A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Functional analysis of *ZjCIGR1* gene in *Zoysia japonica* and *Arabidopsis thaliana*

Yang-Ji Kim

(Supervised by Professor Hyo-Yeon Lee)

Department of Biotechnology

GRADUATE SCHOOL

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ABBREVIATIONS

Bar	phosphinothricin acetyltransferase
GAI	*GIBBERELLIC ACID INSENSITIVE*
HAM	*HAIRY MERISTEM*
LB	left border
LS	*LATERAL SUPPRESSOR*
MS	Murashige and Skoog medium
PAT1	*PHYTOCHROME A SIGNALING TRANSDUCTION 1*
PPT	phosphinothricin
RB	right border
RGA	*REPRESSOR OF GAI*
RT-PCR	reverse transcriptase polymerase chain reaction
SCL	*SCARECROW*-LIKE
SCR	*SCARECROW*
SHR	*SHORT-ROOT*
T3	Transgenic 3 generation
ZjCIGR1	*Zoysia japonica chitin-inducible gibberellin-responsive 1*
35S	CaMV 35S promoter

1. ABSTRACT

Zoyia japonica Steud. is a warm-season lawn grass popular in Korea and cultivated in many places such as river banks, roadside and fields. However, there still is a disadvantage of frequent mowing, and the grass grows poorly under shade and low temperature conditions. To develop a grass variety that circumvents these drawbacks, we cloned *chitin-inducible gibberellins-responsive 1* (*CIGR1*) from *Zoysia japonica* Steud. The full-length of *ZjCIGR1* was obtained by 5'/3' RACE PCR and phylogenetic tree showed that it belongs to the PAT1 group of GRAS protein family. The expression of *ZjCIGR1* in wild-type zoysiagrass was confirmed in roots, meristems, leaves and flowers, especially high in the flowers. The transgenic zoysiagrass was confirmed by PCR using gene-specific primers, phosphinothricin-acetyl-transferase (PAT) strip test and Southern blot analysis. Resistance to abiotic stress was enhanced in the transformants and the plant heights were shorter compared to the wild-type. In addition, the transgenic *Arabidopsis* plant showed delayed aging and enhanced stress resistance. These results suggest that *ZjCIGR1* plays a role in regulating environmental stress resistance and plant height.

2. INTRODUCTION

The GRAS family of proteins are named after the three initially discovered transcription factors *GIBBERELLIC ACID INSENSITIVE (GAI), REPRESSOR OF GAI (RGA) and SCARECROW (SCR*) (Peng *et al*. 1997; Pysh *et al*. 1998; Silverstone *et al*. 1998). The GRAS protein family can be divided into several groups that have been so named after one of their respective members or of functional roles: SHORT-ROOT (SHR), SCR, LATERAL SUPPRESSOR (LS), SCARECROW-LIKE 9 (SCL9), SCL4/7, HAIRY MERISTEM (HAM), DELLA and PHYTOCHROME A SIGNALING TRANSDUCTION 1 (PAT1) (Bolle *et al*. 2004; Lee *et al.* 2008; Sun *et al.* 2012). Both SHR and SCR play a regulatory role in root growth (Hao *et al*. 2012; Koizumi *et al*. 2012; Sun *et al*. 2012). LS is associated with the initiation or maintenance of the axillary meristem (Schumacher *et al*. 1998; Greb *et al*. 2003; Yang *et al*. 2011), whereas SCL9 is a transcriptional regulator associated with the rooting-competent cuttings in response to auxin (Sanchez *et al*. 2007; Fode *et al*. 2008; Czikkel *et al*. 2007; Sun *et al*. 2012). SCL4/7 functions as a transcriptional regulator in response to salt, osmotic shock and drought stresses (Ma *et al*. 2010). HAM acts to maintain shoot meristem and responds to auxin, functioning as a transcriptional co-activator (Gao *et al*. 2004; Kalo *et al*. 2005). DELLA controls gibberellin-responsive genes and modulates light and jasmonate (JA) signaling (Murase *et al*. 2008; Lucas *et al*. 2008; Hou *et al*. 2010). Lastly, PAT1 proteins are involved in phytochrome A specific light signal transduction and also plays a positive regulatory role in phytochrome B dependent red light signaling (Bolle *et al*. 2000; Torres-Galea *et al*. 2013). Recently, they have also been implicated in affecting environmental stress. For instance, overexpression of *VaPAT1* has been reported to confer tolerance to cold, drought and salt stresses in *Vitis amurensis* (Yuan *et al*. 2015). The c*hitin-inducible gibberellins-responsive 1* (*CIGR1*) gene belonging to the PAT1 group plays key transcriptional regulatory roles in plant development and defense (Day *et al*. 2003; Bolle *et al*. 2004; Tian *et al*. 2004). In rice, the *CIGR1* gene is a candidate for a major locus affecting plant height (Kovi *et al*.

