shang et al., 2011). Anthocyanins are also well known for the antioxidant properties, alleviating oxidative stresses in plant tissues by scavenging free radicals, thus often used as a food additive for health benefit (Prior et al., 1998; Tulio et al., 2008).

The anthocyanin biosynthesis pathway is largely conserved among flowering plants. Flavonoid biosynthesis begins with chalcone synthase (CHS) that utilizes a *p*-coumaroyl-CoA and three malonyl-CoAs to form a central flavonoid precursor tetrahydroxychalcone (naringenin chalcone), which is subsequently converted to flavanone naringenin by chalcone isomerase (CHI). Naringenin is either hydroxylated at the C-3 position within the central C-ring by flavanone-3-hydroxylase (F3H) to produce dihydroflavonol, or hydroxylated at the 3’ position of the B-

ring by flavonoid 3’ hydroxylase (F3’H). Dihydroflavonols such as dihydromyricetin, dihydrokaempferol, and dihydroquercetin are subjected to diverse B-ring modifications by the action of flavonoid hydroxylases such as F3’H and flavonoid 3’, 5’-hydroxylase (F3’5’H), which primarily cause color differences among anthocyanin pigments. Consequently, the resulting dihydroflavonols serve as immediate precursors for the synthesis of flavonols and leucoanthocyanidins by flavonol synthase (FLS) and dihydroflavonol 4-reductase (DFR), respectively. Lecuoanthocyanidins are colorless but converted by anthocyanidin synthase (ANS) to colored anthocyanidins, to which a group of glycosyltransferases (GTs) then add diverse sugar moieties forming stable, water-soluble anthocyanin pigments. In addition to the hydroxylation at B-ring, the introduction of a methoxyl group also affects the color of anthocyanins.

To understand the genetic basis of purple pigmentation in zoysiagrass stolons and spikes, the transcriptomes were analyzed in two zoysiagrass cultivars displaying different colorations. *Z. japonica* ‘Anyang-jungji’ (AJ) shows purple coloration in stolons and spikes, while the other cultivar ‘Greenzoa’ (GZ) has green colors. Through HPLC analysis, anthocyanin accumulation was found to be a main cause of different colorations of stolons and spikes between the two cultivars. To dissect the key components of anthocyanin pigmentation in zoysiagrass, a *de novo* transcriptome assembly was performed and two anthocyanin biosynthesis genes *ZjDFR1* and *ZjANS1* were

observed to highly upregulate in purple-colored AJ stolons and spikes, but hardly expressed in GZ stolons and spikes.

MATERIALS AND METHODS

Plant Materials

Zoysiagrass (*Z. japonica*) cultivars AJ and GZ were obtained from Fungi and Plant (FnP) Cooperation (Jeungpyeong, Korea). The two cultivars were grown in greenhouse with 16 h light/8 h dark cycles, 26/22^{o}C (day/night) temperatures, and 60% relative humidity. Other seven zoysiagrass cultivars ‘Meyer’, ‘Senock’, ‘Yaji’, ‘Gumjandi’, ‘Konhee’, ‘Millock’, and ‘Zenith’ were grown in the field of Experimental Farm of Seoul National University (Suwon, Korea).

HPLC Analysis

HPLC analysis was performed to detect major anthocyanidins derived from anthocyanins according to the previous report with minor modifications using spike samples (Zifkin et al., 2012). Briefly, anthocyanin pigments were extracted with a solvent mixture of acetone:methanol:water:formic acid (40:40:20:0.1, v/v/v/v). Extracts were filtered through Sep-Pak C18 cartridge (Waters Scientific, Ontario, CA). For hydrolysis of anthocyanins to aglycones, 3 mL of 2 N HCl in 50% (v/v) aqueous methanol was added to the sample powder. After incubation at 100°C for 1 h, the extracts were injected onto the Eclipse ZOBRAX XDB-C18 Rapid Resolution Threaded Column (4.6 × 150 mm, 5 μm; Agilent Technologies, Palo Alto, CA, USA) on an UltiMate 3000 HPLC system (Thermo Scientific, Hudson, NH, USA), using

delphinidin chloride, cyanidin chloride, peonidin chloride, malvidin chloride (Sigma-Aldrich, St Louis, MO, USA), and petunidin chloride (EXTRASYNTHESE, Lyon, France) as standards. Anthocyanin aglycones were quantified at the wavelength of 520 nm.

RNA Extraction and RNA-Seq

Total RNAs were extracted separately from spike, stolon, young/old leaf and root tissues in the TRIzol® reagent (Life Technologies) according to the manufacturer's protocol. For transcriptome analysis, total RNA from AJ and GZ spike tissues was subjected to RNA-Seq. Sequencing was performed on an Illumina HiSeq 2000 system at the National Instrument Center for Environmental Management (NICEM, http://nicem.snu.ac.kr). The raw sequences were deposited in the NCBI/EBI/DDBJ Short Read Archive (Accession number: DRA001679).

***De novo* Transcriptome Assembly**

The raw reads were filtered by removing the adapter sequences, empty reads, low quality reads, and the reads with more than 20% Q < 20 bases. Transcriptome *de novo* assembly was carried out with three assemblers such as CLC Genomics Workbench (Ver. 3.7.1), Velvet (Ver. 1.1.04)-Oases (Ver. 0.1.21), and Trinity (release 20110519) (Grabherr et al., 2011; Schulz et al., 2012) with various k-mer lengths. A default k-mer value (25-mer) was used for assembly with CLC. For Velvet-Oases and Trinity, different k-mer values were applied to get the best results

(from 21 to 79 for Velvet-Oases and 25 to 33 for Trinity). As Oases does not cluster assembled contigs, CD-HIT-EST was used to cluster the contigs with identity more than 90% and coverage of 100% (Li and Godzik, 2006). All resulting data sets were merged into a single assembly by collapsing identical or near-identical contigs into single unigenes. Assembled transcriptomes are available at http://epigenome.snu.ac.kr/.

Gene Ontology (GO) Annotation

The unigenes were identified by sequence similarity comparison against NCBI non-redundant (NR) protein database (http://www.ncbi.nlm.nih.gov) by running BLAST with a cut-off E-value of $1.0e^{-6}$. Blast2GO was used to obtain GO annotation of unigenes based on BLASTX hit against NR database with a cut-off E-value of $1.0e^{-6}$ (ver. 2.6.5) (Conesa et al., 2005). Obtained GO terms were classified and plotted using WEGO software (Ye et al., 2006).

Phylogenetic Study

A total of 22 plant transcriptome data sets (9 monocots, 9 dicots, 2 bryophytes, and 2 green algae) were obtained from the Ensembl Plants database (http://plants.ensembl.org/). *De novo* assembled AJ transcripts were aligned against these transcriptome sequences using TBLASTX program with a cut-off E-value of $1.0e^{-15}$. To assess the phylogenetic relationship among zoysiagrass and other plant species, phylogenetic analysis was performed using *ACTIN* sequences derived from 22

different plant species (Table 1). Orthologous transcripts of *ZjDFR1* and *ZjANS1* were obtained by TBLASTX search with a cut-off E-value of $1.0e^{-15}$, and sequences were aligned and compared with ClustalX (Ver. 2.1) (Larkin et al., 2007). The phylogenetic tree was constructed by MEGA5.2 (Tamura et al., 2011) using Poisson model and with 1,000 bootstrap replications for each branch. Sequence information of transcripts used in phylogenetic analysis is provided as Figure I-1.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) Analysis

A total of 1 - 2 μg RNA was treated with RNase-Free DNase Set (QIAGEN) to remove contaminating DNA and then subjected to cDNA synthesis using the SuperScript II RT Kit (Life Technologies) according to the manufacturer's instructions. qRT-PCR was performed on a Rotor-Gene Q real-time PCR system (QIAGEN). QuantiFast SYBR Green PCR master mix (QIAGEN) was used for amplification. Zoysiagrass *β-ACTIN* (GU290545.1) sequence was used as an internal control to measure the relative amount of transcripts. Information on oligonucleotide sequences for qRT-PCR analysis is listed in Table 1.

5'-/3'-RACE and Thermal Asymmetric Interlaced (TAIL)-PCR

To obtain full-length sequences of *ZjDFR1* and *ZjANS1*, the region outside the contig was extended with SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratories) according to the manufacturer's protocol. To obtain the sequence of regulatory region

Table 1. NCBI accession number of ACTINs.

Species	Accession number
Arabidopsis thaliana	NM_179953.2
Brachypodium distachyon	XM_003560541.1
Brassica rapa	JN120480.1
Chlamydomonas reinhardtii	D50839.1
Glycine max	NM_001253024.2
Hordeum vulgare	AK251023.1
Medicago truncatula	XM_003621971.1
Oryza sativa	AB047313.1
Physcomitrella patens	XM_001782636.1
Populus trichocarpa	XM_006379531.1
Selaginella moellendorffii	XM_002980705.1
Setaria italica	XM_004981913.1
Solanum lycopersicum	XM_004236699.1
Solanum tuberosum	XM_006351284.1
Sorghum bicolor	X79378.1
Vitis vinifera	XM_002283554.2
Zea mays	NM_001156990.1
Zoysia japonica	GU290545.1

A. Alignment of ZjDFR1 homologs

```
ZjDFR1          VVVTGASGFVGSWLVMKLLQAGYTVRATVRGPANVGKTRPLLDLPGAKERLSIYKADLSD
ZmDFR           VLVTGASGFVGSWLVMKLLQAGYTVRATVRDPANVGKTKPLMDLXGATERLSIWKADLAE
HvDFR           VVVTGASGFVGSWLVMKLLQAGYTVRATVRDPANVEKTKPLLELPGAKERLSIWKADLSE
OsDFR           VVVTGASGFVGSWLVMKLLQAGYTVRATVRDPSNVGKTKPLLELAGSKERLTLWKADLGE
AmDFR           VCVTGAAGFIGSWLVMRLLERGYTVRATVRDPGNMKKVKHLIELPKADTNLTLWKADMTV
GhDFR           VCVTGAAGFIGSWLVMRLLERGYVVHATVRDPGDLKKVKHLLELPKAQTNLKLWKADLTQ
VmDFR           VCVTGAAGFIGSWLIMRLLERGYVVRATVRDPGNLKKVKHLLELPKADTNLTLWKADLNE
PhDFR           VCVTGAAGFIGSWLVMRLLERGYNVHATVRDPENKKKVKHLLELPKADTNLTLWKADLTV
LjDFR2          VCVTGAAGFIGSWLVMRLMERGYMVRATVRDPANMKKVKHLLELPEAKTKLTLWKADLAE
LjDFR3          VCVTGSTGFIGSWLVMRLMEGGYTVRATVRDPDNMKKVKHLLELPGAKTNLTIWNADLTE
LjDFR5          VCVTGAAGFIGSWLVMRLIERGYTVRATIRDPANMKKVKHLLELPDAKTKLSLWKADLAE
RhDFR           VCVTGASGFIGSWLVMRLLDRGYTVRATVRDPANKKKVNHLLDLPKAATHLTLWKADLAE
VvDFR           VCVTGASGFIGSWLVMRLLERGYTVRATVRDPTNVKKVKHLLDLPKAETHLTLWKADLAD
AtDFR           VCVTGASGFIGSWLVMRLLERGYFVRATVRDPGNLKKVQHLLDLPNAKTLLTLWKADLSE
ZjDFR2          VCVTGASGFLASWLIRRLLEAGYHVRGTVRDPGNRQKVAHLWKLPGAKERLQIVRADLLE
ZjDFR3          -CVTGASGYIASWITKLLLDRGYTVRATVRDTADPKKTQHLRDLDGAKDRLHLFKASLLD


ZjDFR1          EGSFDEAIKGCTGVFHVATPMDFESKDPENEVIKPTVEGMMSIMRACKDAGTVKRIVFTS
ZmDFR           EGSFHDAIRGCTGVFHVATPMDFLSKDPENEVIKPTVEGMISIMRACKEAGTVRRIVFTS
HvDFR           DGSFNEAIAGCTGVFHVATPMDFDSQDPENEVIKPTVEGMLSIMRACKEAGTVKRIVFTS
OsDFR           EGSFDAAIRGCTGVFHVATPMDFESEDPENEVVKPTVEGMLSIMRACRDAGTVKRIVFTS
AmDFR           EGSFDEAIQGCEGVFHLATSMEFDSVDPENEVIKPTIDGMLNIIKSCVQAKTVKKFIFTT
GhDFR           EGSFDEAIQGCHGVFHLATPMDFESKDPENEIIKPTIEGVLSIIRSCVKAKTVKKLVFTS
VmDFR           EGSFDEAIEGCVGVFHVATPMDFESKDPENEVIKPTINGVLSIIKSCTKAKTVKRLVFTS
PhDFR           EGSFDEAIQGCQGVFHVATPMDFESKDPENEVIKPTVRGMLSIIESCAKANTVKRLVFTS
LjDFR2          EGSFDEAIKGCTGVFHVATPMDFESKDPENEVIKPTINGVLDIMKACQKAKTVRRLVFTS
LjDFR3          EGSFDEAINGCSGVFHVASPMDFNSKDPENEVIKPSINGVLDIMKACQKAKTVRRLVFTS
LjDFR5          EGSFDEAIRGCTGVFHVATPMDFESKDPENEVIKPTINGLLDILKACEKAKTVRRLVFTS
RhDFR           EGSFDEAIKGCTGVFHVATPMDFESKDPENEVIKPTINGVLDIMQACLKAKTVRRLVFTS
VvDFR           EGSFDEAIKGCTGVFHVATPMDFESKDPENEVIKPTIEGMLGIMKSCAAAKTVRRLVFTS
AtDFR           EGSYDDAINGCDGVFHVATPMDFESKDPENEVIKPTVNGMLGIMKACVKAKTVRRFVFTS
ZjDFR2          EGSFDDAVMACEGVFHTASPVLANCDSSKEETLVPAVHGTLNVLRSCKKNPFLKRVVLTS
ZjDFR3          EGSFDDAVNGCDCVFHTASPFYHNVKDPKAELIDPAVKGTLNVLSSCKK-ASIKRVVVTS


ZjDFR1          SAGTVNIEGRQRPVYDHDNWSDIDFCRRVKMTGWMYFVSKSLAEKAAMAYAAEHGLDLIS
ZmDFR           SAGTVNLEERQRPVYDEESWTDVDFCRRVKMTGWMYFVSKTLAEKAALAYAAEHGLDLVT
HvDFR           SAGSVNIEERPRPAYDQDNWSDIDYCRRVKMTGWMYFVSKALAEKAAMEYASENGLDFIS
OsDFR           SAGTVNIEERQRPSYDHDDWSDIDFCRRVKMTGWMYFVSKSLAEKAAMEYAREHGLDLIS
AmDFR           SGGTVNVEEHQKPVYDETDSSDMDFINSKKMTGWMYFVSKILAEKAGMEAAKENNIDFIS
GhDFR           SAGTVNGQEKQLHVYDESHWSDLDFIYSKKMTAWMYFVSKTLAEKAAWDATKGNNISFIS
VmDFR           SAGAVVDQEHQPLVFDENNWSDVDFLYDKKMTGWTYFVSKTLAERAAMEAAKEISIDFIS
PhDFR           SAGTLDVQEQQKLFYDQTSWSDLDFIYAKKMTGWMYFASKILAEKAAMEEAKKKNIDFIS
LjDFR2          SAGTLNVIEHQKQMFDESCWSDVEFCRRVKMTGWMYFVSKTLAEQEAWKFAKEHGIDFIT
LjDFR3          SAGTLNAVEHQKQMCDESCWSDVEFCRRVKMTGWMYFVSKTLAEQEAWKFAQEHDIDFIT
LjDFR5          SAGTVDVTEHPKPVIDETCWSDIEFCLRVKMTGWMYFVSKTRAEQEAWKYAKEHNIDFVS
RhDFR           SAGSVNVEETQKPVYNESNWSDVEFCRRVKMTGWMYFASKTLAEQEAWKFAKENNIDFIT
VvDFR           SAGTVNIQEHQLPVYDESCWSDMEFCRAKKMTAWMYFVSKTLAEQAAWKYAKENNIDFIT
AtDFR           SAGTVNVEEHQKNVYDENDWSDLEFIMSKKMTGWMYFVSKTLAEKAAWDFAEEKGLDFIS
ZjDFR2          SSSAVRIRDDAQPVLDETTWSSVQLCERMQL---WYALAKVYAEKAAWEFAKENDIDLVT
ZjDFR3          SMAAVAYNGKPRTVVDETWFSDPEICAKLQQ---WYVVSKTLAEEAAWKFARDNGFEIVT
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ZjDFR1          IIPTLVVGPFLSTAMPPSLVTALALVTRNEPHYSILKQVQFVHLDDLCDAEIYLFEHPDA
ZmDFR           IIPTLVVGPFISASMPPSLITALALITGNAPHYSILKQVQLIHLDDLCDAEIFLFENPAA
HvDFR           IIPTLVVGPFLSAGMPPSLVTALALITGNEAHYSILKQVQLVHLDDLCDAMTFLFEHPEA
OsDFR           VIPTLVVGPFISNGMPPSHVTALALLTGNEAHYSILKQVQFVHLDDLCDAEIFLFESPEA
AmDFR           IIPPLVVGPFIMPTFPPSLITALSPITGNEAHYSIIKQCQYVHLDDLCEGHIFLFEYPKA
GhDFR           IIPTLVVGPFITSTFPPSLVTALSLITGNEAHYSIIKQGQYVHLDDLCECHIYLYENPKA
VmDFR           IIPTLVVGPFISPTFPPSLITVLSPITGNEAHYSIIKQCQYVHLDDLCKYLMFLLEHPEA
PhDFR           IIPPLVVGPFITPTFPPSLITALSLITGNEAHYCIIKQGQYVHLDDLCEAHIFLYEHPKA
LjDFR2          IIPPLVVGSFLMPTMPPSLITALSPITGNEAHYSIIKQGQYVHLDDLCLAHIFLFEHPES
LjDFR3          IIPSLVVGSFLMPTLPPSLTTALSPITGNEAHYSIIKQGQYVHLDDLCLAHIFLFEHPKS
LjDFR5          VIPPLVVGPFLMPTMPPSLITALSLITGNEAHYSIIKQGQYVHLDDLCLAHIFLFENPKA
RhDFR           IIPTLVIGPFLMPSMPPSLITGLSPLTGNESHYSIIKQGQFIHLDDLCQSHIYLYEHPKA
VvDFR           IIPTLVVGPFIMSSMPPSLITALSPITGNEAHYSIIRQGQFVHLDDLCNAHIYLFENPKA
AtDFR           IIPTLVVGPFITTSMPPSLITALSPITRNEAHYSIIRQGQYVHLDDLCNAHIFLYEQAAA
ZjDFR2          VLPSFVIGPSLSKELCVTASDVLGLLQGDTARFSSYGRMGYVHIDDVASSHILVYEALEA
ZjDFR3          INPAMVIGPLLQPTLNTSAEAILKLINGSSSTYSNVT-LGWVNVKDVALAHILAYEVPSA

ZjDFR1          AGRYVCSSDDATIHGLAAMLRERYPEYDIPESFPGIDDDLPPVHFSSKKLLDHGFRFRYT
ZmDFR           AGRYVCSSHDVTIHGLAAMLRDRYPEYDVPQRFPGIQDDLQPVRFSSKKLQDLGFTFRYT
HvDFR           NGRYICSSHDATIHGLARMLQDRFPEYDIPQKFAGVDDNLQPIHFSSKKLLDHGFSFRYT
OsDFR           RGRYVCSSHDATIHGLATMLADMFPEYDVPRSFPGIDDHLQPVHFSSWKLLAHGFRFRYT
AmDFR           EGRYICSSHDATIYDIAKLITENWPEYHIPDEFEGIDKDIPVVSFSSKKMIGMGFIFKYT
GhDFR           KGRYICSSHDATIHQLAKIIKDKWPEYYIPTKFPGIDEELPIVSFSSKKLIDTGFEFKYN
VmDFR           EGRYICSSHDATIYDLAKMMRRNGPGTMSPNEFKGIDKELPIMSFSSKKLLVIGFKFKYN
PhDFR           DGRFICSSHHAIIYDVAKMVREKWPEYYVPTEFKGIDKDLPVVSFSSKKLTDMGFQFKYT
LjDFR2          EGRYICSASEATIHDIAKLINSKYPEYNIPTKFKNIPDELELVRFSSKKIKDMGFEFKYS
LjDFR3          EGRYICSASEATIHDIAKLINSKYPEYNVPTKFKNIPDELELVRFSSKKIKDMGFEFKYS
LjDFR5          QGRYMCSAYEATIHEVARMINKKYPEFNVPTKFKDIPDELDIIKFSSKKITDLGFKFKYS
RhDFR           EGRYICSSHDATIHEIAKLLKGKYPEYNVPTTFKGIEENLPKVHFSSKKLLETGFEFKYS
VvDFR           EGRYICSSHDCIILDLAKMLREKYPEYNIPTEFKGVDENLKSVCFSSKKLTDLGFEFKYS
AtDFR           KGRYICSSHDATILTISKFLRPKYPEYNVPSTFEGVDENLKSIEFSSKKLTDMGFNFKYS
ZjDFR2          TGRYLCSSVVLDNNELVSLLAKRYPIFPIPRRLNN-PYGEQSYQLNTSKLQGLGFKFKG-
ZjDFR3          NGRYCIVERVLHYSDVVNVIRKMYPTIPLPDKCADDKFVP-TYQVSKEKIRSLGIELIP-

ZjDFR1          VQDMFDEAIRTCREKGLIP
ZmDFR           LEDMFDAAIRTCQEKGLIP
HvDFR           TEDMFDAAIHTCRDKGLIP
OsDFR           LEDMFEAAVRTCREKGLLP
AmDFR           LEDMVRGAIDTCREKGMLP
GhDFR           LEDMFKGAIDTCREKGLLP
VmDFR           LEDMFRGAIDTWQEKGLLP
PhDFR           LEDMYKGAIDTCRQKQLLP
LjDFR2          LEDMYTGAIDTCKEKGLLP
LjDFR3          LEDMYTGAIDTCKEKGLLP
LjDFR5          LEDMYTGAVETCREKGLLP
RhDFR           LEDMFVGAVDACKEKGLLP
VvDFR           LEDMFTGAVDTCRAKGLLP
AtDFR           LEEMFIESIETCRQKGFLP
ZjDFR2          VQEMFDDCVQSLKDQGHL-
ZjDFR3          LETSIKETIESLKEKGFVS
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B. Alignment of ZjANS1 homologs

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GmANS1          LANNASGQLEWEDYFFHLVFPEDKRDLSIWPKKPDDYIEVTSEYAKRLRGLATKMLEALS
MtANS           LANNASGQLEWEDYFFHCIFPEDKRDLSIWPKTPADYTKVTSEYAKELRVLASKIMEVLS
AtANS           LANNASGQLEWEDYFFHLAYPEEKRDLSIWPKTPSDYIEATSEYAKCLRLLATKVFKALS
MdANS           LANNASGQLEWEDYFFHCVYPEDKRDLSIWPQTPADYIEATAEYAKQLRELATKVLKVLS
VvANS           LANNASGQLEWEDYFFHLIFPEDKRDMTIWPKTPSDYVPATCEYSVKLRSLATKILSVLS
NtANS           LANSACGQLEWEDYFFHCVFPEDKCNLSIWPKTPTDYIPATSEYAKQIRNLATKILAVLS
GhANS           LANNASGQLEWEDYFFHLVFPEEKRDLTIWPTTPSDYTDATTEYAKQLRALATKILPALS
ZjANS1          LATNASGQREWEDYLFHLLHPDGLADHALWPAHPPDYVATTREFGRRVRELASRLLAILS
ZmANS           LATNTCGQREWEDYLFHLVHPDGLADHALWPAYPPDYIAATRDFGRRTRDLASTLLAILS
OsANS           LAANASGKREWEDYLFHLVHPDHLADHSLWPANPPEYVPVSRDFGGRVRTLASKLLAILS
TaANS           LAGSAGGKREWEDYLFHMLHPDARADHARWPAHPPEYVPVTKAFGEHVSALSSRLLAILS
ZjANS2          LANDDS-VLDWRDYLDHHTLPESRRNPSHWPDFVPGYRDTVVKYSNSMKDLAQRLLRIIS
ZjANS3          LVKFEDQTLDWCDRLHLRVEPEAERNCSLWPKHPESFRALLHEYTLSCRRIRDCILQAMA

GmANS1          IGLGLEGGRLEKEVGGMEELLLQLKINYYPICPQPELALGVEAHTDVSSLTFLLHNMVPG
MtANS           LELGLEGGRLEKEAGGMEELLLQMKINYYPICPQPELALGVEAHTDVSSLTFLLHNMVPG
AtANS           VGLGLEPDRLEKEVGGLEELLLQMKINYYPKCPQPELALGVEAHTDVSALTFILHNMVPG
MdANS           LGLGLDEGRLEKEVGGLEELLLQMKINYYPKCPQPELALGVEAHTDVSALTFILHNMVPG
VvANS           LGLGLEEGRLEKEVGGMEELLLQKKINYYPKCPQPELALGVEAHTDVSALTFILHNMVPG
NtANS           IGLRLEEGRLEKEVGGMEDLLLQMKINYYPKCPQPELALGVEAHTDVSALTFILHNMVPG
GhANS           LGLGLEEGRLEKEVGGIEELILQLKINYYPKCPQPELALGVEAHTDVSALTFILHNMVPG
ZjANS1          LGLGLREHKLEDELTNQEDLLLQLKINYYPRCPQPELAVGVEAHTDVSALSFILHNGVPG
ZmANS           MGLGTDGDALEKALTT--DLLLQLKINYYPRCPQPELAVGVEAHTDVSALSFILHNGVPG
OsANS           LGLGLPEETLERRLRG--DLLLQLKINYYPRCPRPDLAVGVEAHTDVSALSFILHNGVPG
TaANS           LGLGVPADTLERRLRL--DLLLKLKINYYPRCPQPELAVGVEAHTDVSALSVILTNGVPS
ZjANS2          ECLNLP-PSYIEEAVG--EVYQNITVSYYSPCPQPDLALGLQSHSDMGAITLLIQDDVGG
ZjANS3          KTLGLNEDYIISHFTD--KAPTFARFNYYPPCPRPDLVFGIKPHSDSGVLTILLVDDVAG

GmANS1          LQLFYQGQWFTAKCVPNSILMHIGDTIEILSNGKYKSILHRGLVNKEKVRISWAMFCEPP
MtANS           LQLFYEGKWVTAKCVPDSILMHIGDTIEILSNGKYKSILHRGLVNKEKVRISWAVFCEPP
AtANS           LQLFYEGKWVTAKCVPDSIVMHIGDTLEILSNGKYKSILHRGLVNKEKVRISWAVFCEPP
MdANS           LQLFYEGKWVTAKCVPNSIVMHIGDTLEILSNGKYKSILHRGMVNKEKVRISWAVFCEPP
VvANS           LQLFYEGKWVTAKCVPNSIIMHIGDTIEILSNGKYKSILHRGLVNKEKVRISWAVFCEPP
NtANS           LQLFYEGQWVTAKCVPNSIIMHIGDTLEILSNGKYKSILHRGVVNKEKIRISWAIFCEPP
GhANS           LQLFYDGQWVSAQCVPDSIILHIGDALEILSNGEYKSILHRGLVNKEKVRISWAVFCEPP
ZjANS1          LQVLHGGRWVTARSEPGTMIVHVGDALEILSNGRYTSVLHRGLVNREAVRVSWVVFCEPP
ZmANS           LQVLHGARWVTARHEPGTIIVHVGDALEILSNGRYTSVLHRGLVNREAVRISWVVFCEPP
OsANS           LQVHHAGSWVTARPEPGTIVVHVGDALEILTNGRYTSVLHRGLVSRDAVRLSWVVFCEPP
TaANS           LQVLHPGNWVTARDEPGTLVVHVGDSLEILSNGRYTSVLHRGLVNRQAVRVSWVVFAQPP
ZjANS2          LEVMKDGMWIPVPPLPDGILVILADQTEIITNGRYRSSVHRAVVNAEHARLSVATFYDPS
ZjANS3          LQILRDDKWHNVPTSPHRLLVNLGDYSEIMSNGIFKSPVHRAVANMEKERISLAMFHGLD

GmANS1          KEKIILQPLPELVTETEPARFPPRTFAQHIHHKLFRK
MtANS           KEKIILKPLPELVTEKEPARFPPRTFAQHIHHKLFRK
AtANS           KDKIVLKPLPEMVSVESPAKFPPRTFAQHIEHKLFGK
MdANS           KEKIILKPLPETVSEDEPAMFPPRTFAEHIQHKLFRK
VvANS           KEKIILKPLPETVSETEPPLFPPRTFSQHIQHKLFRK
NtANS           KEKIILKPLPETITEAEPPRFPPRTFAQHMAHKLFKK
GhANS           KEKIVLKPLPETVSEAEPPLFPPRTFRQHMEHKLFRK
ZjANS1          PDAVLLRPLPELVTEEEPARFTPRTFKEHLDRKLFKK
ZmANS           PDSVLLHPLPELVTEGHPARFTPRTFKQHLDRKLFKK
OsANS           PESVLLQPVQELLADGGKPLFAPRTFKQHVQRKLFKK
TaANS           PDSVLLGPLPELVQGYSRRRMINRTTRLRSRRKVVKK
ZjANS2          KSRKICT-APLLVSNDEPKKYRDIVYGDYVSS-WYSK
ZjANS3          PEKEIEPAVDCYMKSN----------QHGIGN-----
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Figure I-1. Alignment of ZjDFR1 and ZjANS1 homologs used for phylogeny.

upstream of transcription start site, TAIL-PCR (Liu and Whittier, 1995) was performed. The *cis*-acting regulatory element at the promoter region was predicted by homology search against the PlantCARE database (Lescot et al., 2002). Information on oligonucleotide sequences for rapid amplification of cDNA ends (RACE) and TAIL-PCR is provided in Table 2.

Local Bisulfite Sequencing (BS-Seq)

Genomic DNA was extracted from AJ and GZ spikes at stages S5 and S6 (Doyle and Doyle, 1987). The sodium bisulfite treatment and following DNA purification were carried out using EpiTect® Bisulfite Kit (QIAGEN) according to the manufacturer's protocol. Information on oligonucleotide sequences for BS-Seq analysis is provided in Table 2.

Table 2. Primer sequences used in this study.

Purpose	Gene	Sequence
qRT-PCR	ZjPAL1-F	CGAATGTTATTGCCGTCCTT
	ZjPAL1-R	GGTGAGGTGGTCGGTGTACT
	ZjPAL2-F	CCTAAGCAGGACAGGTACGC
	ZjPAL2-R	GTTGTCGTTGACGGAGTTGA
	ZjPAL3-F	CTACAACAACGGGCTGACCT
	ZjPAL3-R	GAGCTCGGAGCAGTAGGATG
	ZjCHS1-F	CCGACTGGAACTCCATCTTC
	ZjCHS1-R	TCCTTGTCGAGCTTGACCTT
	ZjCHS2-F	CAGAGGAGGGCATCAAGTTC
	ZjCHS2-R	GCTCCTTCATCAGCTTCCTG
	ZjCHI1-F	AGGATGCTTGCTGACTCCAT
	ZjCHI1-R	AGCAACACAAGCACAACCTG
	ZjCHI2-F	GAACAGGAGGACACCAAAGG
	ZjCHI2-R	TGGTTTGGTTCCCTCAAAAG
	ZjCHI3-F	GCTGCTGCATTCTATGTGGA
	ZjCHI3-R	CCGGTGCTTTAAAAATGGAG
	ZjCHI4-F	GCCGAGAAGGTGACTGAGAA
	ZjCHI4-R	CTTGAACGCCTCCTTGAACT
	ZjF3H1-F	ATGTCGAACCGGAGCTTATC
	ZjF3H1-R	CACGGGGTACGAGAAGTAGG
	ZjF3H2-F	GGATTCTTCCAGGTGCTGAA
	ZjF3H2-R	GTCGTCGGAGTAGAGCTTGG
	ZjF3’H-F	CCCACTAGAGTTCCGACCAG
	ZjF3’H-R	CGCACCAAACGGAATAAGAT
	ZjF3’5’H-F	ATGTCCAGCTTCTCCTCGC
	ZjF3’5’H-R	TCAGGCCGCAGCGTAG
	ZjDFR1-F	GCCTGGACCTTATCAGCATC
	ZjDFR1-R	AACTGCACCTGCTTGAGGAT
	ZjDFR2-F	GTCCTCGCTAACTGCGATTC
	ZjDFR2-R	GAAACGGGTTCTTCTTGCAC
	ZjDFR3-F	TGGATGGAGCAAAGGATAGG
	ZjDFR3-R	GTGATAGAAGGGCGAAGCAG
	ZjANS1-F	GGATTTCGTCCCTGGTTACA
	ZjANS1-R	ATATAAGAGGGCGGCAGGTT
	ZjANS2-F	CTTCGACCTTTTGGCTGAAC
	ZjANS2-R	ACTGCGGCTGATCCTTCTT
	ZjANS3-F	GACCTGGTGAAATTCGAGGA
	ZjANS3-R	CTCTGAACGATTCGGGATGT
	ZjUFGT1-F	GCTTGGCATCTATGGAGGAG

	ZjUFGT1-R	TGTCGAATCACCCACAGAAA
	ZjUFGT2-F	GCTTGGCATCTATAGAGCAG
	ZjUFGT2-R	TGTCGGATTACCCACAAAAA
	ZjFLS-F	CACTGGTACGACGCCAAGTA
	ZjFLS-R	CCTTGTACTCCCCGTTGCT
	ZjMYB1-F	TGCGCTGGATCAACTATCTG
	ZjMYB1-R	GATCAAGGACCACCTGTTGC
	ZjMYB2-F	CTGCGGTGGATCAACTACCT
	ZjMYB2-R	ACCATTTGTTGCCGACTAGG
	ACT11_F	AAGCTGTTCTTTCCCTCTACGC
	ACT11_R	GGAACAGTGTGACTCACACCATC
RACE	DFR_5’RACE_GSP1	CGTCGGCTTGATCACCTCGTTCTC
	DFR_5’RACE_GSP2	CGTCGGCTTGATCACCTCGTTCTC
	DFR_3’RACE_GSP1	GGCCTGGACCTTATCAGCATCATCC
	DFR_3’RACE_GSP2	GAACGAGCCGCACTACTCGATCCTC
	ANS_5’RACE_GSP1	GCACTGCGGCTGATCCTTCTTGTC
	ANS_5’RACE_GSP2	GGGGTGCAGGAGGTGGAAGAGGTA
TAIL-PCR	DFR_Tail_R1	CGTCGGCTTGATCACCTCGTTCTC
	DFR_Tail_R2	CGTCGGCTTGATCACCTCGTTCTC
	DFR_Tail_R3	GATGGACAGCCGCTCCTTCGCT
	ANS_Tail_R1	CAGCCAAAAGGTCGAAGGCGTCG
	ANS_Tail_R2	GTCGGCGGGTCGGACGTACTC
	ANS_Tail_R3	GCTGCAGCACCGTCGAAGATG
BS-seq	ZjDFR1_BS_F	GAYGGGTGAGATTYATTTYGTG
	ZjDFR1_BS_R	CTTCACCTCCATCGCCTCTTC
	ZjANS1_BS_F	GGYGYTAAGGTATYTTGAGGGTGATG
	ZjANS1_BS_R	CACCTCCACACATACAACCACC
Protein expression	ZjDFR1_pLM302_F	GTCGACATGGGGGAGGTGGT
	ZjDFR1_pLM302_R	AAGCTTTCACACATGCTCCCTTC
	ZjANS1_pLM302_F	GAATTCGATGTCATCTTCGACGG
	ZjANS1_pLM302_R	AAGCTTTCAGTTGGTTTTCGGTG

RESULTS

Purple Pigmentations of Zoysiagrass are Related to Anthocyanin Accumulation