2011). In addition, it functions as a transcriptional regulator in elicitor-induced defense response (Day *et al*. 2003; Day *et al*. 2004).

Zoyia japonica Steud. is a typical warm-season Korean lawn grass, which is more resilient to high temperature conditions than cold-season lawn grass including Kentucky bluegrass (*Poa pratensis*) and creeping bentgrass (*Agrostis stolonifera* subsp.). Because the grass grows fast with disease resistance trait, maintenance cost of the lawn is relatively low (Song *et al*. 2006). In Korea, the zoysiagrass is cultivated in many places such as river banks, roadside, play grounds and golf courses. Recently, it has also been used for landscape gardening in newly developed towns, home and school yards and the Saemangeum reclaimed land all of which contribute to a reduced water pollution (Bae *et al*. 2013; Bae *et al*. 2016). Although acreage of the turfgrass covered areas is steadily increasing worldwide, there still is a disadvantage of entailing frequent mowing as well as poor growth in the shady areas and the cold region. .

This study aims at securing the useful gene(s) to develop stress tolerant and dwarf plants. We report here on the cloning of the *ZjCIGR1* gene in zoysiagrass and carried out analyses of the gene expression profiles and phenotypic traits of the transgenic zoysiagrass and the *Arabidopsis* plant for comparison. In addition, we studied the function of *ZjCIGR1* in abiotic stress tolerance and plant height using the transgenic plants.

3. MATERIALS AND METHODS

3.1. Plant materials and growth conditions

Tissue culture plantlets of *Zoysia japonica* Steud. plant were grown on half-strength (1/2) Murashige and Skoog(MS) medium in a growth chamber at 22-24℃ under long-day conditions (16--h light/8-h dark). To transfer the plantlets into the soil, the grasses were acclimated in water for 3 days and then grown in soil. Seeds of the *Arabidopsis thaliana* plant (ecotype Columbia Col-0) were stored at 4℃ for 3 days and then sown in a growth chamber at 22-24℃ under long-day conditions (16-h light/8-h dark).

3.2. Cloning of *ZjCIGR1* gene and phylogenetic analysis

Stress-responsive genes in zoysiagrass were induced by salt and cold stress treatments of the plant. Among them, partial fragment of *ZjCIGR1* was isolated by RT-PCR, 5'/3'-RACE (Rapid Amplification of cDNA Ends) using sp1(5'–TAGGCACCCAATCGCTGTAT-3'), sp2(5'–ACGATGTTCTGTGGGTCCTC-3'), sp3(5'–GTGATGGTTGCCGTGATTCA-3'), sp4(5'–AGCAGGCTTGTTCTCCTCAT-3'), sp5(5'–TGCTCAAGGGACACAATGGA-3'), sp6(5'–AGTATGCTCGTGGTGAAGGT-3'), sp7(5'–CCCTCTACCTGTACTACGCG-3'), sp8(5' –TGTTCTTGACCGTTGCCTCA-3'), sp9(5'–TCAGGCGCCATTAGAACAGT-3'), sp10(5'–TGCCTGCCTGTCTGTATGAG-3'), sp11(5'–CTGGAGTGTTGCTAAGAGCAGA-3'). The amplified products were inserted into a pGEM T-easy vector (Promega, Madison, WI, USA) and sequence analysis was performed. A Translator server was used to translate the sequences of *ZjCIGR1* into amino acids. Homologues of the gene were identified by NCBI databases (http://www.ncbi.nlm.nih.gov). Sequence alignment was performed using CLUSTALX software

program (Thompson *et al*. 1997) and phylogenetic tree was drawn using MEGA program (v.7.0) using neighbor-joining method (Hall *et al*. 2013). The homologues and their GenBank accession numbers for the phylogenetic analysis are as follows: *Ziziphus jujube* (XM_016042698), *Malus domestica* (XM_008367613), *Vitis vinifera* (XM_002282906), *Citrus sinensis* (XM_006475224), *Theobroma cacao* (XM_007021048), *Gossypium hirsutum* (XM_016847999), *Lupinus angustifolius* (XM_019574573), *Arachis ipaensis* (XM_016315262), *Glycine max* (XM_003543235), *Arachis duranensis* (XM_016081016), *Elaeis guineensis* (XM_010925694), *Musa acuminata* (XM_009420487), *Setaria italica* (XM_004957886), *Oryza sativa Japonica* (AY062209), *Brachypodium distachyon* (XM_003562912) and *Zea mays* (NM_001154467).

3.3. RNA extraction and gene expression analysis

Total RNA's of zoysiagrass and Arabidopsis were extracted using Trizol (Invitrogen, Carlsbad, GA, USA) following the manufacturer's procedure. Two microgram RNA was used for synthesizing the cDNA with M-MLV RT kit (Moloney Murine Leukemia Virus Reverse Transcriptase, Promega). RT-PCR was performed with the synthesized cDNA using *ZjCIGR1*-specific primers (forward, 5' -GCCCCGAAGGTGACTACTTT-3'; reverse, 5'–TGCCTCTCCACTCTGTCCTT- 3'). To ensure equal amounts of cDNA, *18s ribosomal RNA* was used as control (forward, 5'–CTCATGGGATGTGGCTTCTT- 3'; reverse, 5' –GCGTTCAAAAACTCGATGGT-3'). Expression of the genes was identified on electrophoresis. Real-time PCR was performed using MJ Opticon Monitor^{TM} (Bio-Rad, Cambridge, MA, USA) and iQ SYBR Green Supermix (Bio-Rad) following the manufacturer's procedure. For the accuracy of results, experiments were performed in triplicates using the relative quantization method ($2^{-\Delta\Delta Ct}$; Livak *et al*. 2001).

The gene expression patterns of transgenic zoysiagrass were determined with the plant leaves under normal growth conditions. Expression analysis of the gene under stress treatment was performed with the leaves of *ZjCIGR1* transgenic line 7. All the analyses were conducted using *ZjCIGR1*-primers

(forward, 5’-GCCCCGAAGGTGACTACTTT-3’; reverse, 5’–TGCCTCTCCACTCTGTCCTT-3’).

For *Arabidopsis*, total RNA was extracted from the leaves of the plant survived under cold stress. Then, expression analysis of *ZjCIGR1* and stress-related genes in *Arabidopsis thaliana* (*At*) (*AtHKT1*, *AtCOR15A*, *AtProDH1*, *AtRD29A* and *AtCBF3*) was performed. The sequences of primers used in this study were as follows; *AtHKT1* forward (5’-TCAGTGCATATGGAAACGTTGG-3’) and reverse (5’-CCATTGGACTCCATCGTCCTG-3’); *AtCOR15A* forward (5’-AAAGAGGCATTAGCAGATGGTGA-3’) and reverse (5’-TTTTCCTTTCTCCTCCACATACG-3’); *AtProDH1* forward (5’-ACACATAACGCTGATTCGGGGAG -3’) and reverse (5’-GATACGGTATAGCGGTTGCGACG-3’); *AtRD29A* forward (5’- TGGATACGGTGAGGCATCGA-3’) and reverse (5’-ACAGTTCCCGCCACTTGAGTTTG-3’) and *AtCBF3* forward (5’-GATGACGACGTATCGTTATGGA-3’) and reverse (5’-TACACTCGTTTCTCAGTTTTACAAAC-3’).

3.4. Southern blot analysis of *ZjCIGR1*

Genomic DNA was isolated from the young leaves of zoysiagrass and *Arabidopsis* wild type and 10 lines of transgenic zoysiagrass plants, respectively. To calculate the number of copies of the *ZjCIGR1* gene in the wild-type and transgenic plants, 30㎍ of genomic DNA was digested with *Xba* Ⅰ (TAKARA, Japan) then fractionated on 0.8% (w/v) agarose gel. The gel was blotted onto a nylon membrane (Hybond N+, Amersham, Little Chalfont, UK) and then cross-linked. The membrane was hybridized with a Dig-labeled probe (PCR DIG Probe Synthesis Kit, Roche Diagnostics, USA). Dig-labeled probes were designed by PCR using *ZjCIGR1* and *bar* primers. To identify the *ZjCIGR1* gene in zoysiagrass, 35S promoter forward primer (5’-AAACCTCCTCGGATTCCATT-3’) and *ZjCIGR1* reverse primer (5’-TCAGGCGCCATTAGAACAGT-3’) were used and *bar* forward (5’-AAGTCCAGCTGCCAGAAACCCAG-3’) and reverse primers (5’-GTCTGCACCATCGTCAACCACTA-3’) were used for identification of the *bar* gene. To identify the