Two zoysiagrass cultivars AJ and GZ are phenotypically similar to each other except the color of spike and stolons (Figures I-2A, I-3). AJ spikes develop purple color during ripening, whereas GZ spikes keep green color until maturation. To analyze the temporal changes in purple pigmentation, developing spikes were categorized into six stages by their size, color, and floral organ structure (Figure I-2A; Table 3). Developmental stages S1 and S2 were primarily defined by the size of spikes. Small spikes, or spikelets, at stage S1 were 20-25 mm long and hidden under the leaf sheath. Spikelets at stage S2 were 25-35 mm long and partially emerged from the leaf sheath. Stages S3 and S4 were categorized by the degree of coloration in AJ spikes and the equivalent sizes were applied to designate developmental stages of GZ spikelet. AJ spikes at S3 initiate coloration at the distal region, and 40-70% of spike body turned to purple at S4. Stigma and stamen of zoysiagrass emerged at different time points to prevent self-pollination (Chen et al., 2009), and this characteristic was applied to designate remaining stages S5 and S6. The spikes at stage S5 were fully-grown and the feathery stigma appeared, whereas at stage S6, pendulous stamens emerged as the stigma starts to wither. In the case of AJ, deep purple coloration of entire spike body was another characteristic at stages S5 and S6.

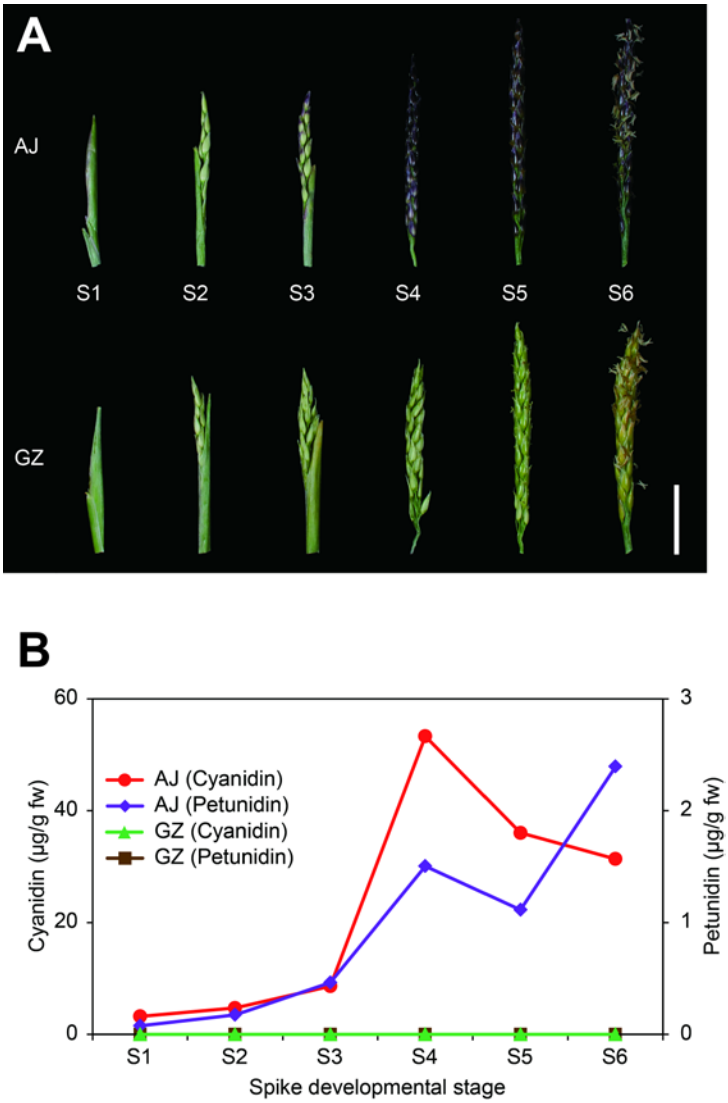


Figure I-2. Differential pigment accumulation in *Zoysia japonica* 'Anyang-jungji' (AJ) and *Zoysia japonica* 'Greenzoa' (GZ) spikes. (A) Developmental stages of zoysiagrass spikes according to the size and the degree of pigmentation. AJ and GZ spikes are categorized into six developmental stages (S1-S6) by size, color, and flower development. (B) Aglycone contents of acid-hydrolyzed anthocyanins in zoysiagrass spikes over developmental stages. Fw, Fresh weight.

Figure I-3. Field-grown phenotypes of zoysiagrass cultivars Anyang-jungji (A) and Greenzoa (B) used in this study. Arrows indicate creeping stolons.

Table 3. Characteristics of AJ and GZ spikes during spike development.

Stage[a]	Average length (mm)		Spike color		Developmental characteristics
	AJ	GZ	AJ	GZ	
S1	21.3	20.9	Green	Green	Spikelet hidden inside the leaf sheath
S2	24.7	24.9	Green	Green	Partial spikelet emergence from the leaf sheath
S3	26.1	26.1	Partially purple (0-30%)	Green	Complete spike emergence and pigmentation initiation (AJ)
S4	30.4	29.8	Partially purple (30-80%)	Green	Floret development and strong pigmentation in the lemma and palea
S5	36.2	35.9	Purple	Green	Emergence of feathery stigma from the floret
S6	36.8	36.5	Purple	Green	Emergence of pendulous stamens with stigma withering

[a]The stages refer to Figure I-2.

Anthocyanins are red and purple pigments abundantly found in a broad range of plant species (Mazza and Miniati, 1993). Anthocyanins are glycoside forms of anthocyanidins, and glycosylation is a major modification responsible for the solubilization and diversification of anthocyanin compounds (Zhang et al., 2004). Therefore, in an effort to identify major sugar-free anthocyanidin (aglycone) molecules that serve as immediate precursors for anthocyanin biosynthesis in zoysiagrass, anthocyanins were acid-hydrolyzed to remove sugar moieties and performed HPLC analysis (Goiffon et al., 1991; Zifkin et al., 2012). The HPLC analysis revealed two major anthocyanin aglycones cyanidin and petunidin from AJ spikes (Figures I-2B, I-4). Even at green stages (S1-S2), relatively low levels of cyanidin and petunidin were detected from AJ spikes, and both accumulated during ripening (Figure I-2B). In accordance with deep coloration, the spikes at stages S4 to S6 displayed high levels of anthocyanin accumulation (Figure I-2B). Red-purple colored cyanidin was most abundant, followed by blue-colored petunidin. By contrast, no apparent anthocyanin accumulation was observed in GZ spikes at any developmental stages. Luteolinidin, a kind of 3-deoxyanthocyanidin that is a 3-deoxy form of cyanidin, was not detected in AJ spikes (Figure I-5), suggesting that zoysiagrass has different profiles of flavonoid pigments compared to its close relatives such as *Sorghum bicolor*, in which yellow luteolinidin specifically accumulates as one of the major flavonoids (Awika et al., 2004). These observations indicate that distinct coloration of AJ and GZ is determined by differential

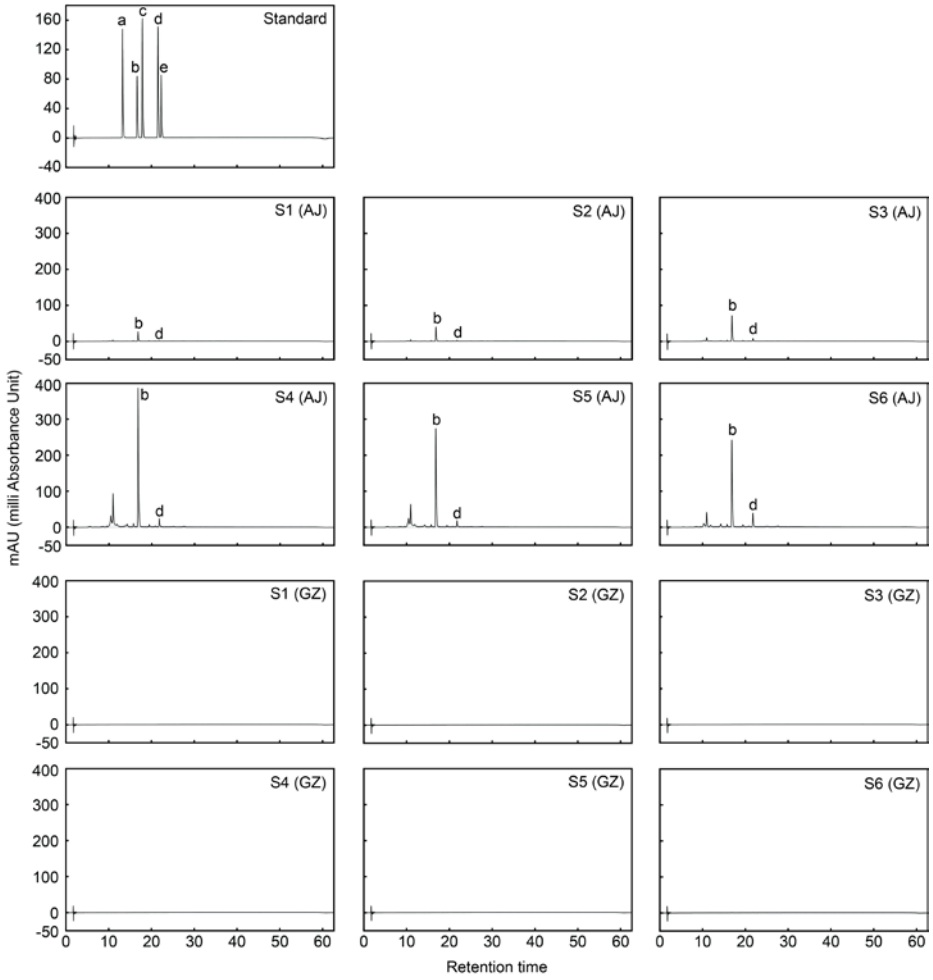


Figure I-4. HPLC analysis of anthocyanin aglycones present in *Zoysia japonica* 'Anyang-jungji' (AJ) and *Zoysia japonica* 'Greenzoa' (GZ) spikes at six developmental stages. The chromatograms were recorded at 520 nm. a, delphinidin; b, cyanidin; c, peonidin; d, petunidin; e, malvidin.

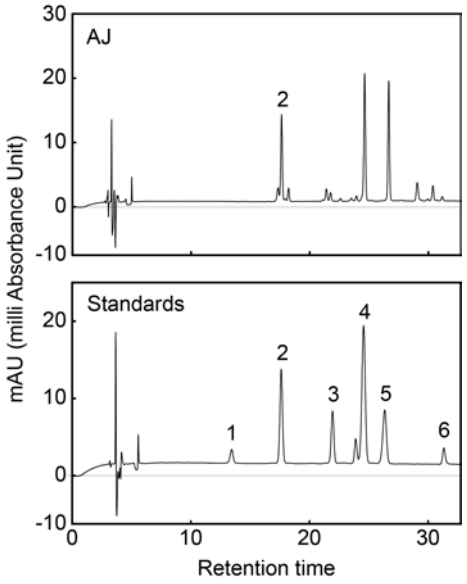


Figure I-5. HPLC analysis of anthocyanins in the *Zoysia japonica* 'Anyang-jungji' (AJ) spikes. The chromatograms were recorded at 475 nm. 1, delphinidin-3-O-glucoside; 2, cyanidin-3-O-glucoside; 3, pelargonidin-3-O-glucoside; 4, peonidin-3-O-glucoside; 5, malvidin-3-O-glucoside; 6, luteolinidin.

accumulation of anthocyanin pigments.

De novo Transcriptome Analysis of Zoysiagrass

As only limited genome information is available for zoysiagrass, next generation sequencing-based transcriptome analysis was performed on mature spike tissues of AJ and GZ to identify candidate genes involved in the anthocyanin biosynthesis (stages S5 to S6; Figure I-2A). From each of AJ and GZ spike tissues, approximately 44 Gbp of nucleotides were obtained and assembled into 28,561 and 28,984 mRNA contigs, respectively (Tables 4-6). In both assemblies, the N50 size of assembled contigs was longer than 1,395 bp, with the longest contig being 15,359 bp long. The average contig lengths were 982.4 bp and 984.9 bp for AJ and GZ, respectively.

From comparative transcriptome analysis between AJ and GZ, orthologous gene sets obtained were composed of 21,275 transcript pairs, where over 98.4% of GZ transcripts were identical to AJ transcripts at the nucleotide level (Figure I-6; Table 7). As expected, the zoysiagrass transcriptome displayed relatively high similarities to those from several monocot species (Figure I-7). Among monocot species, the closest relative of *Z. japonica* appeared to be *Setaria italica*, with approximately 84.8% of transcripts displaying significant homology to zoysiagrass transcriptome, and the next closest was *Sorghum bicolor* (Figure I-7). I also compared the nucleotide sequence of zoysiagrass *ACTIN* with its homologs from various plant species, and their

Table 4. Summary of de novo transcriptome assemblies of *Zoysia japonica* 'Anyang-jungji' (AJ) and *Zoysia japonica* 'Greenzoa' (GZ).

	AJ	GZ
Total length (bp)	28,057,682	28,547,216
Number of contigs	28,561	28,984
Average length (bp)	982.4	984.9
Median length (bp)	721	715
Maximum length (bp)	15,205	15,359
Minimum length (bp)	200	200
N50 length (bp)	1,395	1,415
N80 length (bp)	699	700
GC content (%)	44	43

Table 5. Summary of filtered and assembled RNA-Seq data generated on Illumina HiSeq 2000 platform using RNA isolated from *Zoysia japonica* 'Anyang-jungji' (AJ) and *Zoysia japonica* 'Greenzoa' (GZ) spike tissues.

	AJ	GZ
Total read pairs sequenced	22,228,361,386	22,724,700,838
Number of reads obtained after quality filtering	19,212,882,206	19,253,496,909
Average read size used for assembly (bp)	96.4	96.3
Properly mapped read pairs (%)	81.3	80.6

Table 6. *De novo* transcriptome assembly details using multiple assemblers.

Cultivar	Assembler	Avg. contig size (bp)	Median contig size (bp)	N50 contig size (bp)	Max. size (bp)	Min. size (bp)	Total contig number	Total length (bp)
AJ	Trinity	1,451.2	1,163	2,043	13,605	200	20,338	29,515,235
	Velvet	1,242.5	1,015	1,643	15,279	200	12,758	15,851,569
	CLC	986.6	714	1,420	15,206	200	25,850	25,502,199
	Merged	982.4	699	1,395	15,205	200	28,561	28,057,682
GZ	Trinity	1,516.5	1,233	2,096	14,778	200	18,832	28,558,098
	Velvet	1,252.2	1,010	1,668	13,073	200	12,691	15,891,637
	CLC	989.8	706	1,438	15,359	200	26,445	26,174,934
	Merged	984.9	715	1,415	15,359	200	28,984	28,547,216

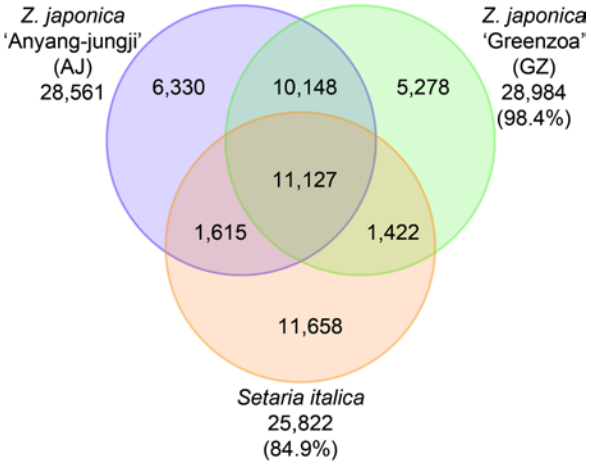


Figure I-6. Transcript homology of two zoysiagrass cultivars and *Setaria italica*. Venn diagram shows the number of orthologous transcripts (E-value < $1.0e^{-15}$). The percentage in the brackets indicates the degree of transcriptome homology relative to AJ.

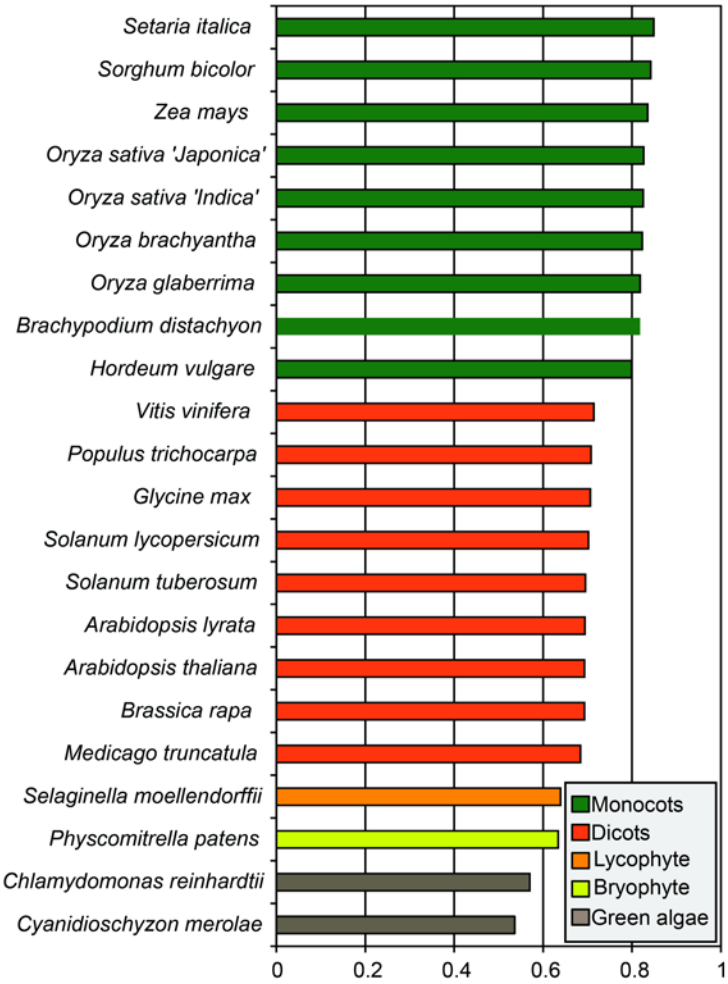


Figure I-7. Relative similarity of zoysiagrass transcriptome with other plants. The zoysiagrass unigenes annotated with the NR database were matched by TBLASTX hit with E-value < $1.0e^{-15}$.

Table 7. Ortholog set of *Zoysia japonica* 'Anyang-jungji' (AJ) and *Zoysia japonica* 'Greenzoa' (GZ).

AJ_transcriptome	GZ_transcriptome	Identity	Hit_length	Annotation
aj_contig_1	gz_contig_21419	100	260	#N/A
aj_contig_2	gz_contig_9606	100	722	gi|414879584|tpg|DAA56715.1|
aj_contig_3	gz_contig_15354	98.1	473	gi|242057859|ref|XP_002458075.1|
aj_contig_5	gz_contig_21764	100	188	gi|357115058|ref|XP_003559309.1|
aj_contig_6	gz_contig_14089	99.38	959	gi|115489472|ref|NP_001067223.1|
aj_contig_7	gz_contig_11591	100	527	gi|115454207|ref|NP_001050704.1|
aj_contig_9	gz_contig_2847	100	1070	gi|115437984|ref|NP_001043429.1|
aj_contig_10	gz_contig_9127	100	623	gi|212276074|ref|NP_001130513.1|
aj_contig_12	gz_contig_3539	95.07	2189	gi|242063612|ref|XP_002453095.1|
aj_contig_13	gz_contig_8608	99.68	923	gi|357137124|ref|XP_003570151.1|
.	.	.	.	.
.	.	.	.	.
.	.	.	.	.
aj_contig_34221	gz_contig_32902	92.22	539	gi|116317884|emb|CAH65912.1|

- Full data set is available at http://ipdgl.snu.ac.kr

phylogenetic relationship was established (Figure I-8; Table 1). Consistently with transcriptome homology, zoysiagrass *ACTIN* is most similar to that of *S. italica*, *S. bicolor*, and other monocot plants. These data support that the zoysiagrass transcriptome was precisely assembled, and also demonstrate that zoysiagrass is genetically closest to *S. italica* among monocot species.

GO Term Analysis and Profiling of Differentially Expressed Genes (DEGs)

Functional annotation of zoysiagrass transcripts was performed using Blast2GO (Götz et al., 2008). From a complete set of *de novo* assembled zoysiagrass transcripts, 17,040 were assigned for their functions (Table 8). They were classified into 44 major GO terms, where both AJ and GZ transcriptomes show very similar GO term distributions (Figure I-9A).

When expression levels of all orthologous transcript pairs were compared between AJ and GZ (Figure I-6; Table 7), a total of 1,448 NR transcript pairs were identified as significant DEGs using DESeq ($P < 0.01$) (Figure I-10A), where 1,307 AJ transcripts were more abundant than in GZ, whereas only 141 transcripts were less abundant. Among them, 836 upregulated DEGs and 81 downregulated DEGs were assigned to GO terms with 39 different categories (Pearson's χ^2 test, $P < 0.05$) (Figures I-9, I-10B). Notably, a significant number of DEGs were assigned to GO term 'pigmentation' (GO:0043473), suggesting that

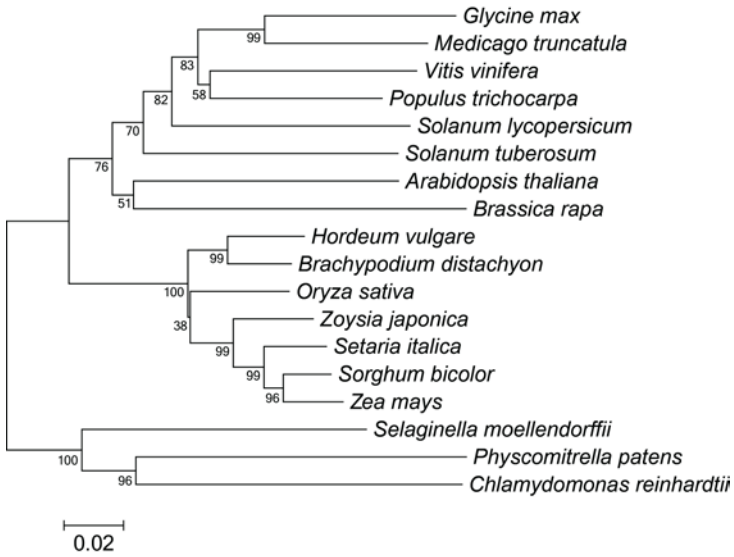
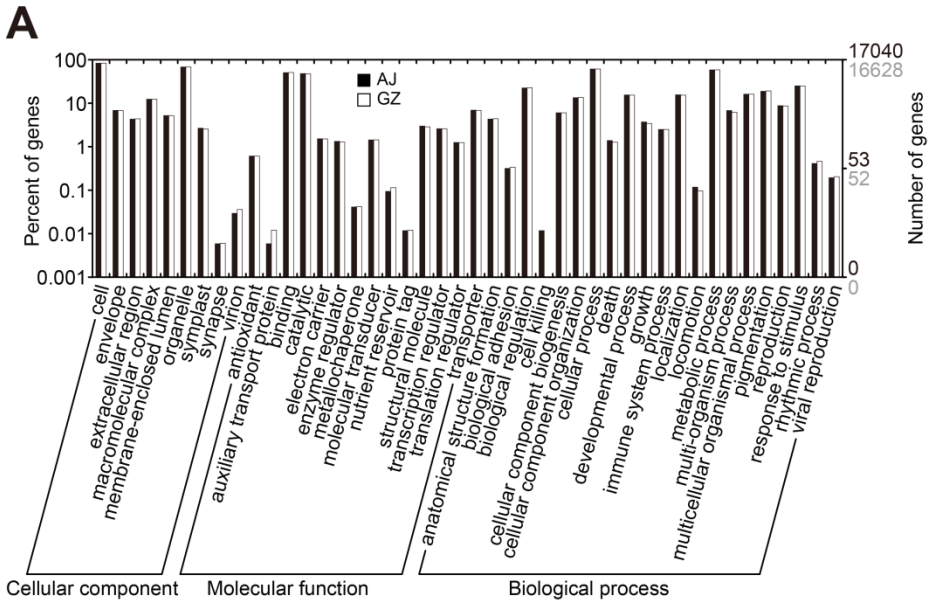


Figure I-8. Phylogenetic tree based upon the *β-ACTIN* sequences from various plant species. The tree was constructed by using the maximum-likelihood method with MEGA 5.2 based on the ClustalX-generated multiple sequence alignment. The topological stability of the tree was evaluated by 1,000 bootstrap replications, and the bootstrapping percentage values are indicated by the numbers at the tree nodes. The GenBank accessions of *β-ACTIN* sequences used for phylogenetic analysis are listed in Table 1.

Table 8. GO statistics of *Zoysia japonica* 'Anyang-jungji' (AJ) transcriptome.

Contig	GO ID							
aj_contig_1	GO:0070838	GO:0005739	GO:0008233	GO:0009507	GO:0006508	GO:0030003		
aj_contig_4	GO:0005829	GO:0016887	GO:0005634	GO:0006511	GO:0005524	GO:0005886	GO:0008540	
aj_contig_6	GO:0000325	GO:0015991	GO:0033179	GO:0005774	GO:0009506	GO:0005794	GO:0046961	GO:0005886
aj_contig_7	GO:0032578	GO:0005886	GO:0022626	GO:0005509	GO:0009705			
aj_contig_8	GO:0016760	GO:0005739	GO:0000139	GO:0016021	GO:0005886	GO:0005783	GO:0030244	
aj_contig_9	GO:0005739							
aj_contig_10	GO:0006468	GO:0006487	GO:0005737	GO:0045727	GO:0004674	GO:0005634	GO:0005524	
.	.							
.	.							
.	.							
aj_contig_28550	GO:0016746							
aj_contig_28551	GO:0005737							

- Full data set is available at http://ipdgl.snu.ac.kr


A
Percent of genes
100
10
1
0.1
0.01
0.001
AJ
GZ
17040
16628
53
52
0
0
Number of genes
cell
envelope
extracellular region
macromolecular complex
membrane-enclosed lumen
organelle
symplast
synapse
virion
antioxidant
auxiliary transport protein
binding
catalytic
electron carrier
enzyme regulator
metallochaperone
molecular transducer
nutrient reservoir
protein tag
structural molecule
transcription regulator
translation regulator
transporter
anatomical structure formation
biological adhesion
biological regulation
cell killing
cellular component biogenesis
cellular component organization
cellular process
death
developmental process
growth
immune system process
localization
locomotion
metabolic process
multi-organism process
multicellular organismal process
pigmentation
reproduction
response to stimulus
rhythmic process
viral reproduction
Cellular component
Molecular function
Biological process

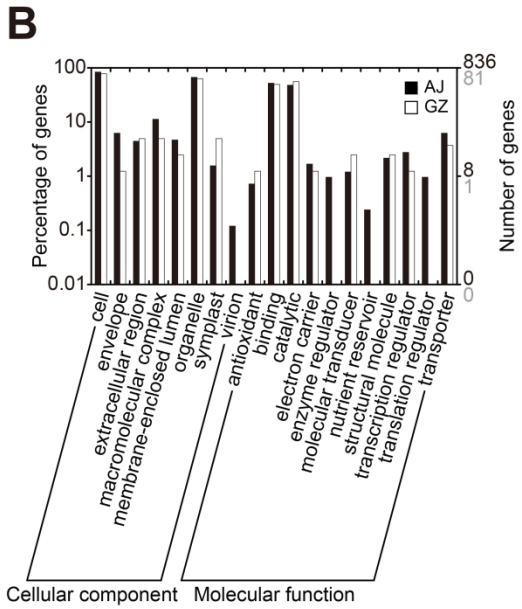

B
Percentage of genes
100
10
1
0.1
0.01
AJ
GZ
836
81
8
1
0
0
Number of genes
cell
envelope
extracellular region
macromolecular complex
membrane-enclosed lumen
organelle
symplast
virion
antioxidant
binding
catalytic
electron carrier
enzyme regulator
molecular transducer
nutrient reservoir
structural molecule
transcription regulator
translation regulator
transporter
Cellular component
Molecular function

Figure I-9. Gene ontology classification of zoysiagrass transcriptome. GO terms for each zoysiagrass (*Zoysia japonica* 'Anyang-jungji' (AJ) and *Zoysia japonica* 'Greenzoa' (GZ)) transcripts were assigned based on significant TBLASTX hits (E-value < $1.0e^{-15}$) against the NR database. (A) The results are summarized in three main categories (biological process, molecular function, and cellular component) and 44 subcategories. (B) GO classification of DEGs between AJ and GZ.

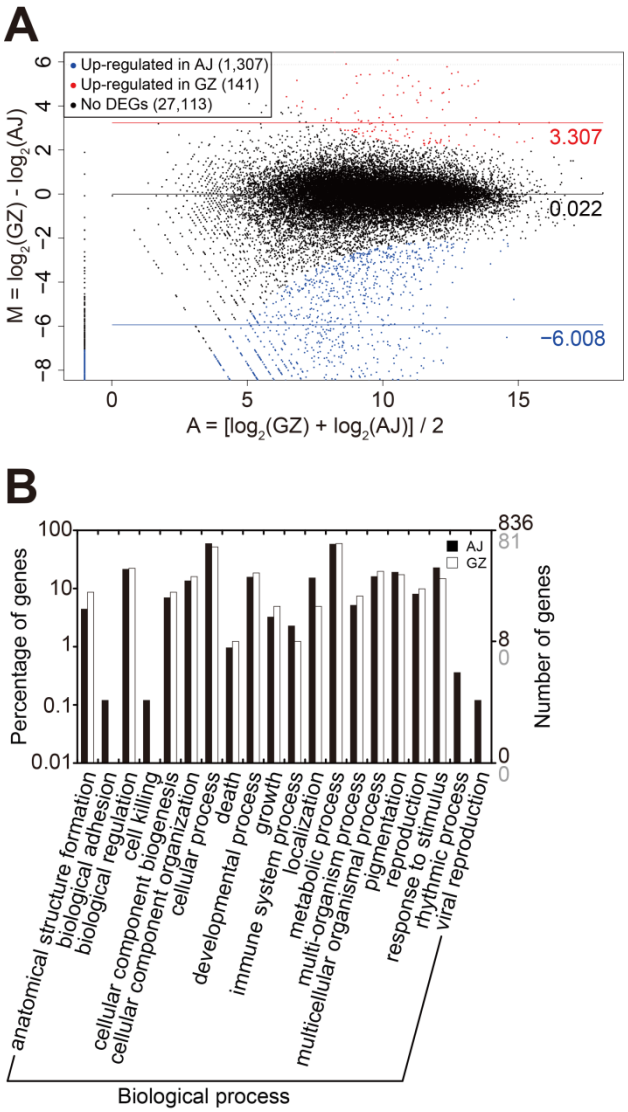

A
Up-regulated in AJ (1,307)
Up-regulated in GZ (141)
No DEGs (27,113)
3.307
0.022
−6.008
M = log2(GZ) - log2(AJ)
A = [log2(GZ) + log2(AJ)] / 2
B
AJ
GZ
Percentage of genes
Number of genes
anatomical structure formation
biological adhesion
biological regulation
cell killing
cellular component biogenesis
cellular component organization
cellular process
death
developmental process
growth
immune system process
localization
metabolic process
multi-organism process
multicellular organismal process
pigmentation
reproduction
response to stimulus
rhythmic process
viral reproduction
Biological process

Figure I-10. Differentially expressed genes between *Zoysia japonica* ‘Anyang-jungji’ (AJ) and *Zoysia japonica* ‘Greenzoa’ (GZ) spike tissues. (A) MA plot of differentially expressed transcripts identified in AJ and GZ. The X axis represents the mean expression level, and the Y axis represents the $\log_2$ fold-change of GZ transcripts over AJ. Red and blue dots represent DEGs that are significantly abundant in GZ and AJ, respectively, at *P*-value < 0.05. Horizontal lines and the values to the right represent the average M-values of corresponding groups of DEGs and non-DEGs. (B) GO classification of the DEGs. GO terms of *Z. japonica* unigenes are based on significant hits against the NR database.

these DEGs are responsible for differential anthocyanin accumulation between AJ and GZ spikes (Figure I-10B). To understand the genetic machinery of differential anthocyanin accumulation between AJ and GZ, expression profiles of anthocyanin biosynthesis-related genes were further examined.

Anthocyanin Biosynthesis Genes in Zoysiagrass

The anthocyanin biosynthesis pathway is branched from the general phenylpropanoid pathway that is carried out by sequential actions of a number of enzymes such as CHS, CHI, F3H, DFR, ANS, and several GTs (Lu and Rausher, 2003). Major genes involved in the anthocyanin biosynthesis pathway have been characterized in several plant species, and the overall pathway appears to be highly conserved in angiosperms. Anthocyanin biosynthesis in zoysiagrass was also postulated to take place in a similar manner to other *Gramineae* species (Figure I-11A).

Accumulation of anthocyanins such as cyanidin and petunidin in AJ spikes allowed investigating whether different coloration between AJ and GZ resulted from differential expression of anthocyanin biosynthesis genes. According to the Blast2GO annotation, 22 candidates were predicted to encode anthocyanin biosynthesis enzymes in zoysiagrass–(Figure I-11B; Table 9). As showed in Figure 11B, expression levels of *PAL*s, *CHI*s, *F3H*s, and *UFGT*s were not significantly different between AJ and GZ. *ZjDFR1* and *ZjANS1* were more highly expressed in AJ relative to GZ, whereas the expressions

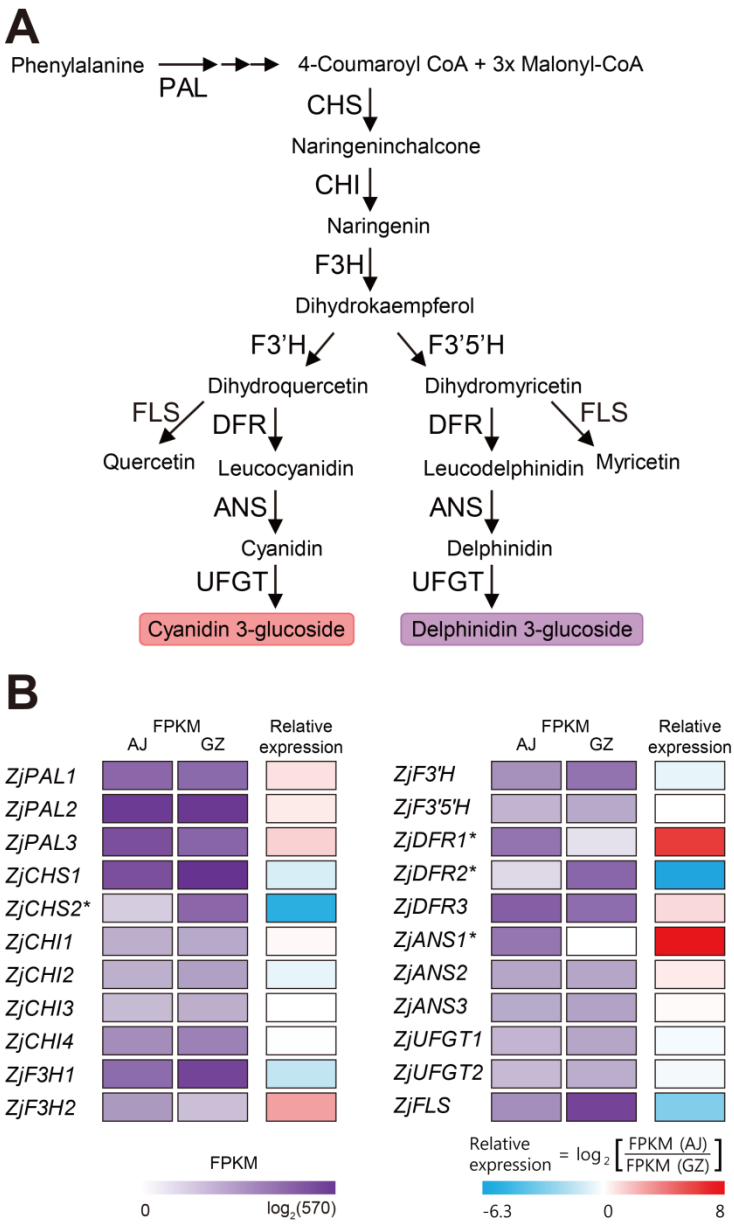

A
Phenylalanine
PAL
4-Coumaroyl CoA + 3x Malonyl-CoA
CHS
Naringeninchalcone
CHI
Naringenin
F3H
Dihydrokaempferol
F3'H
F3'5'H
Dihydroquercetin
Dihydromyricetin
FLS
DFR
DFR
FLS
Quercetin
Leucocyanidin
Leucodelphinidin
Myricetin
ANS
ANS
Cyanidin
Delphinidin
UFGT
UFGT
Cyanidin 3-glucoside
Delphinidin 3-glucoside
B
FPKM
AJ
GZ
Relative expression
ZjPAL1
ZjPAL2
ZjPAL3
ZjCHS1
ZjCHS2*
ZjCHI1
ZjCHI2
ZjCHI3
ZjCHI4
ZjF3H1
ZjF3H2
ZjF3'H
ZjF3'5'H
ZjDFR1*
ZjDFR2*
ZjDFR3
ZjANS1*
ZjANS2
ZjANS3
ZjUFGT1
ZjUFGT2
ZjFLS
FPKM
0
log2(570)
Relative expression = log2 [FPKM (AJ) / FPKM (GZ)]
-6.3
0
8

Figure I-11. Zoysiagrass transcripts involved in general anthocyanin biosynthesis pathway. (A) A schematic view of the core anthocyanin biosynthesis pathway. ANS, anthocyanidin synthase; CHS, chalcone synthase; DFR, dihydroflavonol reductase; F3H, flavanone 3-hydroxylase; F3'H, flavonol 3'-hydroxylase; F3'5'H, flavonol 3',5'-hydroxylase; FLS, flavonol synthase; PAL, phenylalanine ammonia lyase; UFGT, UDP-glucose:flavonoid 3-*O*-glucosyltransferase. (B) Heatmap of digital expression of candidate transcripts related to anthocyanin biosynthesis pathway in zoysiagrass. The log2-transformed FPKM values and relative expression (AJ vs. GZ) values are represented by the color map. Asterisks indicate statistical DEGs.

of *ZjCHS2*, *ZjDFR2*, and *ZjFLS* were significantly lower in AJ (Table 10). These findings suggest that *ZjDFR1* and *ZjANS1* are strong candidates leading to anthocyanin biosynthesis and purple pigmentation in AJ, and that the lack of their expression, or little if any, may result in the failure of anthocyanin accumulation in GZ.

Spatiotemporal Expression of Anthocyanin Biosynthesis Genes in Zoysiagrass

To verify the expression levels of DEGs identified from transcriptome analysis, qRT-PCR analyses on AJ and GZ spike tissues were performed at six developmental stages (Figures I-12, I-13). In AJ, in accordance with the increasing level of anthocyanin pigmentation (Figure I-2A), expressions of both *ZjDFR1* and *ZjANS1* significantly increased as the spike developed, whereas their expression was contrastingly low in developing GZ spikes until stage S5 (Figure I-12). This also supports transcriptome data, in which both *ZjDFR1* and *ZjANS1* transcripts were found to be highly abundant only in purple AJ spikes (Figure I-11B). By contrast, *ZjANS2*, *ZjANS3*, *ZjDFR2*, and *ZjDFR3* did not display meaningful expression patterns throughout the developmental stages (Figure I-12). For example, *ZjANS2* and *ZjDFR3* were constantly expressed at all stages, but *ZjDFR2* expression was highest in early spikelets and gradually decreased in both AJ and GZ spikes toward maturation (Figure I-12). Therefore, both *ZjDFR1* and *ZjANS1* were hypothesized developmentally to be regulated to control

Table 9. **Zoysiagrass unigenes used for qRT-PCR analysis.**

Gene	REFSEQ match	Accession No.	E-value	ID[a]	Region of ID[b]	% Cov[c]	Amplicon region[d]
ZjPAL1	*SiPAL*	XP_004953154.1	0	93	*1-718*	99	551-639 (1,884)
ZjPAL2	*ZmPAL*	NP_001241797.1	0	94	*1-701*	99	997-1,113 (2,106)
ZjPAL3	*SiPAL*	XP_004976238.1	0	87	230-702*	99	576-681 (1,425)
ZjCHS1	*SiCHS*	XP_004979391.1	0	95	*1-399*	99	896-983 (1,206)
ZjCHS2	*ZmCHS*	NP_001149508.1	1.00E-127	84	178-412*	98	263-355 (690)
ZjCHI1	*AtCHI*	NP_567140.1	5.00E-37	34	72-275*	48	526-633 (1,284)
ZjCHI2	*ZmCHI*	NP_001149585.1	8.00E-88	84	112-274*	99	232-338 (495)
ZjCHI3	*BdCHI*	XP_003571233.1	3.00E-100	77	68-265*	99	139-256 (627)
ZjCHI4	*SiCHI*	XP_004981264.1	3.00E-100	84	54-233*	98	160-261 (537)
ZjF3H1	*ZmF3H1*	NP_001105695.1	0	88	*1-336*	90	310-429 (1,116)
ZjF3H2	*SiF3H*	XP_004985921.1	0	89	55-336*	98	22-141 (855)
ZjF3'H	*BdF3'H*	XP_003577475.1	0	77	21-529	96	1,287-1,383 (1,599)
ZjF3'5'H	*SiF3'5'H*	XP_004984278.1	1.00E-32	97	456-517	92	98-201 (201)
ZjDFR1	*SiDFR*	XP_004969260.1	0	86	*1-365*	96	578-716 (1,116)
ZjDFR2	*ZmDFR1*	NP_001105644.1	0	89	*1-331*	99	265-364 (990)
ZjDFR3	*BdDFR*	XP_003567727.1	0	83	10-329*	97	144-262 (982)
ZjANS1	*ZmANS*	NP_001106074.1	5.00E-180	74	*1-395*	99	132-244 (1,179)
ZjANS2	*SiANS*	XP_004964770.1	1.00E-137	90	117-343*	99	100-216 (685)

ZjANS3	*ZmANS*	NP_001152138.1	8.00E-142	63	*1-314*	95	379-496 (987)
ZjUFGT1	*SiUFGT1*	XP_004955859.1	6.00E-147	72	187-470*	99	320-407 (855)
ZjUFGT2	*SiUFGT1*	XP_004955859.1	1.00E-143	70	187-470*	99	321-408 (856)
ZjFLS	*SiFLS*	XP_004954034.1	0	79	*3-333*	98	736-823 (1,011)
ZjMYB1	*AtMYB-IF35*	NP_001105092.1	2.00E-67	94	*1-109	99	164-276 (329)
ZjMYB2	*AtMYB4*	NP_195574.1	9.00E-68	98	*1-105	69	297-402 (451)

[a]Percentage sequence identity (ID), based on amino acid sequence.

[b]Asterisks at left and right of the region indicate the presence of predicted start and stop codons, respectively.

[c]Percentage coverage, the percentage of total predicted protein length present in unigene sequence.

[d]The nucleotide region within each unigene that qRT-PCR primers were designed to amplify, with total unigene coding sequence length in parentheses.

Table 10. FPKM value of anthocyanin biosynthesis related transcripts in *Zoysia japonica* 'Anyang-jungji' (AJ) and *Zoysia japonica* 'Greenzoa' (GZ).

Gene	AJ transcript	GZ transcript	AJ FPKM*	GZ FPKM*
ZjPAL1	aj_contig_963	gz_contig_1100	120.11	106.28
ZjPAL2	aj_contig_171	gz_contig_514	551.02	514.24
ZjPAL3	aj_contig_93	gz_contig_334	255.84	132.20
ZjCHS1	aj_contig_1774	gz_contig_821	255.10	674.06
ZjCHS2	aj_contig_28560	gz_contig_3068	2.80	117.62
ZjCHI1	aj_contig_17328	gz_contig_11760	11.01	14.02
ZjCHI2	aj_contig_20572	gz_contig_5945	10.25	20.68
ZjCHI3	aj_contig_20378	gz_contig_12665	6.19	10.68
ZjCHI4	aj_contig_22952	gz_contig_12577	36.57	54.30
ZjF3H1	aj_contig_11511	gz_contig_2786	99.24	367.83
ZjF3H2	aj_contig_12276		26.01	5.09
ZjDFR1	aj_contig_14208		78.95	1.12
ZjDFR2	aj_contig_28561	gz_contig_117	1.73	122.93
ZjDFR3	aj_contig_2500	gz_contig_6946	153.48	96.34
ZjANS1	aj_contig_7856		72.92	0.29
ZjANS2	aj_contig_898	gz_contig_19130	14.30	15.52
ZjANS3	aj_contig_6803	gz_contig_4405	13.96	17.99
ZjFLS	aj_contig_14235	gz_contig_2392	35.69	361.12
ZjUFGT1	aj_contig_9283	gz_contig_4733	7.95	16.06
ZjUFGT2	aj_contig_9283	gz_contig_4735	6.72	11.22
ZjF3'H	aj_contig_8021	gz_contig_5629	33.48	80.64
ZjF3'5'H	aj_contig_24141	gz_contig_24420	8.20	14.28
ZjMYB1	aj_contig_13033	gz_contig_11866	15.94	38.77
ZjMYB2	aj_contig_13209	gz_contig_7425	19.57	9.27

* FPKM, Fragments per kilobase of exon per million fragments mapped.

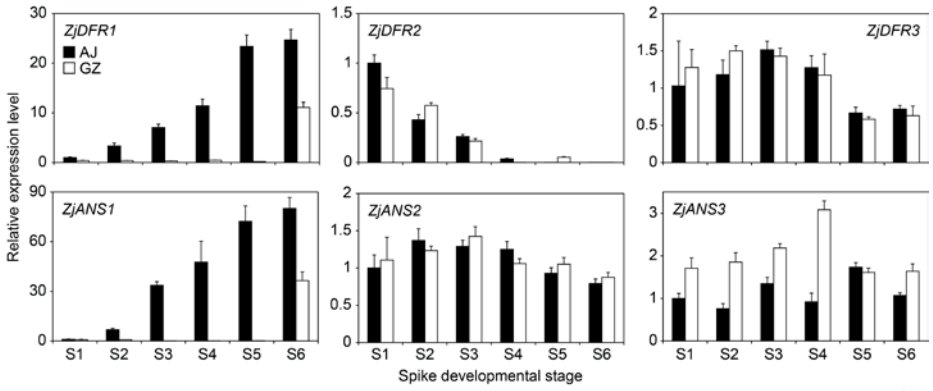


Figure I-12. Expression levels of DFR and ANS genes in *Zoysia japonica* 'Anyang-jungji' (AJ) and *Zoysia japonica* 'Greenzoa' (GZ) spikes at different developmental stages. The relative expression level of each transcript was determined by qRT-PCR, in which all values are normalized relative to the mean abundance of *β-ACTIN*. The expression levels are presented relative to the AJ gene abundance at stage S1. Bars represent means ± SD from triplicate biological repeats.

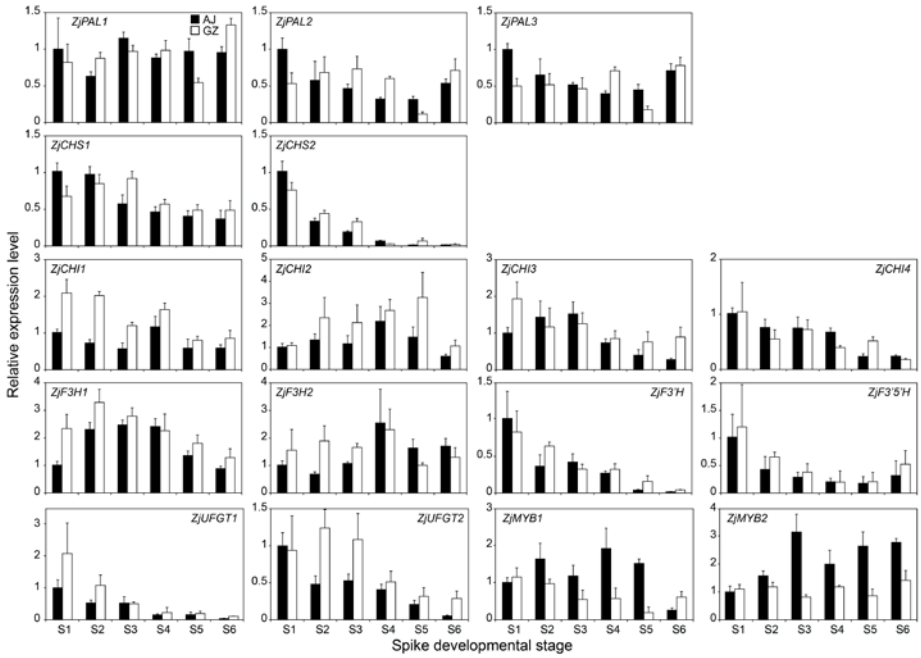


Figure I-13. Expression levels of anthocyanin biosynthesis genes at different developmental stages of *Zoysia japonica* 'Anyang-jungji' (AJ) and *Zoysia japonica* 'Greenzoa' (GZ) spikes. The relative expression level of each transcript was determined by qRT-PCR. All values are normalized relative to the mean abundance of *β-ACTIN* at each stage. Bars represent means ± SD from triplicate biological repeats.

the synthesis of anthocyanin pigments in purple zoysiagrass spikes but the absence of their expression leads to no pigmentation in the green spike.

Tissue-specific expressions of zoysiagrass anthocyanin synthesis genes were examined in other tissues such as stolon, young and old leaves, and root. As in the spike, *ZjDFR1* was also highly expressed in the AJ stolon, another tissue showing strong purple pigmentation, while low levels of its expression were observed in the other tissues (Figure I-14). *ZjANS1* was also expressed in the AJ stolon, although not as much as in the AJ spike, but its expression was rarely detectable in other tissues of AJ and GZ (Figure I-14). Three *PAL* genes (*ZjPAL1~3*) were highly expressed in the AJ root, but their expression probably had no relationship with tissue-specific anthocyanin biosynthesis, as the AJ root did not accumulate purple pigments. The quantitative expression analysis revealed that besides *ZjDFR1* and *ZjANS1*, no other anthocyanin biosynthesis-related genes were likely involved in the tissue-specific pigmentation in zoysiagrass (Figure I-14).

ZjDFR1 and *ZjANS1* Confer Different Anthocyanin Accumulation in Zoysiagrass

Several anthocyanin biosynthesis genes in angiosperm have been reported to exist in multiple copies. In particular, *DFR* and *ANS* homologs are categorized into several phylogenetic subclades and each subclade is expected to have different functional activity in the pathway

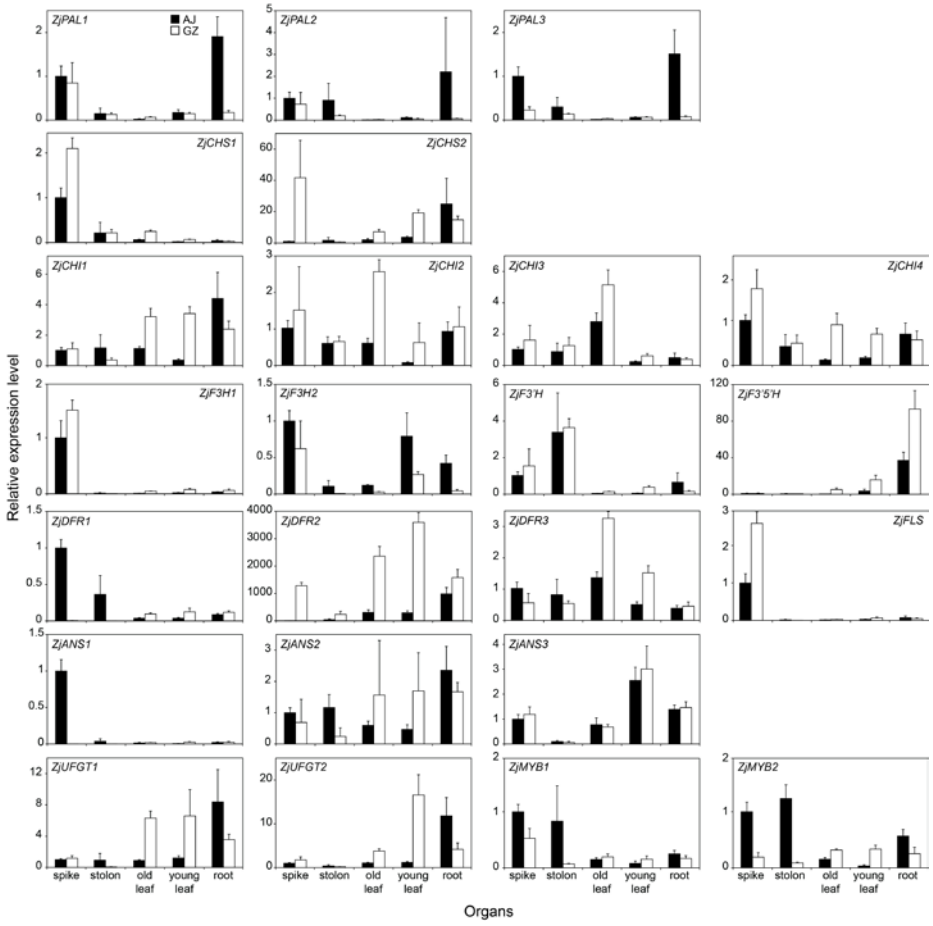


Figure I-14. Expression levels of anthocyanin biosynthesis genes in diverse tissues of *Zoysia japonica* 'Anyang-jungji' (AJ) and *Zoysia japonica* 'Greenzoa' (GZ). The relative expression level of each transcript was determined by qRT-PCR. All values are normalized relative to the mean abundance of *β-ACTIN*. Bars represent means ± SD from triplicate biological repeats.

(Des Marais and Rausher, 2008; Zhou et al., 2013). In an effort to assess the distinct functions of zoysiagrass DFR and ANS in anthocyanin biosynthesis, the phylogenetic relationships of these proteins were analyzed with other homologs that have been previously characterized (Table 11). When compared with DFR protein sequences from 11 other plant species, ZjDFR1 was assigned to the same group with other DFR homologs, whereas ZjDFR2 and ZjDFR3 were distantly related (Figure I-15A; Table 11). Similarly, ZjANS1 was grouped into the same clade with 10 other ANS homologs, but ZjANS2 and ZjANS3 were in the outgroup (Figure I-15B; Table 11). These results indicated that ZjDFR1 and ZjANS1 had similar structures and functions, and thus, their proper spatiotemporal expression may be required for the anthocyanin biosynthesis in zoysiagrass.

Since differential anthocyanin accumulations in the spike and stolon tissues were also observed in other zoysiagrass cultivars, tissue-specific upregulation of *ZjDFR1* and *ZjANS1* was examined to find out whether that is a common phenomenon in zoysiagrass plants with purple-colored spikes and stolons. Nine zoysiagrass cultivars with different spike and stolon colors were collected and analyzed for the anthocyanin contents (Figures I-16A, I-17A; Table 12). HPLC analysis revealed that significant amounts of anthocyanins accumulated in the spikes of six purple-pigmented cultivars, whereas no anthocyanin was detected in the other three green colored ones (Figure I-18). The green stolon cultivars had significantly low levels of ZjDFR1 and ZjANS1

Table 11. **Accession number of DFR and ANS homologous proteins.**

Gene product[a]	Accession number
AmDFR	P14721.1
AtDFR	NP_199094.1
GhDFR	P51105.1
HvDFR	P51106.1
LjDFR2	BAE19949.1
LjDFR3	BAE19950.1
LjDFR5	BAE19953.1
OsDFR	BAA36183.1
PhDFR	P14720.2
RhDFR	BAA12723.1
VmDFR	AAL89714.1
VvDFR	AAX12423.1
ZmDFR	NP_001152467.1
AtANS	NP_194019.1
GhANS	AAY15744.1
GtANS	BAE44202.1
MdANS	P51091.1
NtANS	BAM37963.1
OsANS	CAA69252.1
PcANS	ABB70119.1
TaANS	BAE98276.1
VvANS	NP_001268147.1
ZmANS	P41213.1

[a]Am, *Antirrhinum majus*, At, *Arabidopsis thaliana*; Gh, *Gerbera hybrid*; Gt, *Gentiana triflora*; Hv, *Hordeum vulgare*; Lj, *Lotus japonicus*; Md, *Malus domestica*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Pc, *Pyrus communis*; Ph, *Petunia hybrid*; Rh, *Rosa hybrid*; Ta, *Triticum aestivum*; Vm, *Vaccinium macrocarpon*; Vv, *Vitis vinifera*; Zm, *Zea mays*.

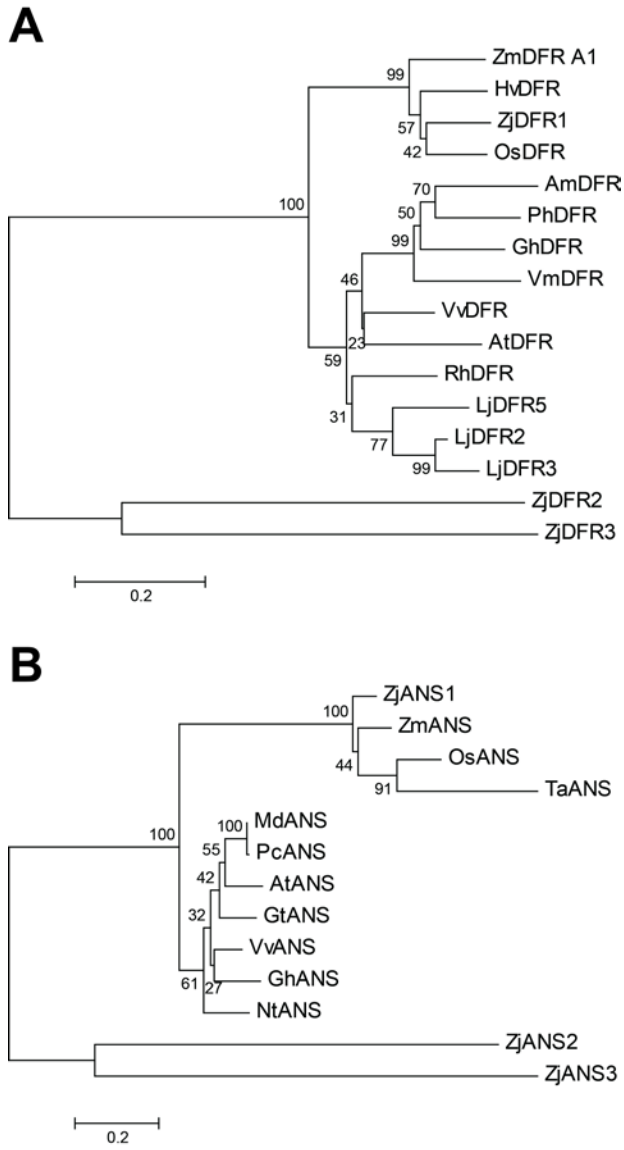

A
ZmDFR A1
HvDFR
ZjDFR1
OsDFR
AmDFR
PhDFR
GhDFR
VmDFR
VvDFR
AtDFR
RhDFR
LjDFR5
LjDFR2
LjDFR3
ZjDFR2
ZjDFR3
99
57
42
70
50
99
46
23
59
31
77
99
100
0.2
B
ZjANS1
ZmANS
OsANS
TaANS
MdANS
PcANS
AtANS
GtANS
VvANS
GhANS
NtANS
ZjANS2
ZjANS3
100
44
91
100
55
42
32
61
27
100
0.2

Figure I-15. Phylogenetic trees of the DFR and ANS protein families. (A) Phylogeny of the DFR protein family. (B) Phylogeny of the ANS protein family. Am, *Antirrhinum majus*, At, *Arabidopsis thaliana*; Gh, *Gerbera hybrid*; Gt, *Gentiana triflora*; Hv, *Hordeum vulgare*; Lj, *Lotus japonicus*; Md, *Malus domestica*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Pc, *Pyrus communis*; Ph, *Petunia hybrid*; Rh, *Rosa hybrid*; Ta, *Triticum aestivum*; Vm, *Vaccinium macrocarpon*; Vv, *Vitis vinifera*; Zm, *Zea mays*. Accession numbers of the proteins analyzed are listed in Table 11.