ZjCIGR1 gene in wild-type *Arabidopsis*, *ZjCIGR1* forward (5'-AGATCTATGGACTTGCAGCAGTTATT-3') and reverse primers (5'-TCAGGCGCCATTGAACAGT-3') were used (Fig. 1).

Hybridization was carried out at 42℃ overnight in high -SDS buffer containing 50% formamide, 5X SSC, 50mM sodium phosphate (pH 7.0), 2% blocking reagent and 0.1% N-lauroylsarcosine. The blots were washed twice with 2X SSC, 0.1% SDS for 15 min at 37℃, then washed twice with 0.1X SSC, 0.1% SDS for 20 min at 60℃. Hybridization signals were detected by chemiluminescence (CDP-star, Amersham, Little Chalfont, UK) and visualized by LAS4000 luminescent image analyzer (Fujifilm, Tokyo, Japan).

3.5. Vector construction for plant transformation

Enzyme sites were identified, which were not in the full sequence of *ZjCIGR1*. Among them, *Bgl* Ⅱ and *BstE* Ⅱ were used with a binary vector pCAMBIA 3301 containing 35S CaMV (cauliflower mosaic virus) promoter. The full coding sequence of *ZjCIGR1* was amplified with primers containing the two enzyme sites [forward, 5'-AGATCT ATGGACTTGCAGCAGTTATT-3' (the *Bgl* Ⅱ site is underlined); reverse. 5'–GGTNACC GTGCCATGCTGAAGCTGATA-3' (the *BstE* Ⅱ site is underlined)] and inserted into the binary vector. The expression vectors of *ZjCIGR1* were introduced into *Agrobacterium tumefaciens* EHA105 for zoysiagrass and GV3101 for *Arabidopsis*.