transcripts (Figures I-16, I-17), although their expression levels varied among purple-pigmented cultivars. These observations suggest that differential regulation of *ZjDFR1* and *ZjANS1* at the transcription level determines the rate of anthocyanin accumulation in zoysiagrass, which is expressed as distinct pigmentation in the spike and stolon tissues.

Isolation of Genes Encoding DFR and ANS Protein from Zoysiagrass

To verify whether differential anthocyanin pigmentation is exclusively determined at the transcription level, the structures of *ZjDFR1* and *AjANS1* genes were compared between AJ and GZ. *ZjDFR1* consisted of 1,458 bp of open reading frame (ORF) with 1,113 bp of coding sequence, whereas *ZjANS1* contained only 1,179 bp of ORF without intronic sequences (Figure I-19). Overall structures of these genes are highly similar to those of other monocot species such as maize, rice, and sorghum (Figure I-19). Furthermore, they shared higher than 98% of sequence identity at the amino acid level among different zoysiagrass cultivars (Figures I-20, I-21). Each of *ZjDFR1* and *ZjANS1* was found to have exactly the same coding sequence between AJ and GZ (Figure I-22), indicating that structures and functions of their gene products are the same, and therefore, the regulatory elements for transcriptional control might be more important for differential anthocyanin biosynthesis between AJ and GZ.

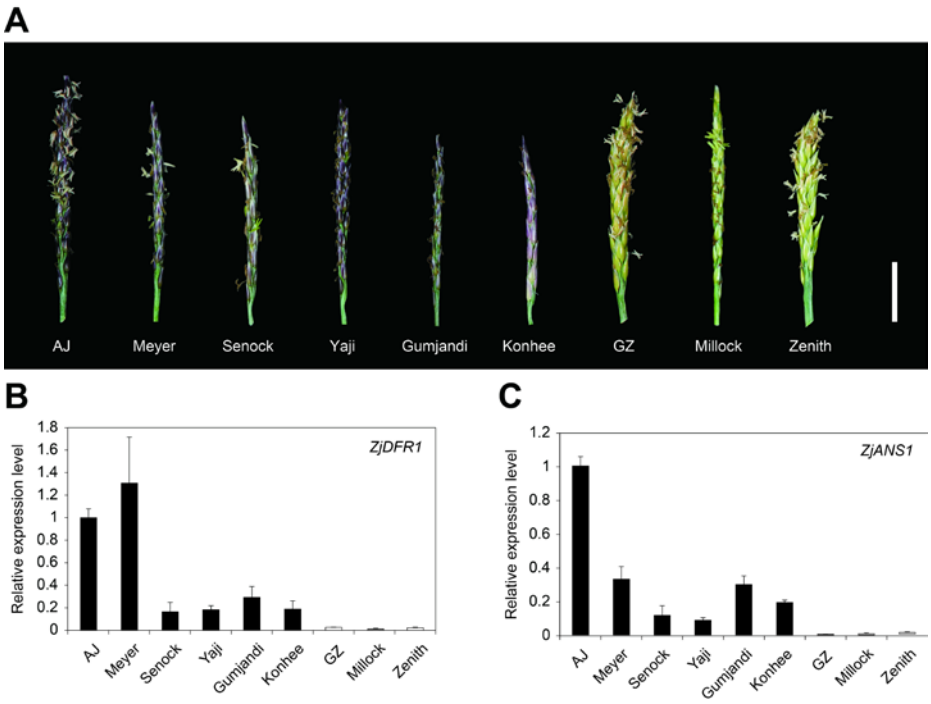


Figure I-16. Analysis of *ZjDFR1* and *ZjANS1* expression among zoysiagrass cultivars. (A) Representative spikes of nine zoysiagrass cultivars at developmental stage S6. Bar = 10 mm. Expression levels of *ZjDFR1* (B) and *ZjANS1* (C) genes in spike tissues determined by qRT-PCR. All values are normalized relative to the mean abundance of *β-ACTIN*. Bars represent means ± SD from triplicate biological repeats. AJ, *Zoysia japonica* 'Anyang-jungji'; GZ, *Zoysia japonica* 'Greenzoa'.

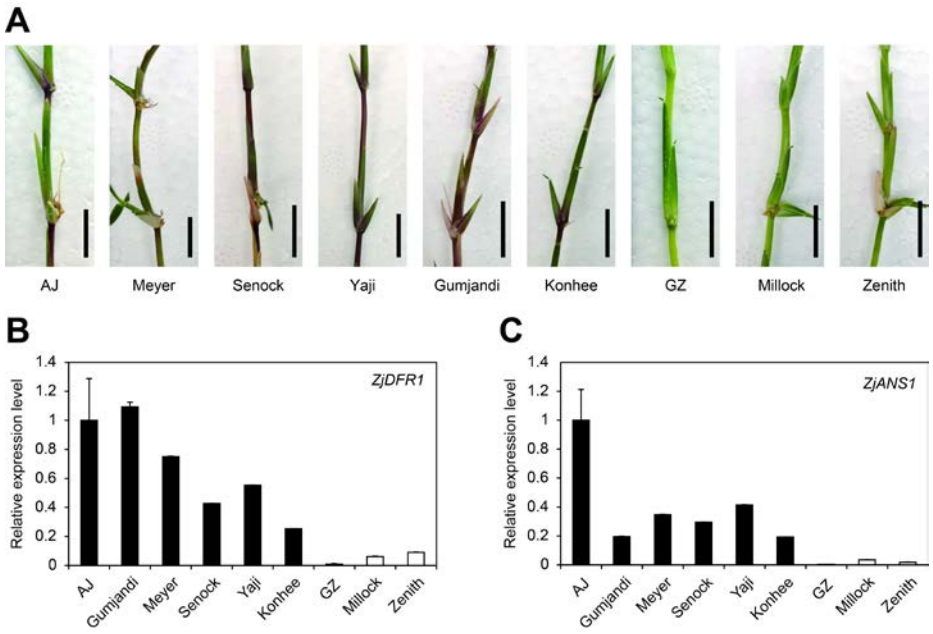


Figure I-17. Expression analysis of *ZjDFR1* and *ZjANS1* genes in stolon tissues of nine zoysiagrass cultivars. (A) Representative pictures of the stolon of nine zoysiagrass cultivars. Bar = 10 mm. Expression patterns of *ZjDFR1* (B) and *ZjANS1* (C) at stolon determined by qRT-PCR. All values are normalized relative to the mean abundance of *β-ACTIN*. Bars represent means ± SD from triplicate biological repeats. AJ, *Zoysia japonica* 'Anyang-jungji'; GZ, *Zoysia japonica* 'Greenzoa'.

Table 12. **Characteristics of *Zoysia* species.**

Species	Cultivar	Leaf color	Stolon color
Z. japonica	AJ	Green	Purple
	GZ	Green	Green
	Meyer	Green	Purple
	Yaji	Green	Purple
	Zenith	Green	Green
Z. matrella	Gumjandi	Green	Purple
Z. sinica × *Z. matrella*	Senock	Green	Purple
Zoysia cultivar	Konhee	Green	Purple
	Millock	Green	Green

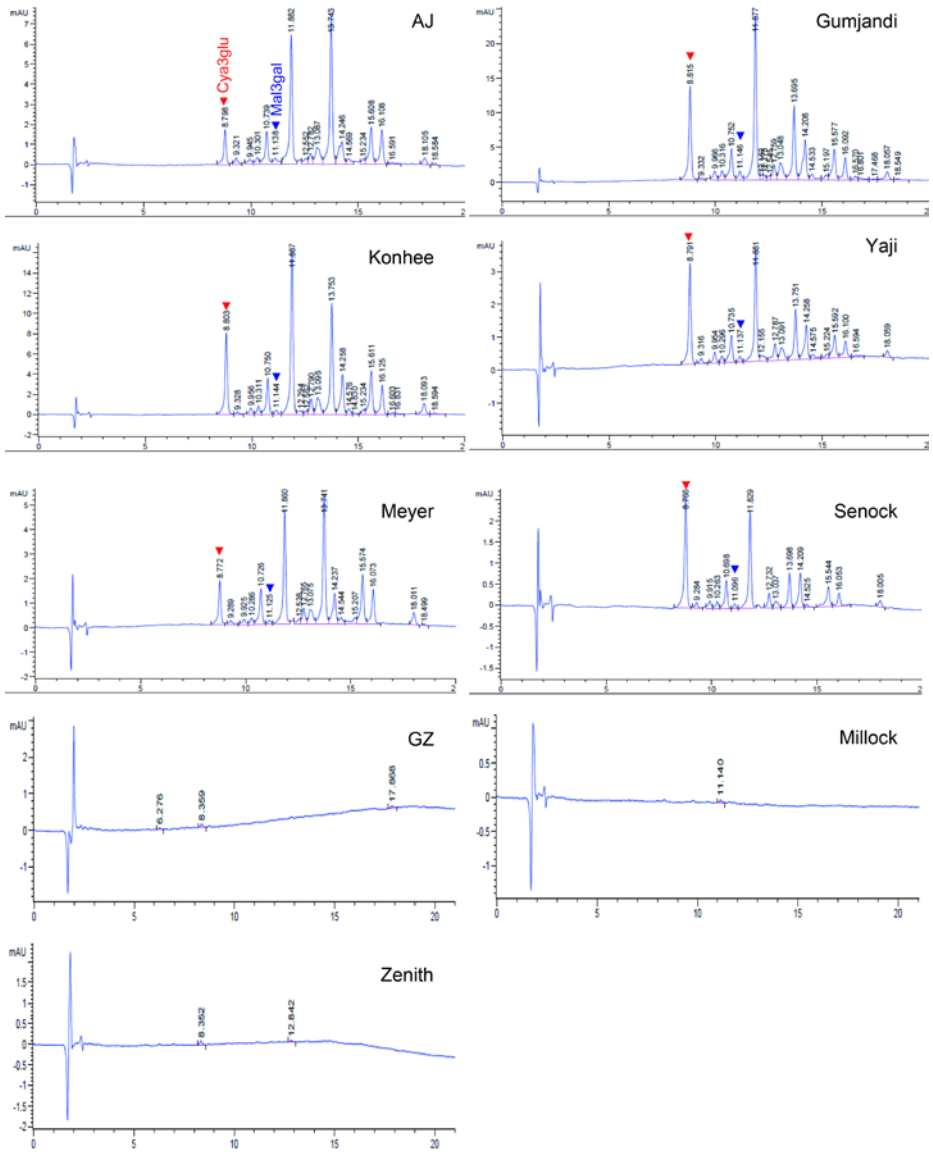


Figure I-18. HPLC profiles of anthocyanins present in stolon tissues of nine zoysiagrass cultivars. The chromatograms were recorded at 520 nm. Red and blue arrowheads indicate cyanidin-3-O-glucoside and malvidin-3-O-glucoside, respectively. AJ, *Zoysia japonica* ‘Anyang-jungji’; GZ, *Zoysia japonica* ‘Greenzoa’.

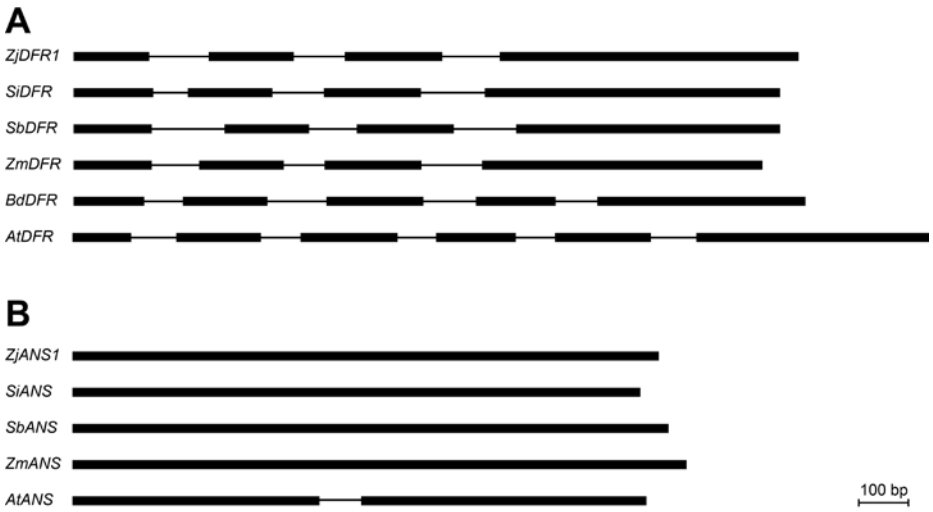


Figure I-19. Schematic representations of exon-intron structures of DFR and ANS proteins derived from different species. Closed bars and lines represent exons and introns, respectively. Zj, *Zoysia japonica*; Si, *Setaria italica*; Sb, *Sorghum bicolor*; Zm, *Zea mays*; Bd, *Brachypodium distachyon*; At, *Arabidopsis thaliana*.

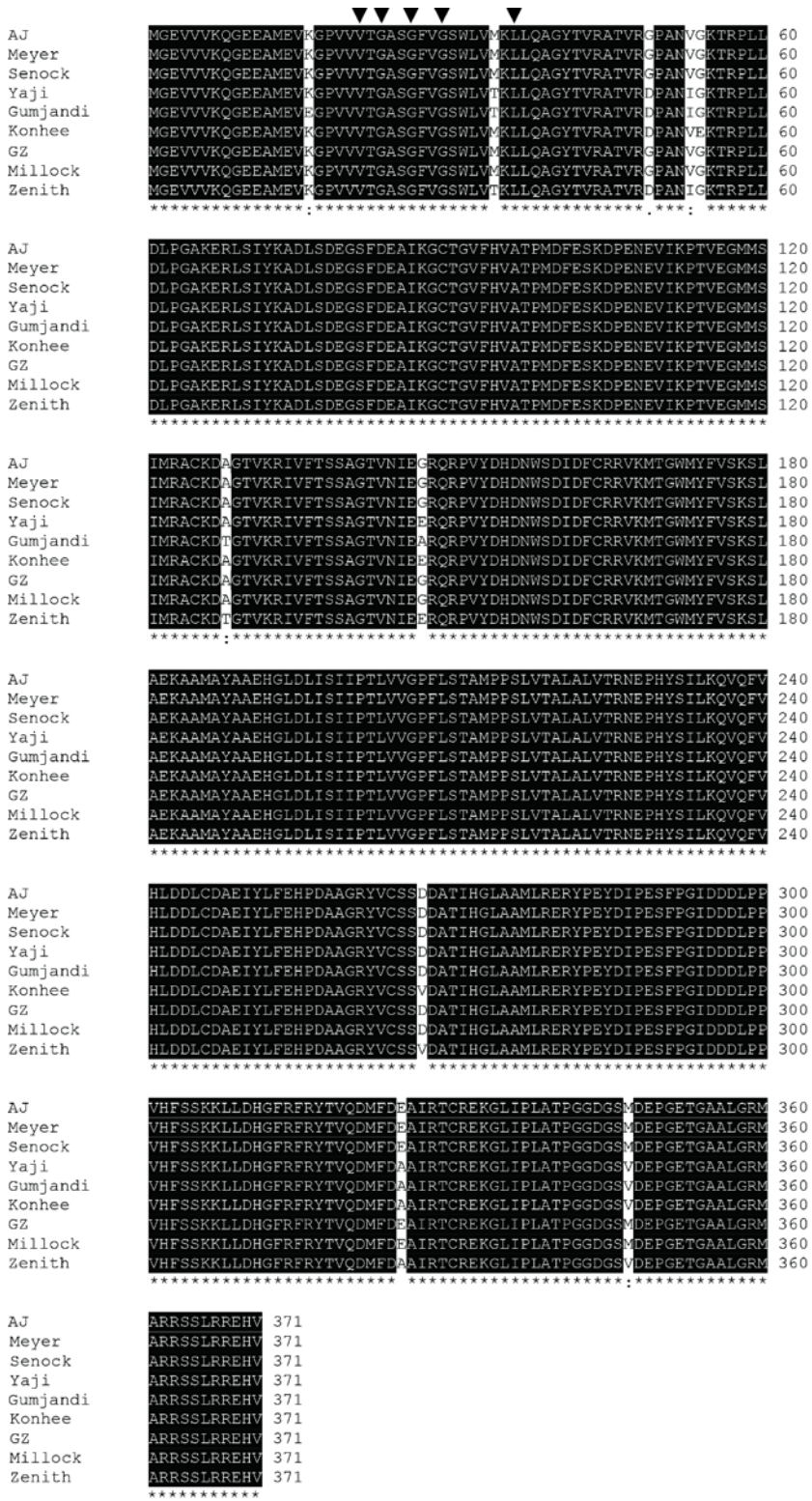
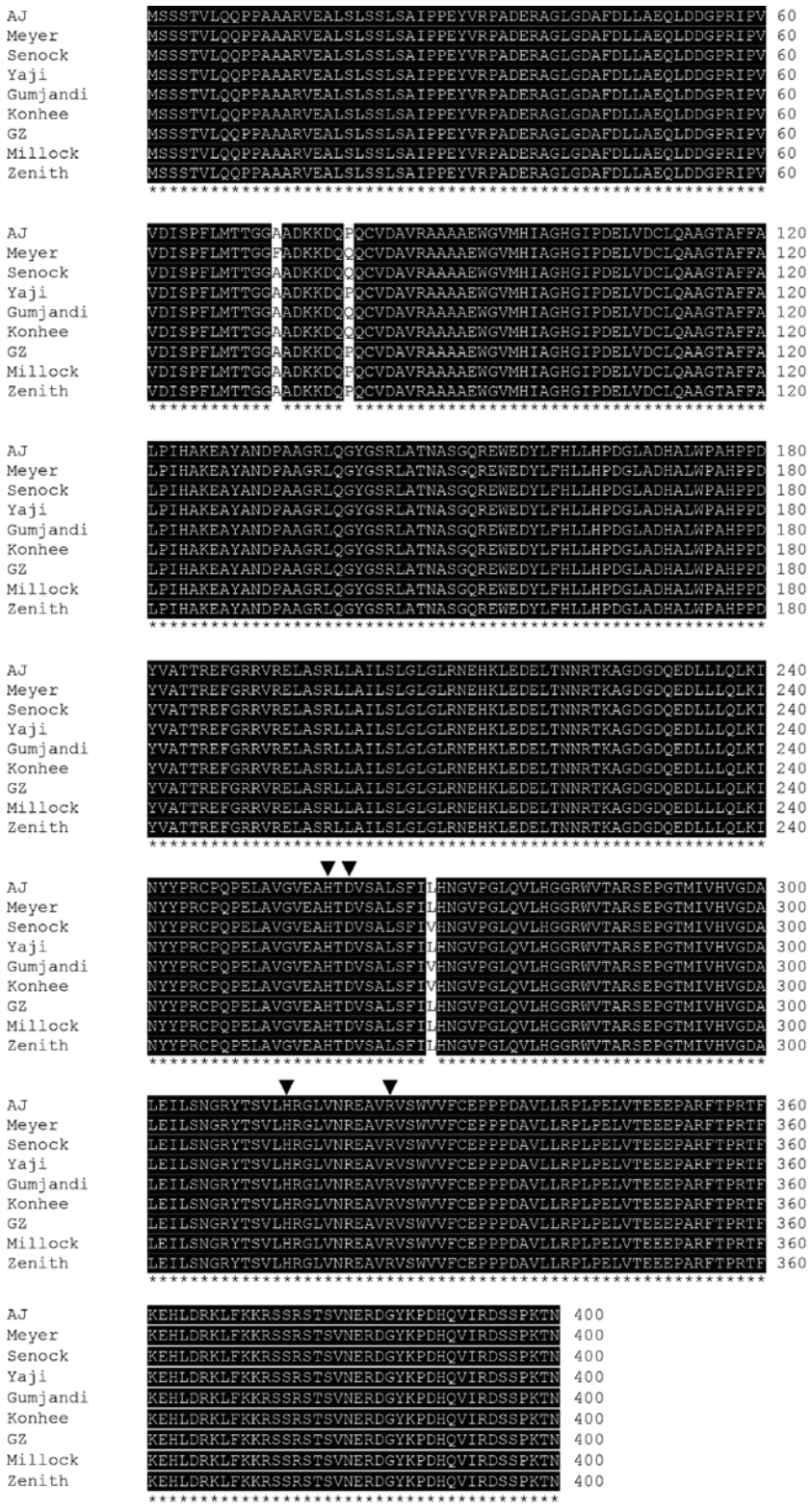

AJ
Meyer
Senock
Yaji
Gumjandi
Konhee
GZ
Millock
Zenith

Figure I-20. Sequence alignment of predicted ZjDFR1 proteins among nine zoysiagrass cultivars. Arrowheads on ZjDFR1 indicate conserved amino acid residues in the hydroxysteroid dehydrogenase/DFR superfamily (Tanaka et al., 1995).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Meyer MSSSTVLQQPPAAARVEALSLSSLSAIPPEYVRPADERAGLGDAFDLLAEQLDDGPRIPV 60
Senock MSSSTVLQQPPAAARVEALSLSSLSAIPPEYVRPADERAGLGDAFDLLAEQLDDGPRIPV 60
Yaji MSSSTVLQQPPAAARVEALSLSSLSAIPPEYVRPADERAGLGDAFDLLAEQLDDGPRIPV 60
Gumjandi MSSSTVLQQPPAAARVEALSLSSLSAIPPEYVRPADERAGLGDAFDLLAEQLDDGPRIPV 60
Konhee MSSSTVLQQPPAAARVEALSLSSLSAIPPEYVRPADERAGLGDAFDLLAEQLDDGPRIPV 60
GZ MSSSTVLQQPPAAARVEALSLSSLSAIPPEYVRPADERAGLGDAFDLLAEQLDDGPRIPV 60
Millock MSSSTVLQQPPAAARVEALSLSSLSAIPPEYVRPADERAGLGDAFDLLAEQLDDGPRIPV 60
Zenith MSSSTVLQQPPAAARVEALSLSSLSAIPPEYVRPADERAGLGDAFDLLAEQLDDGPRIPV 60
AJ VDISPFLMTTGGAADKKDQPQCVDAVRAAAAEWGVMHIAGHGIPDELVDCLQAAGTAFFA 120
Meyer VDISPFLMTTGGPADKKDQQQCVDAVRAAAAEWGVMHIAGHGIPDELVDCLQAAGTAFFA 120
Senock VDISPFLMTTGGAADKKDQQQCVDAVRAAAAEWGVMHIAGHGIPDELVDCLQAAGTAFFA 120
Yaji VDISPFLMTTGGAADKKDQPQCVDAVRAAAAEWGVMHIAGHGIPDELVDCLQAAGTAFFA 120
Gumjandi VDISPFLMTTGGAADKKDQQQCVDAVRAAAAEWGVMHIAGHGIPDELVDCLQAAGTAFFA 120
Konhee VDISPFLMTTGGAADKKDQQQCVDAVRAAAAEWGVMHIAGHGIPDELVDCLQAAGTAFFA 120
GZ VDISPFLMTTGGAADKKDQPQCVDAVRAAAAEWGVMHIAGHGIPDELVDCLQAAGTAFFA 120
Millock VDISPFLMTTGGAADKKDQPQCVDAVRAAAAEWGVMHIAGHGIPDELVDCLQAAGTAFFA 120
Zenith VDISPFLMTTGGAADKKDQPQCVDAVRAAAAEWGVMHIAGHGIPDELVDCLQAAGTAFFA 120
AJ LPIHAKEAYANDPAAGRLQGYGSRLATNASGQREWEDYLFHLLHPDGLADHALWPAHPPD 180
Meyer LPIHAKEAYANDPAAGRLQGYGSRLATNASGQREWEDYLFHLLHPDGLADHALWPAHPPD 180
Senock LPIHAKEAYANDPAAGRLQGYGSRLATNASGQREWEDYLFHLLHPDGLADHALWPAHPPD 180
Yaji LPIHAKEAYANDPAAGRLQGYGSRLATNASGQREWEDYLFHLLHPDGLADHALWPAHPPD 180
Gumjandi LPIHAKEAYANDPAAGRLQGYGSRLATNASGQREWEDYLFHLLHPDGLADHALWPAHPPD 180
Konhee LPIHAKEAYANDPAAGRLQGYGSRLATNASGQREWEDYLFHLLHPDGLADHALWPAHPPD 180
GZ LPIHAKEAYANDPAAGRLQGYGSRLATNASGQREWEDYLFHLLHPDGLADHALWPAHPPD 180
Millock LPIHAKEAYANDPAAGRLQGYGSRLATNASGQREWEDYLFHLLHPDGLADHALWPAHPPD 180
Zenith LPIHAKEAYANDPAAGRLQGYGSRLATNASGQREWEDYLFHLLHPDGLADHALWPAHPPD 180
AJ YVATTREFGRRVRELASRLLAILSLGLGLRNEHKLEDELTNNRTKAGDGDQEDLLLQLKI 240
Meyer YVATTREFGRRVRELASRLLAILSLGLGLRNEHKLEDELTNNRTKAGDGDQEDLLLQLKI 240
Senock YVATTREFGRRVRELASRLLAILSLGLGLRNEHKLEDELTNNRTKAGDGDQEDLLLQLKI 240
Yaji YVATTREFGRRVRELASRLLAILSLGLGLRNEHKLEDELTNNRTKAGDGDQEDLLLQLKI 240
Gumjandi YVATTREFGRRVRELASRLLAILSLGLGLRNEHKLEDELTNNRTKAGDGDQEDLLLQLKI 240
Konhee YVATTREFGRRVRELASRLLAILSLGLGLRNEHKLEDELTNNRTKAGDGDQEDLLLQLKI 240
GZ YVATTREFGRRVRELASRLLAILSLGLGLRNEHKLEDELTNNRTKAGDGDQEDLLLQLKI 240
Millock YVATTREFGRRVRELASRLLAILSLGLGLRNEHKLEDELTNNRTKAGDGDQEDLLLQLKI 240
Zenith YVATTREFGRRVRELASRLLAILSLGLGLRNEHKLEDELTNNRTKAGDGDQEDLLLQLKI 240
AJ NYYPRCPQPELAVGVEAHTDVSALSFILHNGVPGLQVLHGGRWVTARSEPGTMIVHVGDA 300
Meyer NYYPRCPQPELAVGVEAHTDVSALSFILHNGVPGLQVLHGGRWVTARSEPGTMIVHVGDA 300
Senock NYYPRCPQPELAVGVEAHTDVSALSFIVHNGVPGLQVLHGGRWVTARSEPGTMIVHVGDA 300
Yaji NYYPRCPQPELAVGVEAHTDVSALSFILHNGVPGLQVLHGGRWVTARSEPGTMIVHVGDA 300
Gumjandi NYYPRCPQPELAVGVEAHTDVSALSFIVHNGVPGLQVLHGGRWVTARSEPGTMIVHVGDA 300
Konhee NYYPRCPQPELAVGVEAHTDVSALSFIVHNGVPGLQVLHGGRWVTARSEPGTMIVHVGDA 300
GZ NYYPRCPQPELAVGVEAHTDVSALSFILHNGVPGLQVLHGGRWVTARSEPGTMIVHVGDA 300
Millock NYYPRCPQPELAVGVEAHTDVSALSFILHNGVPGLQVLHGGRWVTARSEPGTMIVHVGDA 300
Zenith NYYPRCPQPELAVGVEAHTDVSALSFILHNGVPGLQVLHGGRWVTARSEPGTMIVHVGDA 300
AJ LEILSNGRYTSVLHRGLVNREAVRVSWVVFCEPPPDAVLLRPLPELVTEEEPARFTPRTF 360
Meyer LEILSNGRYTSVLHRGLVNREAVRVSWVVFCEPPPDAVLLRPLPELVTEEEPARFTPRTF 360
Senock LEILSNGRYTSVLHRGLVNREAVRVSWVVFCEPPPDAVLLRPLPELVTEEEPARFTPRTF 360
Yaji LEILSNGRYTSVLHRGLVNREAVRVSWVVFCEPPPDAVLLRPLPELVTEEEPARFTPRTF 360
Gumjandi LEILSNGRYTSVLHRGLVNREAVRVSWVVFCEPPPDAVLLRPLPELVTEEEPARFTPRTF 360
Konhee LEILSNGRYTSVLHRGLVNREAVRVSWVVFCEPPPDAVLLRPLPELVTEEEPARFTPRTF 360
GZ LEILSNGRYTSVLHRGLVNREAVRVSWVVFCEPPPDAVLLRPLPELVTEEEPARFTPRTF 360
Millock LEILSNGRYTSVLHRGLVNREAVRVSWVVFCEPPPDAVLLRPLPELVTEEEPARFTPRTF 360
Zenith LEILSNGRYTSVLHRGLVNREAVRVSWVVFCEPPPDAVLLRPLPELVTEEEPARFTPRTF 360
AJ KEHLDRKLFKKRSSRSTSVNERDGYKPDHQVIRDSSPKTN 400
Meyer KEHLDRKLFKKRSSRSTSVNERDGYKPDHQVIRDSSPKTN 400
Senock KEHLDRKLFKKRSSRSTSVNERDGYKPDHQVIRDSSPKTN 400
Yaji KEHLDRKLFKKRSSRSTSVNERDGYKPDHQVIRDSSPKTN 400
Gumjandi KEHLDRKLFKKRSSRSTSVNERDGYKPDHQVIRDSSPKTN 400
Konhee KEHLDRKLFKKRSSRSTSVNERDGYKPDHQVIRDSSPKTN 400
GZ KEHLDRKLFKKRSSRSTSVNERDGYKPDHQVIRDSSPKTN 400
Millock KEHLDRKLFKKRSSRSTSVNERDGYKPDHQVIRDSSPKTN 400
Zenith KEHLDRKLFKKRSSRSTSVNERDGYKPDHQVIRDSSPKTN 400

Figure I-21. Sequence alignments of predicted ZjANS1 protein among nine zoysiagrass cultivars. Arrowheads on ZjANS1 indicate conserved His and Asp residues required for ferrous-iron coordination, and Arg for putative 2-oxoglutarate binding site (Singh et al., 2008).

Figure I-22. Sequence alignments of *ZjDFR1* (A) and *ZjANS1* (B) CDS between *Zoysia japonica* 'Anyang-jungji' (AJ) and *Zoysia japonica* 'Greenzoa' (GZ).

DISCUSSION

Anthocyanins are one of the major color pigments in plants, which have diverse physiological functions (Neill and Gould, 2003). However, from an aesthetic point of view, purple coloration in spikes and stolons by anthocyanin accumulation brings about blotchy appearance mixed with otherwise uniform green colors, which often lowers the commercial values of zoysiagrass as a lawn. Therefore, developing the cultivars with even green color is one of the breeding goals in zoysiagrass (Choi and Yang, 2006; Ruemmele and Engelke, 1990).

In this study, anthocyanin profiles were compared between two zoysiagrass cultivars AJ and GZ, whose spikes and stolons develop purple and green colors, respectively (Figures I-2A, I-3). Cyanidin and petunidin were detected as major aglycone forms of anthocyanins in developing spikes of AJ, but not in green spikes of GZ (Figure I-2B), indicating that anthocyanin biosynthesis differed between the two cultivars in a tissue-specific manner.

De novo transcriptome analysis on developing spike tissues identified > 28,000 unigenes expressed in AJ and GZ (Figure I-6), where approximately 5% of genes were found to be differentially expressed (Figure I-10A). Particularily, two anthocyanin biosynthesis genes *ZjDFR1* and *ZjANS1* were highly upregulated in purple AJ spike tissues (Figures I-11B, I-12). Further expression analysis on purple and green spike tissues from various zoysiagrass cultivars revealed a strong

correlation between purple pigmentation and expression levels of *ZjDFR1* and *ZjANS1* (Figure I-16). This implies that anthocyanin biosynthesis and corresponding purple pigmentation in spike and stolon tissues in zoysiagrass are primarily regulated by the upregulation of *ZjDFR1* and *ZjANS1* genes.

Deep purple coloration of developing AJ spikes and stolons was due to the accumulation of cyanidin (Figure I-2), presumably in the form of cyanidin-3-O-glucoside. Its accumulation is significantly larger than that of petunidin (Figure I-2B). This suggests that dihydroquercetin, a precursor of leucocyanidin, is more preferentially formed from dihydrokaempferol (Figure I-11A). The transcriptome analysis revealed that the expression of F3’H that catalyzes the conversion of dihydrokaempferol to dihydroquercetin is significantly higher than F3’5’H in developing spikes (Figure I-11B), suggesting that the downstream synthesis of leucocyanidin and cyanidin primarily takes places in purple-pigmented zoysiagrass tissues. By contrast, the synthesis of petunidin, which requires the supply of dihydromyricetin that is produced by F3’5’H, is relatively small. Considering dihydrokaempferol serves as a common precursor for dihydroquercetin and dihydromyricetin, whose syntheses are catalyzed by F3’H and F3’5’H, respectively, upregulation of F3’H may divert the metabolic flow at the branch point to the synthesis of cyanidin pigments rather than petunidin.

The importance of ANS and DFR in anthocyanin biosynthesis has been reported in both monocots and dicots. In maize, for example, the

a1 and *a2* genes encode DFR and ANS, respectively, and they are essential for anthocyanin pigmentation in the aleurone layer of the kernel (Menssen et al., 1990; O'Reilly et al., 1985; Reddy et al., 1987; Schwarz-Sommer et al., 1987). In Arabidopsis, *TRANSPARENT TESTA3* (*TT3*) and *TT18* encode corresponding enzymes, respectively, and their mutants have a pale yellow seed coat due to a lack of condensed tannins (proanthocyanidins) (Shikazono et al., 2003; Shirley et al., 1995).

In line with the previous studies, putative *ANS* and *DFR* genes in zoysiagrass were also found to be upregulated according to the level of anthocyanin biosynthesis. For example, both *ZjDFR1* and *ZjANS1* genes were highly expressed in developing AJ spikes, whereas their expressions were relatively low in green GZ tissues (Figure I-12). However, there appeared to be no correlation between expression of other *ANS* and *DFR* genes (*ZjDFR2*, *ZjDFR3*, *ZjANS2*, and *ZjANS3*) and anthocyanin biosynthesis in developing purple AJ spikes (Figure I-12). This indicates that a specific set of anthocyanin biosynthesis genes such as *ZjDFR1* and *ZjANS1* are developmentally regulated in a tissue-specific manner. How are these *ZjDFR1* and *ZjANS1* genes specifically upregulated only in purple AJ spike and stolon tissues? The coding sequences of *ZjDFR1* and *ZjANS1* genes from AJ and GZ are identical to each other (Figure I-22), implying that functions of these gene products are not determined by their structures but rather by a transcriptional control.

In summary, a number of anthocyanin biosynthesis genes were identified and their expression patterns were found to be with tissue-specific pigmentation in various zoysiagrass cultivars. This study will not only provide a mechanistic insight into the regulation of flavonoid biosynthesis in zoysiagrass, but also facilitate extensive genome studies in related species.

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CHAPTER 2

Functional Characterization of Dihydroflavonol 4-Reductase and Anthocyanidin Synthase in *Zoysia japonica*

ABSTRACT