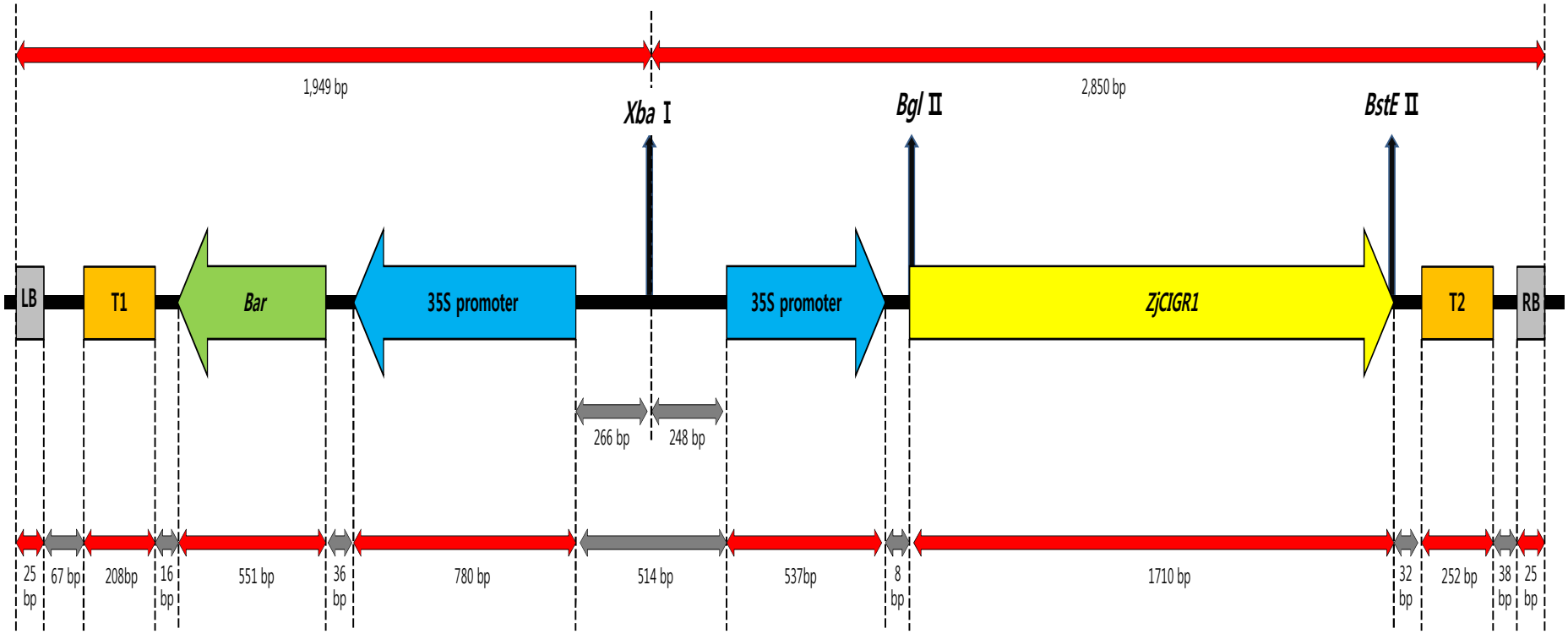


Fig. 1. Vector map of *35S::ZjCIGR1*. LB, left border; T1, CaMV poly(A) signal; T2, NOS terminator; RB, right border.

3.6. Transformation of zoysiagrass and *Arabidopsis*

Zoysiagrass transformation was performed by the method of Toyama *et al*. (2003), with minor modifications. After removing the testa of mature zoysiagrass seeds, the seeds sterilized in 100% ethanol, followed by sodium hypochlorite solution with 0.1% Tween 20 and cleaned three times with sterilized water. The seeds were cultured in medium and callus were induced and selected. *A. tumefaciens* strain EHA105 with the binary vector construct pCAMBIA3301-ZjCIGR1 was grown at 28 ℃ in liquid Yep medium (Lee *et al*. 2008) containing kanamycin and rifampicin. The selected calluses were transferred to liquid infection medium containing *Agrobacterium* suspension and cultured at 25℃ for 24 hours with shaking (110rpm). And then the infected calluses transferred to a solid co-cultivation medium and were cultured in the dark for 3 days and then placed on the selection medium with PPT for 3 weeks. The PPT resistant calluses were transferred to shoot induction media and cultured under light (Toyama *et al*. 2003). After 3 months, elongated shoots were transferred to a root induction medium (Toyama *et al*. 2003; Kim *et al*. 2007) and selected putative transgenic plantlets. The plants were grown at 25 ℃ under long-day conditions (16h-h light/8-h dark).

Arabidopsis transformation was performed by the floral dip method (Clough and Bent 1998). By spraying BASTA solution, transgenic Arabidopsis were selected. T3 homozygous lines were used for stress-tolerance experiments.

3.7. Identification of plant transformants

To determine the insertion of the transgene into zoysiagrass, genomic DNA PCR was performed using 35S promoter, *ZjCIGR1* and *bar* primers: 35S promoter forward (5’-AAACCTCCTCGGATTCCATT-3’) and *ZjCIGR1* reverse (5’-ATCAGGCGCCATTAGAACAG-3’), *ZjCIGR1* forward (5’-AGATCT ATGGACTTGCAGCAGTTATT-3’) and *ZjCIGR1* reverse (5’-GGTNACC GTGCCATGCTGAAGCTGATA-3’) and *bar* forward (5’-AAGTCCAGCTGCCAGAAACCCAC-3’) and *bar* reverse primers (5’-

GTCTGCACCATCGTCAACCACTA-3'). The transformants were also confirmed by phosphinothricin-transferase (PAT) strip test (Koczula and Gallotta 2016) and Southern blot analysis.

For *Arabidopsis*, there was no *ZjCIGR1* gene in wild-type *Arabidopsis* according to Southern blot analysis, and transgenic *Arabidopsis* plants were selected by spraying BASTA solution. Also, to confirm the insertion of the *ZjCIGR1* gene in transgenic *Arabidopsis* plants, genomic DNA PCR was conducted by using *ZjCIGR1* primers; *ZjCIGR1* forward (5'-AGATCTATGGACTTGCAGCAGTTATT-3') and *ZjCIGR1* reverse (5'-GGTNACCGTGCCATGCTGAAGCTGATA-3').

3.8. Abiotic stress tolerance assay on transgenic plants

For zoysiagrass, all leaves were cut into about 3cm pieces and acclimated in sterilized water for 3 hours. For the dark stress treatment, the cut leaves were protected from light by covering the whole plate with aluminum foil for 11 days in sterilized water. For the salt stress, the cut leaves were dipped in 250mM NaCl for 11 days.

Three-week-old wild type and T_3 generation transgenic *Arabidopsis* seedlings were used in cold stress tolerance experiments. Cold stress treatment was performed as reported previously (Miura *et al*. 2011), with minor modifications. The seedlings were treated in a cold treatment chamber maintained at 4℃ for 1 day and the temperature was decreased at a rate of 1℃ h^{-1} from 0℃ to -8℃, each temperature point (0, -2, -4, -6, -8℃), kept for 1 hour, respectively. After that, the seedlings were incubated at 22-24℃ for recovery.

4. RESULTS AND DISDUSSION

4.1. Isolation and sequence analysis of *ZjCIGR1* from zoysiagrass

To clone the *CIGR1* gene from the zoysiagrass plant, total RNA was extracted from leaves of the grass (Fig. 2A). RT-PCR was performed to obtain the gene fragment from the extracted total RNA. A nucleotide sequence of about 730bp was obtained (Fig. 2B). Based on the sequence, RACE PCR primer was designed and performed. The first 5'RACE and 3'RACE resulted in about 800bp (Fig. 2C) containing stop codon and about 700bp (Fig. 2D), respectively. To identify the start codon, second PCR was performed, yielding a ~500bp fragment containing the start codon (Fig. 2E). Full-length PCR was performed to minimize errors in the nucleotide sequence obtained by duplicate RACE PCR (Fig. 2F). Sequence analysis showed the open reading frame (ORF) of *ZjCIGR1* was 1710bp long, encoding a protein of 570 amino acid residues (Fig. 2G).