Zoysia japonica is a warm-season turfgrass species commonly used as a fairway and rough turf. The diverse range of pigment accumulation in zoysiagrass is due to the different regulation of key anthocyanin biosynthesis genes such as *ZjDFR1* and *ZjANS1*. To investigate the underlying molecular mechanism of different pigment accumulation, the promoter regions of ZjDFR1 and ZjANS1 were compared between *Zoysia japonica* ‘Anyang-jungji’ and *Zoysia japonica* ‘Greenzoa’. The promoter regions have a high similarity of sequences and methylation level. In addition, *ZjDFR1* and *ZjANS1* from zoysiagrass are isolated and functionally expressed in *E. coli* and confirmed the expression *in vitro*. Although no pelargonidin derivative was identified in *Z. japonica*, ZjDFR1 reduced three types of dihydroflavonols and ZjANS1 synthesized all types of anthocyanidins. Also, complementation of the genes for the *DFR* and *ANS* in zoysiagrass was also studied in *tt3* (dihydroflavonol 4-reductase) and *tt18* (anthocyanidin synthase) mutants of Arabidopsis. Transgenic Arabidopsis overexpressing *ZjDFR1* and *ZjANS1* showed visible increase of anthocyanin accumulation in stems in comparison with mutant lines. These findings suggest that *ANS* and *DFR* are responsible for tissue-specific anthocyanin biosynthesis and differential pigmentation in zoysiagrass.

Keywords: anthocyanidin, dihydroflavonol, leucoanthocyanidin, *tt18*, *tt3*, ZjDFR1, ZjANS1, *Zoysia japonica*

INTRODUCTION

The flavonoid biosynthesis pathway which synthesizes anthocyanidin 3-glucosides is conserved in various plant species and has been extensively reported (Holton and Cornish, 1995). Flavonoids are phenolic compounds composed of 15 carbons synthesized by many different plants. One of the most important functions of flavonoids is to make diverse colored pigments in flowers and other tissues. Flavonoid biosynthesis is well-known secondary metabolic pathways in plants, and genes encoding flavonoid biosynthesis enzymes have been characterized in various plant species. The isolation of flavonoid biosynthesis genes has been reported in model plants such as *Zea mays*, *Petunia hybrida*, *Antirrhinum majus*, and Arabidopsis by characterization of mutations (Dooner and Robbins, 1991; Shirley et al., 1995).

In anthocyanin biosynthesis pathway, at least two enzyme activities are needed for the conversion of dihydroflavonols into anthocyanins: dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS). DFR enzyme activity has been described in several species. The maize *A1* gene encoding DFR was isolated with the transposable element *Spm*/*En* (O'Reilly et al., 1985). *In vitro* analysis of the protein encoding *A1* cDNA was found to have DFR activity (Reddy et al., 1987). In addition, transformation of *A1* gene in a mutant line of *Petunia hybrid* showed accumulation of kaempferol and dighdrokaempferol in flowers

(Meyer et al., 1987). In *Matthiola incana*, the enzyme preferentially catalyzes the conversion of dihydrokaempferol to leucopelargonidin (Heller et al., 1985). ANS enzyme which catalyzes the conversion of leucoanthocyanidin to anthocyanidin has been identified and isolated in several species including maize and *Perilla frutescens*. The maize *A2* gene encoding ANS was isolated by transposon tagging with transposable elements *rcy* and *dSpm* (Menssen et al., 1990). In *Perilla frutescens*, the cDNA encoding putative ANS has been isolated and confirmed to catalyze the reaction from the colorless leucoanthocyanidins to the colored anthocyanidins (Saito et al., 1999.).

In this study, the promoter sequences of *ZjDFR* and *ZjANS* were compared between *Zoysia japonica* ‘Anyang-jungji’ (AJ) and *Zoysia japonica* ‘Greenzoa’ (GZ) to find genetic or epigenetic factors affecting different expression of the genes. *In vitro* assays were performed with recombinant ZjDFR1 and ZjANS1 proteins showing that they sequentially catalyze the conversions of dihydroflavonols to anthocyanidins, forming leucoanthocyanidins as intermediates. In addition, the function of *ZjDFR1* and *ZjANS1* as enzymes for pigment accumulation was demonstrated in transgenic Arabidopsis.

MATERIALS AND METHODS

Expression of ZjDFR1 and ZjANS1 in *E. coli*

Cloning and expression of ZjDFR1 and ZjANS1 genes in *E. coli* were performed according to the previous study with minor modifications (Lee et al., 2014). Briefly, the cDNA fragments of ZjDFR1 and ZjANS1 were cloned into the pLM302 vector and the constructs were transformed into *E. coli* Rosetta2 (DE3) strain (EMD Millipore, Bedford, MA, USA). Protein expression was induced with 0.1 mM IPTG at 16°C overnight with shaking. Cells were harvested by centrifugation and the pellet was resuspended in 10 mL of lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, 0.1 mM dithiothreitol, 0.1 mM PMSF). After sonication and centrifugation, the supernatant was collected and used for *in vitro* analysis.

In vitro Assay of ZjDFR1 and ZjANS1

In vitro biochemical assay of ZjDFR1 and ZjANS1 was performed according to the previous study with minor modifications (Xie et al., 2004). As substrates, naringenin and dihydroflavonols (dihydroquercetin, DHQ; dihydrokaempferol, DHK; dihydromyricetin, DHM) (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in methanol at 1 mg/mL concentration. For ZjDFR1 reaction, each substrate (10 μg) was reacted with *E. coli* cell extracts (100 μg of total protein) expressing ZjDFR1 in the reaction buffer (100 mM Tris-HCl,

pH 7.0, 1 mM NADPH) at 30°C for 30 min. Reactions were terminated by adding 1 mL of ethyl acetate, and the extracts were evaporated with a stream of nitrogen gas. Remaining solid compounds were dissolved in methanol and subjected to HPLC analysis. The ZjANS1 assay followed the same procedure by using equal amounts of *E. coli* cell extracts expressing ZjDFR1 and ZjANS1, respectively, in the same reaction.

HPLC Analysis

For detection of leucoanthocyanidins and anthocyanidins from *in vitro* assay, the bacterial cell extracts were separated in the Inno C18 column (4.6 mm × 250 mm, 5 μm; Innopia, Seongnam, Korea) on an UltiMate 3000 HPLC system (Thermo scientific, Waltham, MA, USA) with detection at 280 nm. To detect major anthocyanins of T1 transplants, HPLC analysis was performed. Briefly, anthocyanin pigments were extracted with a solvent mixture of methanol:water:HCl (39:60:1, v/v/v). Extracts were filtered through Sep-Pak C18 cartridge (Waters Scientific, Ontario, CA).

The extracts were injected onto the Eclipse ZOBRAX XDB-C18 Rapid Resolution Threaded Column (4.6 × 150 mm, 5 μm; Agilent Technologies, Palo Alto, CA, USA) on an UltiMate 3000 HPLC system (Thermo Scientific), using delphinidin-3-O-glucoside, luteolinidin, malvidin-3-O-glucoside, pelargonidin-3-O-glucoside, cyanidin-3-O-glucoside, malvidin-3-O-galactoside, petunidin-3-O-glucoside, peonidin-3-O-glucoside (Sigma-Aldrich) as standards. Anthocyanins were quantified at the wavelength of 520 nm.

LC-MS Analysis

LC-MS analysis was performed using a ThermoFinnigan LCQ Deca XP plus ion trap mass spectrometer (Thermo Scientific) with an ESI interface at positive ion mode scanned from *m/z* 100 to 600. The capillary temperature was maintained at 275°C, the source voltage was 5 kV, and the capillary voltage was set at 45 V.

Generating Transgenic Arabidopsis

Coding sequence of ZjDFR1 and ZjANS1 were amplified from zoysiagrass by PCR using the primer sequences, DFR1_SalI_F (5'-GTCGACATGGGGGAGGTGGT)/ DFR1_SacI_R (5'-GAGCTCCAC ATGCTCCCTTCTAA), ANS_KpnI_F (5'-GGTACCATGTCATCTTC GACGG)/ ANS_XbaI_R (5'-TCTAGAGTTGGTTTTCGGTGATG) containing the SalI and SacI (ZjDFR1) and KpnI and XbaI (ZjANS1) enzyme sites (underlined) for subsequent cloning. PCR was performed with 30 cycles of 95^{o}C for 10 s, 60^{o}C for 5 s, and 72^{o}C for 90 s. 35S:HA:NOS terminator cassette in the pBI101 vector was replaced with the SalI/ SacI (DFR1), KpnI/ XbaI (ANS1) digested fragments of ZjDFR1 and ZjANS1, respectively.

The plasmids were introduced into *Agrobacterium tumefaciens* GV3101 strain. Transgenic plants were produced by the 'floral-dip' method in *A. thaliana* mutants *tt3* and *tt18* (Clough and Bent, 1998). The selected T1 plants on the MS medium containing 50 μg/mL

kanamycin were transplanted to soil. For staining of proanthocyanidins (condensed tannin), T2 seeds were treated with dimethylaminocinnaldehyde (DMACA) (Sigma-Aldrich) reagent (2% w/v DMACA in 3 M HCl/50% w/v methanol) for 1 week, followed by washing in 70% ethanol for three times.

Quantitative Real-time PCR (qRT-PCR) Analysis

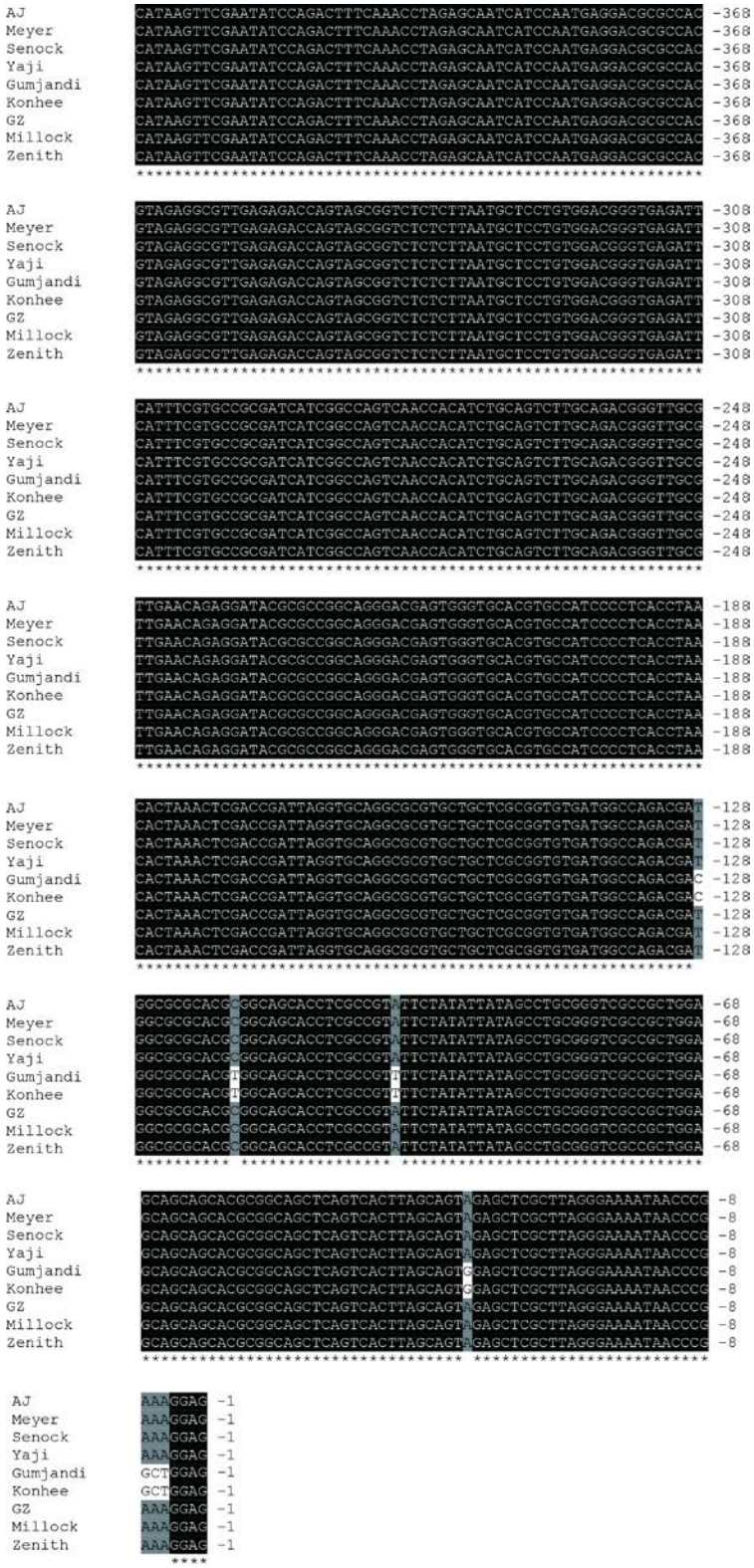
A total of 1 - 2 μg RNA was treated with RNase-Free DNase Set (QIAGEN) to remove contaminating DNA and then subjected to cDNA synthesis using the SuperScript II RT Kit (Life Technologies) according to the manufacturer's instructions. qRT-PCR was performed on a Rotor-Gene Q real-time PCR system (QIAGEN). QuantiFast SYBR Green PCR master mix (QIAGEN) was used for amplification. *AtACTIN11* (NM112046) sequence was used as an internal control to measure the relative amount of transcripts. Information on oligonucleotide sequences for qRT-PCR analysis is listed in Table 2.

RESULTS

Sequence Analysis and DNA Methylation Status of ZjDFR1 and ZjANS1 Promoter Regions

In order to find out the differences in the regulatory region, the sequences of 427 and 475 bp upstream of translation start sites of *ZjDFR1* and *ZjANS1*, respectively, were compared (Figures II-1, II-2). As the same as in the open reading frame regions, these genes were found to have the identical sequence in the promoter region between AJ and GZ (Figure I-22). They were predicted to have several *cis*-regulatory sequences such as an ABA response element (ABRE), G-box, and MYB binding site (MBS) motifs (Figure II-3), suggesting that differential regulation of these genes between AJ and GZ spikes is controlled by certain transcription factors.

Since the possibility of epigenetic modifications for transcriptional control could not be ruled out, differentially methylated regions (DMRs) were examined at the promoters of AJ and GZ spike tissues by bisulfite sequencing (BS-Seq). The promoter regions of both *ZjDFR1* and *ZjANS1* had nearly identical DNA methylation patterns between AJ and GZ (Figure II-4). For example, all cytosine residues in three different sequence contexts (CG, CHG, and CHH, where H = A, C, or T) were hypomethylated in both AJ and GZ *ZjDFR1* genes. By contrast, the *ZjANS1* gene had common CG and CHG methylation sites in both AJ and GZ spike tissues. However, the lack of DMRs between AJ and GZ


AJ CATAAGTTCGAATATCCAGACTTTCAAACCTAGAGCAATCATCCAATGAGGACGCGCCAC -368
Meyer CATAAGTTCGAATATCCAGACTTTCAAACCTAGAGCAATCATCCAATGAGGACGCGCCAC -368
Senock CATAAGTTCGAATATCCAGACTTTCAAACCTAGAGCAATCATCCAATGAGGACGCGCCAC -368
Yaji CATAAGTTCGAATATCCAGACTTTCAAACCTAGAGCAATCATCCAATGAGGACGCGCCAC -368
Gumjandi CATAAGTTCGAATATCCAGACTTTCAAACCTAGAGCAATCATCCAATGAGGACGCGCCAC -368
Konhee CATAAGTTCGAATATCCAGACTTTCAAACCTAGAGCAATCATCCAATGAGGACGCGCCAC -368
GZ CATAAGTTCGAATATCCAGACTTTCAAACCTAGAGCAATCATCCAATGAGGACGCGCCAC -368
Millock CATAAGTTCGAATATCCAGACTTTCAAACCTAGAGCAATCATCCAATGAGGACGCGCCAC -368
Zenith CATAAGTTCGAATATCCAGACTTTCAAACCTAGAGCAATCATCCAATGAGGACGCGCCAC -368
AJ GTAGAGGCGTTGAGAGACCAGTAGCGGTCTCTCTTAATGCTCCTGTGGACGGGTGAGATT -308
Meyer GTAGAGGCGTTGAGAGACCAGTAGCGGTCTCTCTTAATGCTCCTGTGGACGGGTGAGATT -308
Senock GTAGAGGCGTTGAGAGACCAGTAGCGGTCTCTCTTAATGCTCCTGTGGACGGGTGAGATT -308
Yaji GTAGAGGCGTTGAGAGACCAGTAGCGGTCTCTCTTAATGCTCCTGTGGACGGGTGAGATT -308
Gumjandi GTAGAGGCGTTGAGAGACCAGTAGCGGTCTCTCTTAATGCTCCTGTGGACGGGTGAGATT -308
Konhee GTAGAGGCGTTGAGAGACCAGTAGCGGTCTCTCTTAATGCTCCTGTGGACGGGTGAGATT -308
GZ GTAGAGGCGTTGAGAGACCAGTAGCGGTCTCTCTTAATGCTCCTGTGGACGGGTGAGATT -308
Millock GTAGAGGCGTTGAGAGACCAGTAGCGGTCTCTCTTAATGCTCCTGTGGACGGGTGAGATT -308
Zenith GTAGAGGCGTTGAGAGACCAGTAGCGGTCTCTCTTAATGCTCCTGTGGACGGGTGAGATT -308
AJ CATTTCGTGCCGCGATCATCGGCCAGTCAACCACATCTGCAGTCTTGCAGACGGGTTGCG -248
Meyer CATTTCGTGCCGCGATCATCGGCCAGTCAACCACATCTGCAGTCTTGCAGACGGGTTGCG -248
Senock CATTTCGTGCCGCGATCATCGGCCAGTCAACCACATCTGCAGTCTTGCAGACGGGTTGCG -248
Yaji CATTTCGTGCCGCGATCATCGGCCAGTCAACCACATCTGCAGTCTTGCAGACGGGTTGCG -248
Gumjandi CATTTCGTGCCGCGATCATCGGCCAGTCAACCACATCTGCAGTCTTGCAGACGGGTTGCG -248
Konhee CATTTCGTGCCGCGATCATCGGCCAGTCAACCACATCTGCAGTCTTGCAGACGGGTTGCG -248
GZ CATTTCGTGCCGCGATCATCGGCCAGTCAACCACATCTGCAGTCTTGCAGACGGGTTGCG -248
Millock CATTTCGTGCCGCGATCATCGGCCAGTCAACCACATCTGCAGTCTTGCAGACGGGTTGCG -248
Zenith CATTTCGTGCCGCGATCATCGGCCAGTCAACCACATCTGCAGTCTTGCAGACGGGTTGCG -248
AJ TTGAACAGAGGATACGGCGCGGCAGGGACGAGTGGGTGCACGTGCCATCCCCTCACCTAA -188
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Senock TTGAACAGAGGATACGGCCGGCAGGGACGAGTGGGTGCACGTGCCATCCCCTCACCTAA -188
Yaji TTGAACAGAGGATACGCGCCGGCAGGGACGAGTGGGTGCACGTGCCATCCCCTCACCTAA -188
Gumjandi TTGAACAGAGGATACGCGCCGGCAGGGACGAGTGGGTGCACGTGCCATCCCCTCACCTAA -188
Konhee TTGAACAGAGGATACGGCCGGCAGGGACGAGTGGGTGCACGTGCCATCCCCTCACCTAA -188
GZ TTGAACAGAGGATACGCGCCGGCAGGGACGAGTGGGTGCACGTGCCATCCCCTCACCTAA -188
Millock TTGAACAGAGGATACGCGCCGGCAGGGACGAGTGGGTGCACGTGCCATCCCCTCACCTAA -188
Zenith TTGAACAGAGGATACGCGCCGGCAGGGACGAGTGGGTGCACGTGCCATCCCCTCACCTAA -188
AJ CACTAAACTCGACCGATTAGGTGCAGGCGCGTGCTGCTCGCGGTGTGATGGCCAGACGAT -128
Meyer CACTAAACTCGACCGATTAGGTGCAGGCGCGTGCTGCTCGCGGTGTGATGGCCAGACGAT -128
Senock CACTAAACTCGACCGATTAGGTGCAGGCGCGTGCTGCTCGCGGTGTGATGGCCAGACGAT -128
Yaji CACTAAACTCGACCGATTAGGTGCAGGCGCGTGCTGCTCGCGGTGTGATGGCCAGACGAT -128
Gumjandi CACTAAACTCGACCGATTAGGTGCAGGCGCGTGCTGCTCGCGGTGTGATGGCCAGACGAC -128
Konhee CACTAAACTCGACCGATTAGGTGCAGGCGCGTGCTGCTCGCGGTGTGATGGCCAGACGAC -128
GZ CACTAAACTCGACCGATTAGGTGCAGGCGCGTGCTGCTCGCGGTGTGATGGCCAGACGAT -128
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Gumjandi GGCGCGCACGTGGCAGCACCTCGCCGTTTTCTATATTATAGCCTGCGGGTCGCCGCTGGA -68
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GZ GCAGCAGCACGCGGCAGCTCAGTCACTTAGCAGTAGAGCTCGCTTAGGGAAAATAACCCG -8
Millock GCAGCAGCACGCGGCAGCTCAGTCACTTAGCAGTAGAGCTCGCTTAGGGAAAATAACCCG -8
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Meyer AAAGGAG -1
Senock AAAGGAG -1
Yaji AAAGGAG -1
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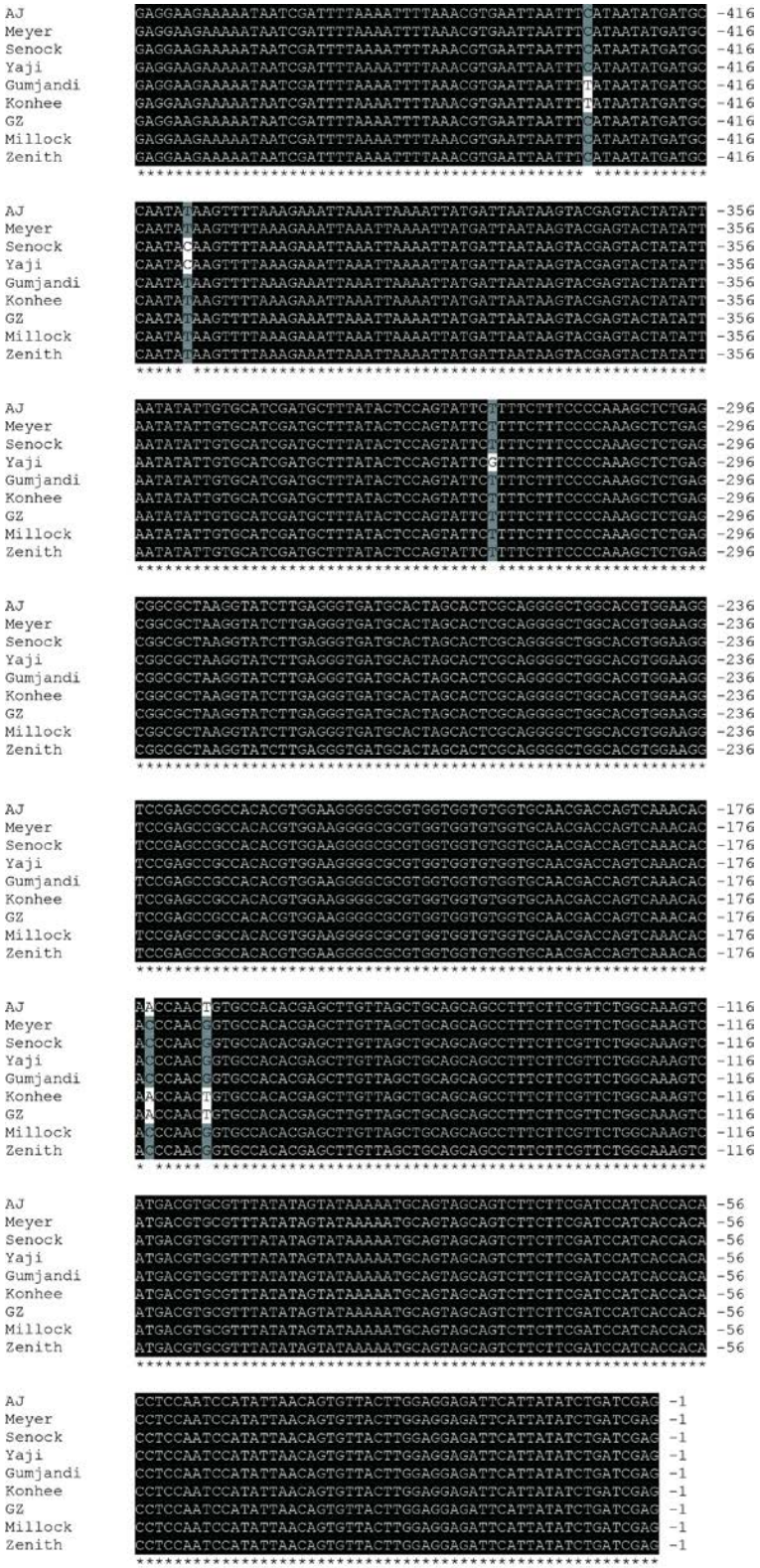
Konhee GCTGGAG -1
GZ AAAGGAG -1
Millock AAAGGAG -1
Zenith AAAGGAG -1

Figure II-1. Sequence alignments of the promoter region of *ZjDFR1* among nine zoysiagrass cultivars.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GZ GAGGAAGAAAAATAATCGATTTTAAAATTTTAAACGTGAATTAATTTGATAATATGATGC -416
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GZ AATATATTGTGCATCGATGCTTTATACTCCAGTATTCTTTCTTTCCCCAAAGCTCTGAG -296
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Zenith ATGACGTGCGTTTATATAGTATAAAAATGCAGTAGCAGTCTTCTTCGATCCATCACCACA -56
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Yaji CCTCCAATCCATATTAACAGTGTTACTTGGAGGAGATTCATTATATCTGATCGAG -1
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Millock CCTCCAATCCATATTAACAGTGTTACTTGGAGGAGATTCATTATATCTGATCGAG -1
Zenith CCTCCAATCCATATTAACAGTGTTACTTGGAGGAGATTCATTATATCTGATCGAG -1

Figure II-2. Sequence alignments of the promoter regions of *ZjANS1* among nine zoysiagrass cultivars.

A