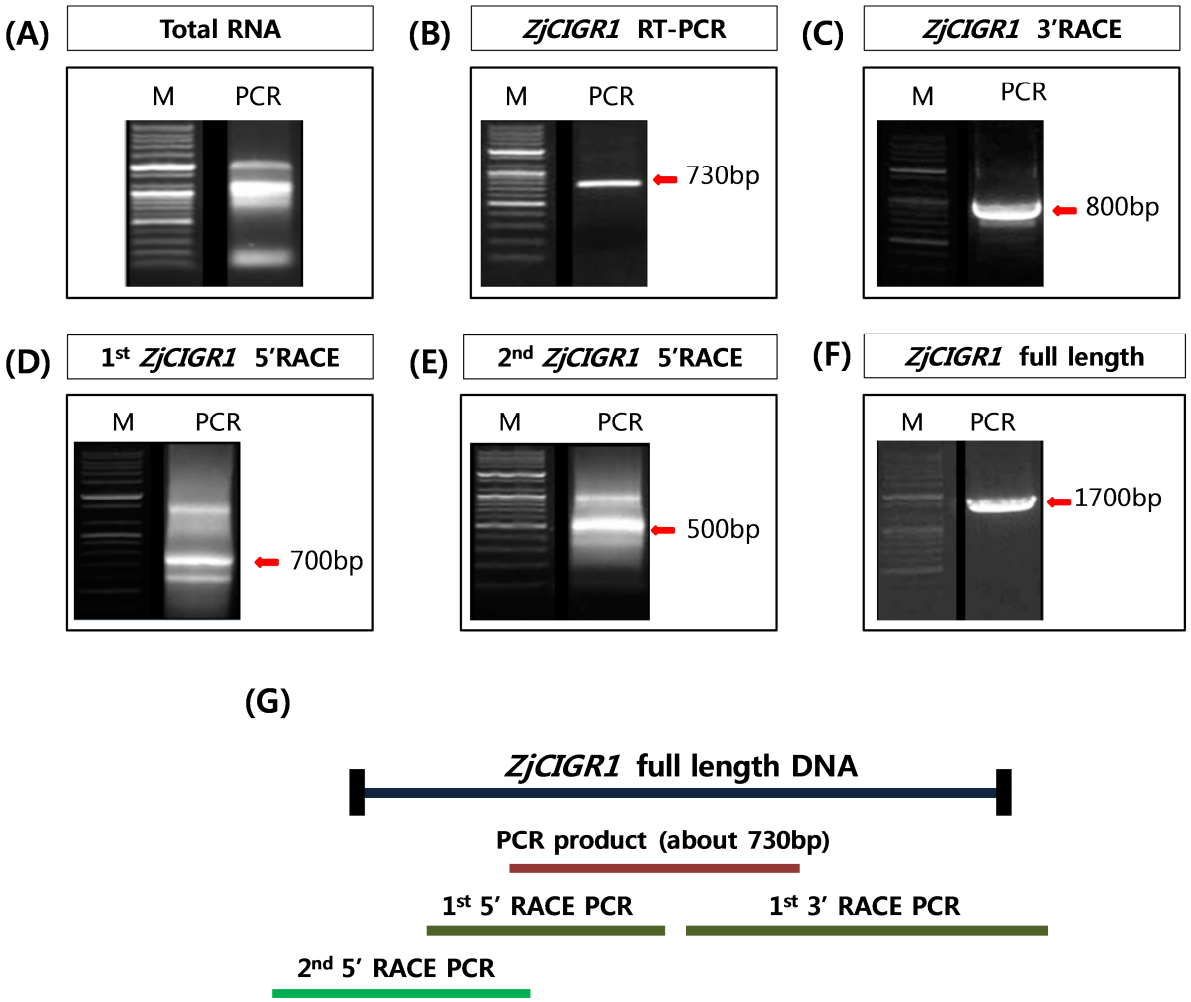


Fig. 2. Cloning of *ZjCIGR1* gene from *Zoysia japonica* plants. (A) Total RNA from *Zoysia japonica* plants ; (B) RT-PCR product ; (C) 3' RACE PCR product ; (D) 1st 5' RACE PCR product ; (E) 2nd 5' RACE PCR product ; (F) Full length DNA PCR product ; (G) Diagram of *ZjCIGR1* gene cloning ; M, Bioneer size maker.

Alignment of ZjCIGR1 protein with their homologues from other plant species revealed a variable N-terminal and a conserved motif typical among the GRAS family proteins in the C-terminal as shown below (Fig. 3). Thus, VHIID motif mediates protein: DNA interactions, and LXXLL motif has been identified to mediate the binding of steroid receptor: co-activator complexes to cognate nuclear receptors in mammals (Heery *et al*. 1997; Sun *et al*. 2012). PFYRE, RVER and SAW are additional peptide residues in most or all members of the GRAS protein family (Bolle 2004). These motifs may have a regulatory function (Itoh *et al*. 2002). With the presence of these motifs, we suggest that ZjCIGR1 belongs to the GRAS protein family and further propose that ZjCIGR1 protein interacts with factors involved in transcriptional regulation.