```
CATAAGTTCGAATATCCAGACTTTCAAACCTAGAGCAATCATCCAATGAGGACGCGCCAC      - 368
                                                          ABRE
GTAGAGGCGTTGAGAGACCAGTAGCGGTCTCTCTTAATGCTCCTGTGGACGGGTGAGATT      - 308

CATTTCGTGCCGCGATCATCGGCCAGTCAACCACATCTGCAGTCTTGCAGACGGGTTGCG      - 248

TTGAACAGAGGATACGCGCCGGCAGGGACGAGTGGGTGCACGTGCCATCCCCTCACCTAA      - 188
            AP2 binding                 G-BOX
CACTAAACTCGACCGATTAGGTGCAGGCGCGTGCTGCTCGCGGTGTGATGGCCAGACGAT      - 128

GGCGCGCACGCGGCAGCACCTCGCCGTATTCTATATTATAGCCTGCGGGTCGCCGCTGGA      - 68
                                    TATA-BOX
GCAGCAGCACGCGGCAGCTCAGTCACTTAGCAGTAGAGCTCGCTTAGGGAAAATAACCCG      - 8

AAAGGAGATGGGGGAGGTGGTGGTGAAGCA                                    + 23
```

B

```
GAGGAAGAAAAATAATCGATTTTAAAATTTTAAACGTGAATTAATTTTATAATATGATGC      - 416
                                    G-BOX
CAATATAAGTTTTAAAGAAATTAAATTAAAATTATGATTAATAAGTACGAGTACTATATT      - 356

AATATATTGTGCATCGATGCTTTATACTCCAGTATTCTTTTCTTTCCCCAAAGCTCTGAG      - 296

CGGCGCTAAGGTATCTTGAGGGTGATGCACTAGCACTCGCAGGGGCTGGCACGTGGAAGG      - 236
                                                      ABRE
TCCGAGCCGCCACACGTGGAAGGGGCGCGTGGTGGTGTGGTGCAACGACCAGTCAAACAC      - 176
              ABRE
AACCAACTGTGCCACACGAGCTTGTTAGCTGCAGCAGCCTTTCTTCGTTCTGGCAAAGTC      - 116
  MBS
ATGACGTGCGTTTATATAGTATAAAAATGCAGTAGCAGTCTTCTTCGATCCATCACCACA      - 56
 G-BOX          TATA-BOX
CCTCCAATCCATATTAACAGTGTTACTTGGAGGAGATTCATTATATCTGATCGAGATGTC      + 5

ATCTTCGACGGTGCTGCAGCAGCCGCCGGC                                    + 35
```

Figure II-3. Predicted *cis*-acting elements on *ZjDFR1* and *ZjANS1* promoter regions. The bold 'ATG' indicates the translational start site. Predicted *cis*-regulatory elements are underlined and identified according to the PlantCare database (Lee et al., 2014). ABRE, Abscisic acid response element; MBS, MYB binding site; AP2, Apetala2.

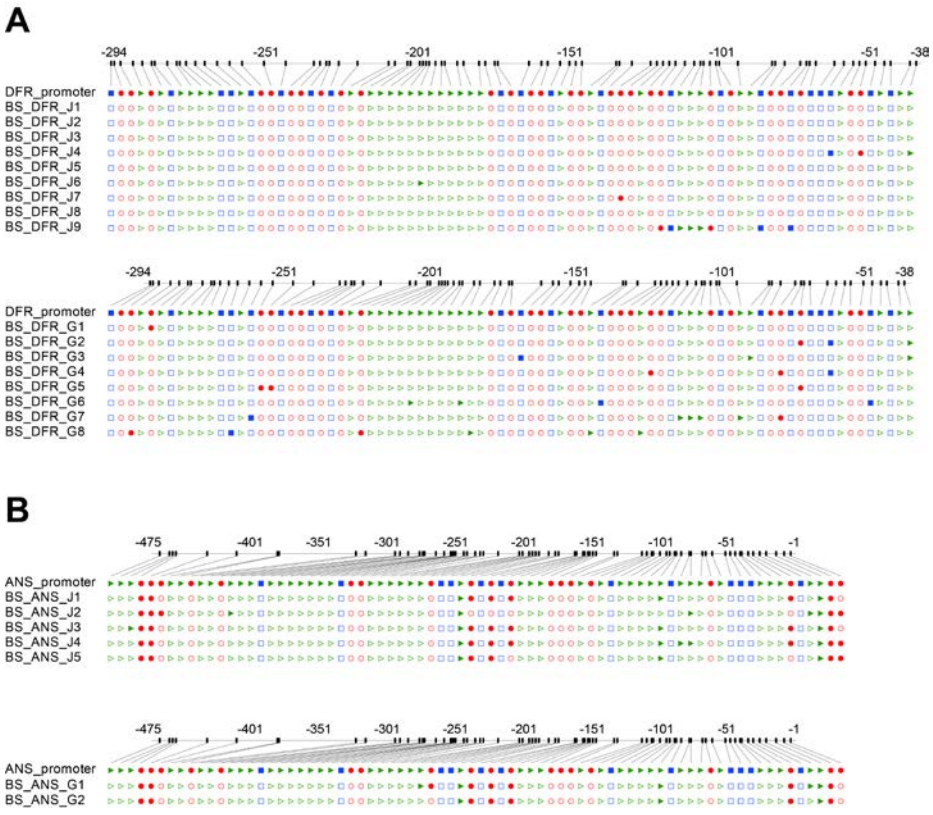

Figure II-4. DNA methylation profiles of *ZjDFR1* (A) and *ZjANS1* (B) promoter region from *Zoysia japonica* 'Anyang-jungji' (AJ) and *Zoysia japonica* 'Greenzoa' (GZ). Numbers are from the translational start site. 5-Methylcytosines in the CG (circle), CHG (triangle), and CHH (square) contexts were displayed by CyMATE (Hetzl et al., 2007). Open and closed symbols indicate unmethylated and methylated cytosines, respectively. Primers used for BS-Seq are listed in Table 2.

suggests that differential tissue-specific expression of *ZjDFR1* and *ZjANS1* is primarily regulated by genetic control rather than by epigenetic factors.

Dihydroflavonol Reductase and Leucocyanidin Oxygenase Activities of Recombinant ZjDFR1 and ZjANS1 Proteins

Although ZjDFR1 and ZjANS1 may primarily regulate tissue-specific anthocyanin pigmentation in purple zoysiagrass cultivars, there is a possibility that they may have other catalytic functions, or some other genes not revealed in this study may encode proteins that actually catalyze essential steps in the anthocyanin biosynthesis pathway. Therefore, recombinant ZjDFR1 and ZjANS1 proteins were expressed in *E. coli* (Figure II-5) and *in vitro* studies were performed to reveal their biochemical activities. In the conventional anthocyanin biosynthesis pathway, DFR catalyzes the conversion of dihydroflavonols to leucoanthocyanidins, and in the next step, ANS acts to convert leucoanthocyanidins into anthocyanidins (Figure I-11A) (Jaakola, 2013). Bacterial cell extracts expressing recombinant ZjDFR1 protein were incubated with dihydroflavonols such as DHM, DHK, and DHQ, and the reaction products were analyzed by HPLC (Figure II-6). As compared to the HPLC profile of control reaction, an additional peak was observed when each dihydroflavonol was reacted with bacterial cell extracts expressing ZjDFR1 (Figure II-6). These peaks are supposedly leucoanthocyanidins such as leucodelphinidin, leucocyanidin, and leucopelargonidin produced by ZjDFR1, as the

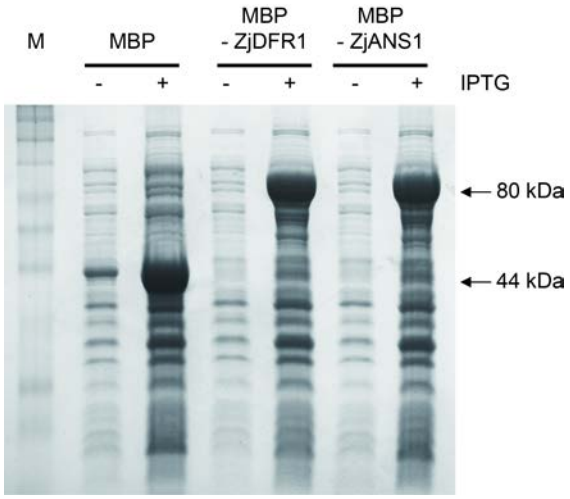

Figure II-5. Expression of recombinant ZjDFR1 and ZjANS1 proteins fused with an N-terminal maltose binding protein (MBP) fragment in *E. coli*. Total proteins were separated on a 10% SDS-PAGE gel and visualized by Coomassie Brilliant Blue staining. Expected molecular weights of MBP, MBP-ZjDFR1, and MBP-ZjANS1 are indicated at the right side of the panel. M, Size marker.

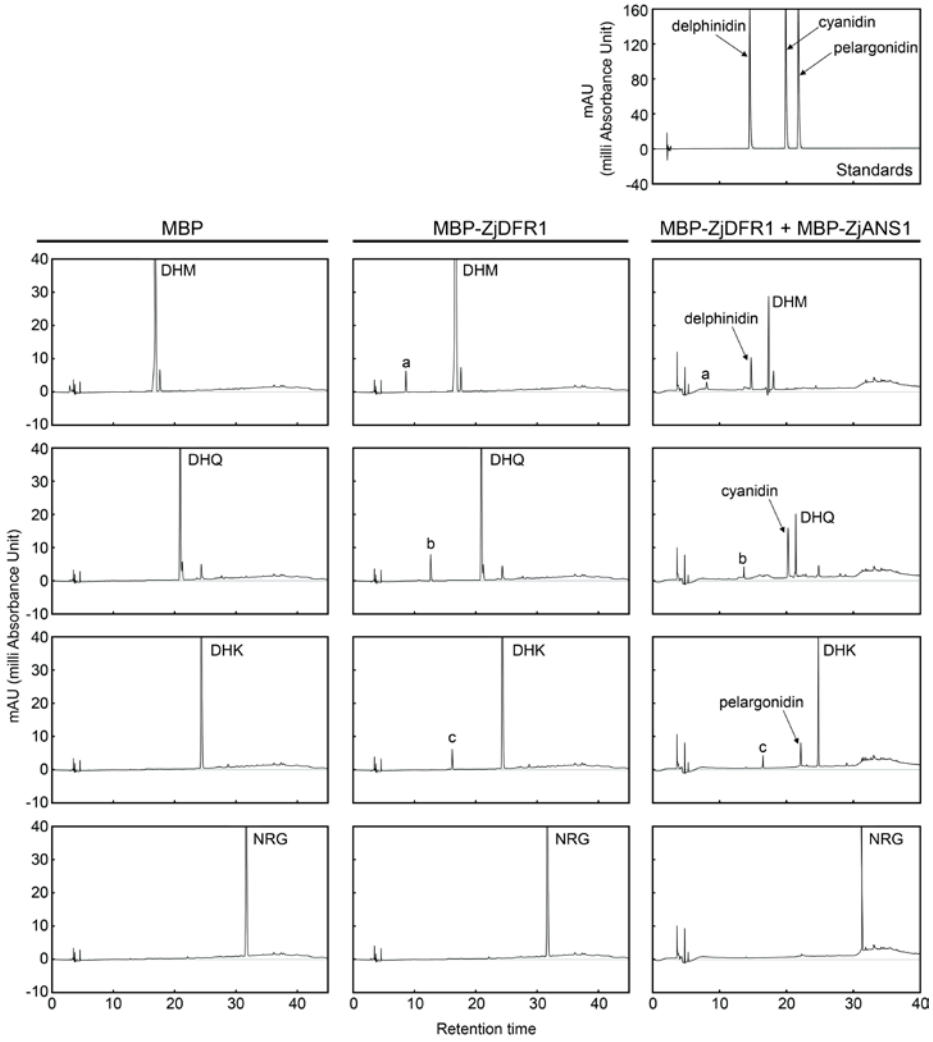

Figure II-6. HPLC analysis of leucoanthocyanidins and anthocyanidins produced by recombinant ZjDFR1 and ZjANS1 proteins in *E. coli*. The chromatograms were recorded at 280 nm. a, leucodelphinidin; b, leucocyanidin; c, leucopelargonidin.

subsequent LC-MS analysis revealed the appearance of another close peak with a two mass unit difference from substrate (dihydroflavonol) due to the addition of two hydrogen atoms by DFR activity (Figure II-7). No reaction product was detected when naringenin was used as substrate, indicating that ZjDFR1 activity is specific to dihydroflavonols (Figure II-7). When dihydroflavonols were reacted with the mixture of bacterial cell extracts expressing ZjDFR1 and ZjANS1, respectively, another peaks were conspicuously detected (Figure II-6). These peaks were found to correspond to delphinidin, cyanidin, and pelargonidin, suggesting that ZjANS1 is able to catalyze the conversion of leucoanthocyanidins into anthocyanidins.

Overexpression of *ZjDFR1* and *ZjANS1* Induces Anthocyanin Biosynthesis in Arabidopsis

The flavonoid gene homologs (i.e., *ZjDFR1* and *ZjANS1*) have not been functionally characterized in planta previously. To verify the function of anthocyanin related genes *in vivo*, the above two zoysiagrass genes were transformed into the appropriate *Arabidopsis transparent testa* (*tt*) mutants, lacking *DFR* and *ANS* activity, for complementation analysis. Each plant contained *ZjDFR1* and *ZjANS1* with the cauliflower mosaic virus 35S promoter included. The transgenic Arabidopsis mutants with *ZjDFR1* and *ZjANS1* accumulated proanthocyanidins (p-dimethylaminocinnamaldehyde (DMACA)-positive) (Figure II-8). In addition, the T1 plants with different seed color showed purple pigmentation along the stems (Figure II-8).

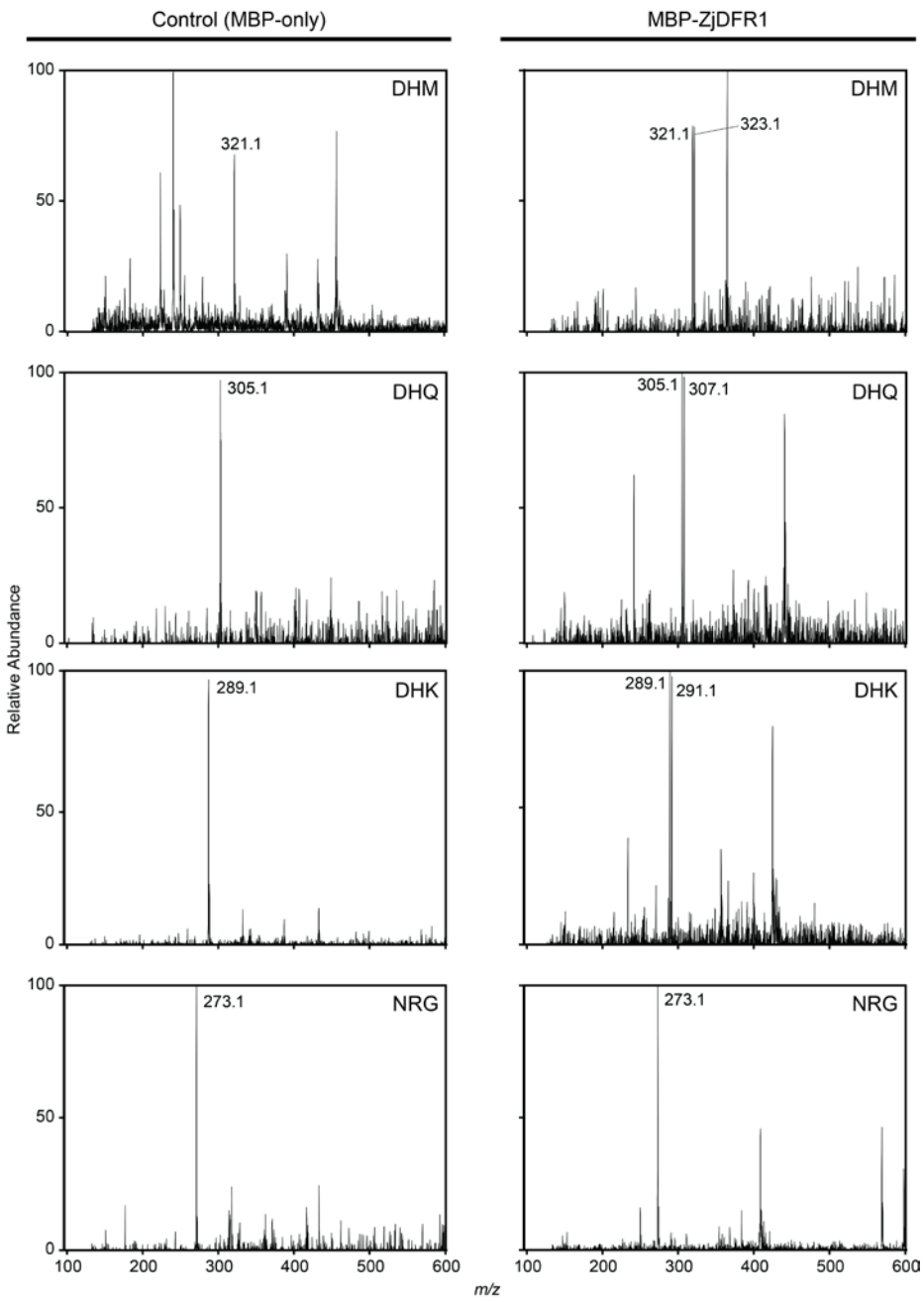
Control (MBP-only)
MBP-ZjDFR1
DHM
321.1
321.1
323.1
DHQ
305.1
305.1
307.1
DHK
289.1
289.1
291.1
NRG
273.1
273.1
Relative Abundance
m/z
100
50
0
100
200
300
400
500
600

Figure II-7. LC-MS analysis of the reaction mixtures of dihydroflavonols incubated with *E. coli* cell extracts expressing ZjDFR1. Mass spectra obtained for bacterial cell extracts incubated with four substrates and the [M+] (*m/z*) values of the corresponding peaks: dihydromyricetin (DHM; *m/z* = 321), dihydroquercetin (DHQ; *m/z* = 305), dihydrokaempferol (DHK; *m/z* = 289), and naringenin (NRG; *m/z* = 273).

Results showed different transgenic Arabidopsis mutants were able to accumulate proanthocyanidins (DMACA-positive) in seed coat (Figure II-8). In addition, approximately 60% (9/16) of *35S:ZjDFR1* and 80% (13/16) of *35S:ZjANS1* T1 plants showed purple pigmentation on stems. To further characterize the flavonoids synthesized by the Arabidopsis mutants expressing zoysiagrass genes, HPLC experiments were conducted using extracts from whole aerial parts of 4-week-old plants. The transgenic plants complemented with *35S:ZjDFR1* and *35S:ZjANS1* showed higher amount of cyanidin 3-O-glucoside, as compared to Arabidopsis *tt3* and *tt18* mutants (Figure II-9).

The expression levels of *ZjDFR1* and *ZjANS1* genes were analyzed by qRT-PCR in mutant (*dfr* and *ans*) and transgenic (*35S:ZjDFR1* and *35S:ZjANS1*) T1 plants, respectively. Little or no *ZjDFR1* and *ZjANS1* expression were detected in non-transgenic plants, whereas *ZjDFR1* and *ZjANS1* were highly expressed in every pigment accumulated transgenic plant (Figure II-10). These results indicate that anthocyanin accumulation was primarily caused by ectopic expression of *ZjDFR1* and *ZjANS1* genes, suggesting that they are all functional orthologs of Arabidopsis *DFR* and *ANS* even though their differential pigment accumulation activities.

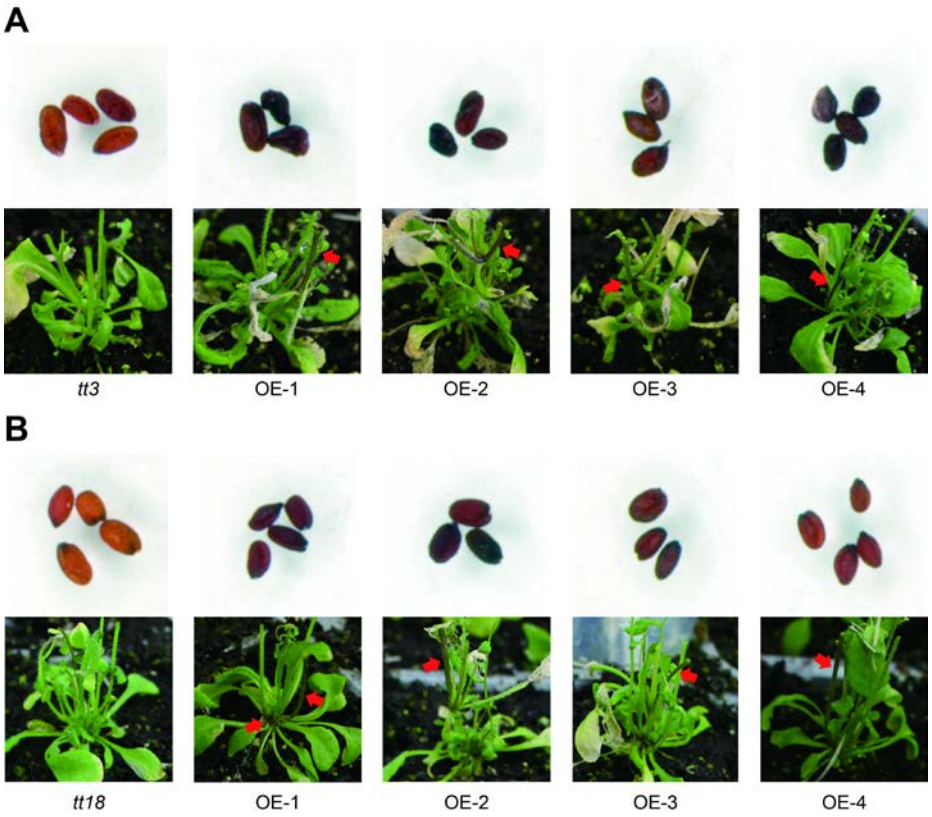

Figure II-8. **Pigment accumulating phenotype of T2 seeds and T1 transgenic plants overexpressing (OE) *ZjDFR1* (A) and *ZjANS1* (B) genes.** Arrows indicate pigment accumulated regions.

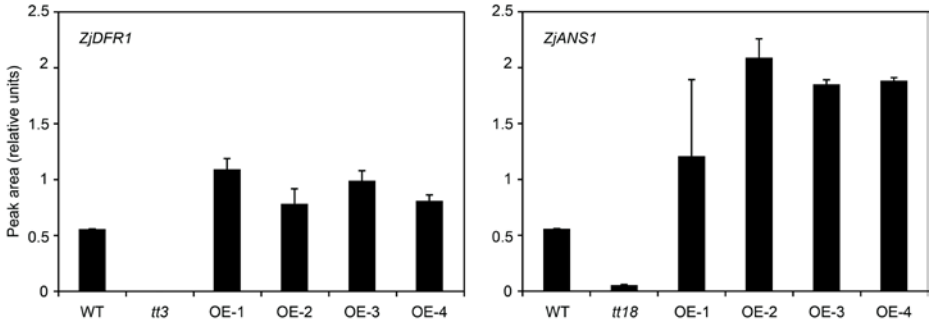

Figure II-9. Analysis of anthocyanins in extracts of *ZjDFR1* and *ZjANS1* transgenic Arabidopsis lines. The relative area of anthocyanins in T1 whole aerial parts was analyzed by HPLC. The error bars indicate means ± SD of three technical replicates.

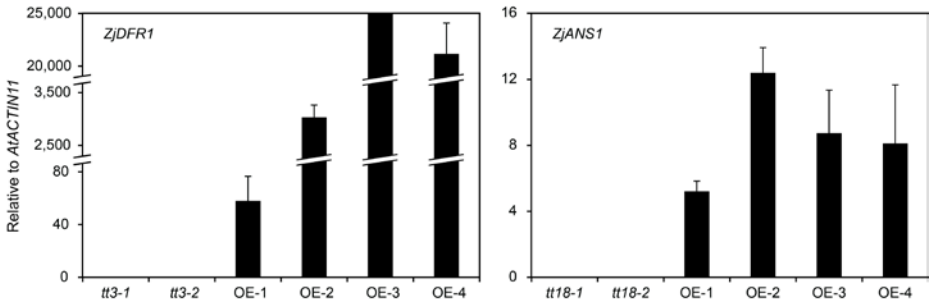

Figure II-10. **Ectopic expression of anthocyanin biosynthesis genes of zoysiagrass inducing pigment accumulation in Arabidopsis.** Expression levels of *ZjDFR1* and *ZjANS1* transgenes in representative individuals. Overexpressed (OE) plants were transformed with *35S:ZjDFR1* (left) or *35S:ZjANS1* (right). Ectopic transgene expression was determined by qRT-PCR relative to *AtACTIN11*. Columns with error bars indicate means ± SD of three technical replicates.

DISCUSSION

Breeding efforts to create a wide variety of novel and desirable flower colors have been highly successful in some species (Johnson et al., 1999; Mol et al., 1995). In zoysiagrass, anthocyanin accumulation of stolons and spikes vary among species. From previous study, two anthocyanin biosynthesis genes, *ZjDFR1* and *ZjANS1* were found to be related to different accumulation of anthocyanin in zoysiagrass. To find the factor of different expression between the cultivars with coloration, the upstream of *ZjDFR1* and *ZjANS1* coding regions was analyzed. As a result, the regulatory sequences upstream of the translational start sites did not significantly differ, and BS-Seq analysis demonstrated that promoter regions of *ZjDFR1* and *ZjANS1* had similar levels and patterns of DNA methylation between AJ and GZ (Figure II-4). This indicated that differential expressions of *ZjDFR1* and *ZjANS1* between AJ and GZ were not determined by DNA methylation, and thus the epigenetic control, at least DNA methylation, is unlikely a central mechanism for their transcriptional regulation.

Plants generally respond to developmental and environmental signals by regulating specific transcription factors. In recent years, MYB transcription factors have been extensively studied for their roles in the regulation of pigmentation in plants (Dixon et al., 2013; Yang et al., 2013). R2R3-type MYB proteins and the MYB-bHLH-WD40 complex have been known to activate the transcription of early (*CHS*,

CHI, *F3'H*, and *FLS*) and late (*DFR*, *ANS*, and *ANR*) flavonoid biosynthesis genes, respectively (Li, 2014). The light-dependent anthocyanin accumulation in the skin of apple fruit can be also explained by the activation of a MYB transcription factor. For instance, MdMYB1, which controls apple anthocyanin pathway genes, is repressed under darkness by interacting with MdCOP1 and subsequent ubiquitin-dependent protein degradation (Jaakola, 2013). Two zoysiagrass *MYB* gene (*ZjMYB1* and *ZjMYB2*) transcripts were found to be more abundant in purple AJ spike and stolon tissues than in green GZ tissues (Figure I-14), and both *ZjDFR1* and *ZjANS1* contain several *cis*-regulatory elements such as ABRE, G-box, MBS, and AP2 binding motifs which might serve as putative MYB binding sites (Figure II-3). These findings suggest that two MYB transcription factors ZjMYB1 and ZjMYB2 specifically activate late flavonoid biosynthesis genes such as *ZjDFR1* and *ZjANS1*, albeit their direct role in anthocyanin biosynthesis regulation awaits further investigation.

Besides purple pigmentation in spikes and stolons, mature seeds of AJ have a dark brown seed coat, whereas those of GZ have pale brown colors (Figure II-11). This is probably due to differential accumulation of condensed tannins between AJ and GZ seed coats, which is reminiscent of the seed coat color differences between wild type and *tt* mutants in *Arabidopsis* (Shikazono et al., 2003; Shirley et al., 1995). Therefore, both monocots and dicots may be evolutionarily divergent but probably have maintained similar mechanisms to regulate flavonoids biosynthesis and their accumulation in the seeds. In

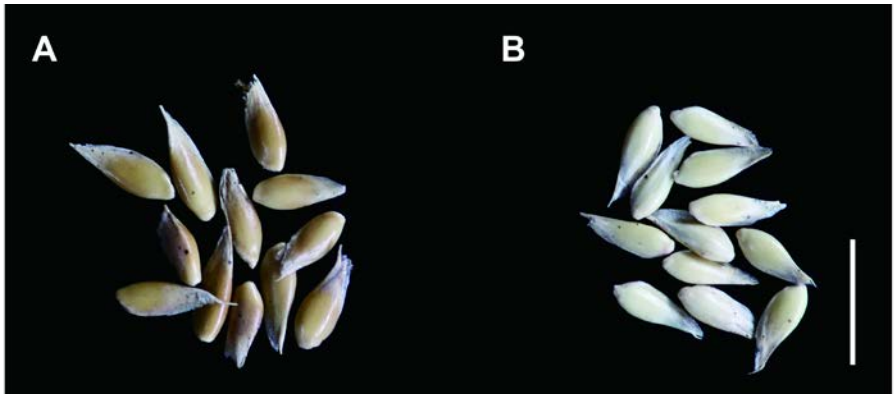

Figure II-11. Seed colors of *Zoysia japonica* 'Anyang-jungji' (AJ) (A) and *Zoysia japonica* 'Greenzoa' (GZ) (B). Bar = 0.5 mm.

zoysiagrass, tissue-specific accumulation of anthocyanin is controlled by key genes such as *ZjDFR1* and *ZjANS1*. However, the function of key genes that catalyze anthocyanin has not been demonstrated. In this study, the activities of ZjDFR1 and ZjANS1 which encodes DFR and ANS, respectively, were confirmed *in vitro* and *in vivo*.

ZjDFR1 and *ZjANS1* cDNA clones were isolated from zoysiagrass transcriptome database and confirmed the presence in zoysiagrass. The two clones were functionally expressed in *E. coli* to encode active proteins followed by *in vitro* assay, confirming that ZjDFR1 and ZjANS1 had essential biochemical activities to convert dihydroflavonols into leucoanthocyanidins and leucoanthocyanidins into anthocyanidins, respectively (Figure II-6). ZjDFR1 and ZjANS1 had substrate specificity for dihydroflavonols but not naringenin. In addition, the isolated *ZjDFR1* and *ZjANS1* were transformed into appropriate Arabidopsis mutants for transgene activity test. HPLC analysis showed that zoysiagrass ZjDFR1 and ZjANS1 complement pigmentation of Arabidopsis *tt3* and *tt18* mutants, respectively (Figure II-9). The transgenes showed ectopic expression in pigmented lines, which follows the pigment accumulation. Thus, the products of the zoysiagrass *ZjDFR1* and *ZjANS1* genes can fully complement Arabidopsis *tt3* and *tt18* mutants, implying that enzymes involved in secondary metabolite accumulation are exchangeable between distantly related plants.

In anthocyanin biosynthesis, DFR catalyzes several phenolic compounds as substrates. They are three kinds of dihydroflavonols,

such as DHQ, DHK, and DHM which are finally synthesized into cyanidin, pelargonidin, and delphinidin derivatives, respectively. Naringenin is synthesized into 3-deoxyanthocyanidin by DFR and ANS enzymes. Especially, the substrate specificity of DFR is reported in other plants. In *Zea mays* DFR activity was tested with DHQ as a substrate which was converted into leucocyanidin (Reddy et al., 1987). *Petunia hybrida* with cyanidin and delphinidin derivatives as main anthocyanins had DFR activity with dihydroquercetin and more efficiently with dihydromyricetin as substrates (Beld et al., 1989). In *Cymbidium*, DFR could not efficiently reduce DHK to produce palargonidin in the transgenic petunia (Johnson et al., 1999). In contrast, ZjDFR1 has specificity with diverse substrates except naringenin. The molecular mechanism of substrate specificity of DFR is unknown. The hydroxyl groups on the B ring of dihydroflavonols and C ring of naringenin may produce hydrogen bonding with DFR enzyme. As the only difference of three dihydroflavonols and naringenin is position of hydroxyl group, different mutations in DFR may change binding activity with substrates. Further studies of DFR structure are needed.

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ABSTRACT IN KOREAN

조이시아속 잔디(Zoysiagrass, *Zoysia* sp.)는 전 세계적인 온난기후에서 주로 서식하고 균일한 녹색을 띄고 있다. 그러나 몇몇 잔디 품종에서 이삭 및 포복경에 붉은 색소를 띄는데 이는 심미적 가치를 떨어뜨린다. 두 개의 조이시아속 잔디 품종인 안양중지(AJ)와 그린조아(GZ)는 이삭과 포복경에서 각각 보라색과 녹색을 띄는데 시아니딘과 페츄니딘이 착색의 주 성분임을 알 수 있었다. 두 잔디 품종간에 서로 다르게 발현되는 유전자를 확인하기 위하여 신규전사체 분석을 수행하였다. 이중 두 개의 안토시아닌 생합성 관련 유전자인 안토시아니딘 합성 효소 *ZjANS1*(*Z. japonica* anthocyanidin synthase 1)와 디하이드로플라보놀 4-환원 효소 *ZjDFR1*(*Z. japonica* dihydroflavonol 4-reductase 1)이 안양중지 이삭에서 높게 발현되었다. 또한 이 유전자들은 여러 다른 조이시아속 잔디 품종들 중 보라색을 띄는 잔디 품종에서 높게 발현되었고, 반면 녹색을 띄는 잔디 품종에서는 낮게 발현되었다. 잔디의 *ZjDFR1* 과 *ZjANS1* 유전자가 암호화하는 효소 활성이 실제 안토시아닌 생합성에 작용하는 것을 확인하였다. 또한 *ZjDFR1* 과 *ZjANS1* 유전자를 각각 *DFR* 과 *ANS* 유전자가 없는 애기장대 돌연변이체에 형질전환하여 T1

형질전환 개체에서의 안토시아닌 생합성을 확인하였다. 이를 통하여 잔디의 *DFR*, *ANS* 유전자의 기능을 확인하였고 이는 단자엽작물이 아닌 애기장대에서도 기능하는 것을 확인할 수 있다. 본 연구를 통하여 잔디에서의 *ANS* 와 *DFR* 유전자의 높은 발현이 조직 특이적인 안토시아닌 생합성을 통한 착색에 관여함을 알 수 있었다.