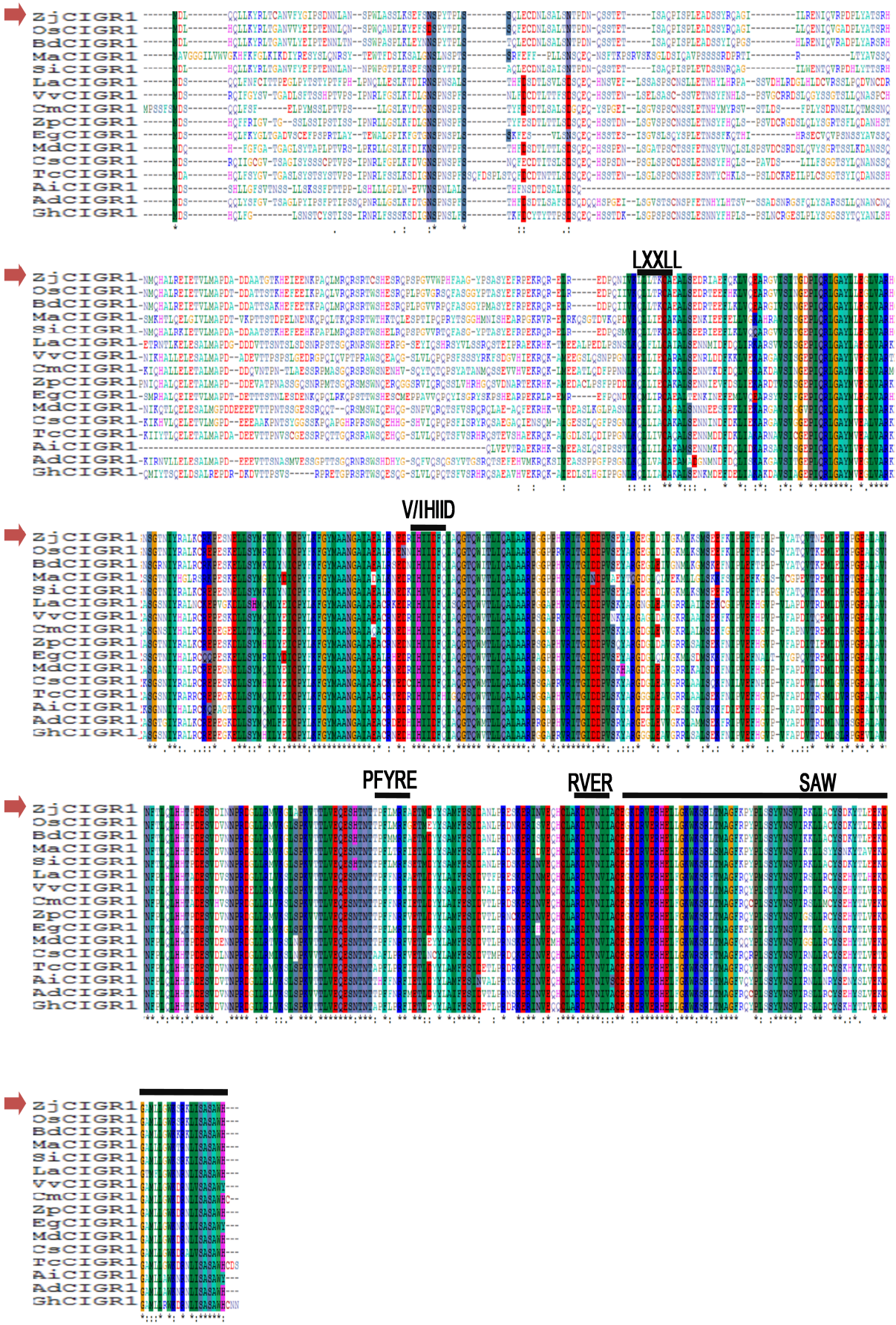


Fig. 3. Alignment of the amino acid sequence of ZjCIGR1 with CIGR1 proteins of other crops. Zp, *Ziziphus jujuba* ; Md, *Malus domestica* ; Vv, *Vitis vinifera* ; Cs, *Citrus sinensis* ; Tc, *Theobroma cacao* ; Gh, *Gossypium hirsutum* ; La, *Lupinus angustifolius* ; Ai, *Arachis ipaensis* ; Gm, *Glycine max* ; Ad, *Arachis duranensis* ; Eg, *Elaeis guineensis* ; Ma : *Musa acuminata* ; Si, *Setaria italica* ; Os, *Oryza sativa Japonica* ; Bd, *Brachypodium distachyon* ; Zm, *Zea mays*

Phylogenetic analysis was performed based on the conserved domains (Fig. 4). The blue circles are monocots and the pink circles are dicots. As shown in Fig. 4, monocots and dicots are clearly separated into two groups, and *CIGR1* and *CIGR2* were readily distinguishable. *ZjCIGR1* appears to be evolutionarily closely related to *SiCIGR1* from *Setaria italica* and *OsCIGR1* from *Oryza sativa*, consistent with NCBI blast results (90% and 89% identities, respectively). The *CIGR1* gene functions as a transcriptional regulator in plant development and defense responses (Day *et al*. 2003; Bolle *et al*. 2004; Tian *et al*. 2004). In rice, the *CIGR1* gene is located in the major locus affecting plant height (Kovi *et al*. 2011), and also functions as a transcriptional regulator in elicitor-induced defense responses (Day *et al*. 2003; Day *et al*. 2004). We also constructed a phylogenetic tree with other GRAS proteins and confirmed that the ZjCIGR1 protein is a member of the PAT1-group of GRAS protein family (Fig. 5). The PAT1 group is involved in the phytochrome A signaling pathway and plays a positive regulatory role in phytochrome B-mediated red-light signaling (Bolle *et al*. 2000; Sun *et al*. 2012). In addition to response to light, the PAT1 group was recently identified as positive components that functioned in stress resistance (Yuan *et al*. 2015). These results suggest that *ZjCIGR1* plays a similar role in stress responses.

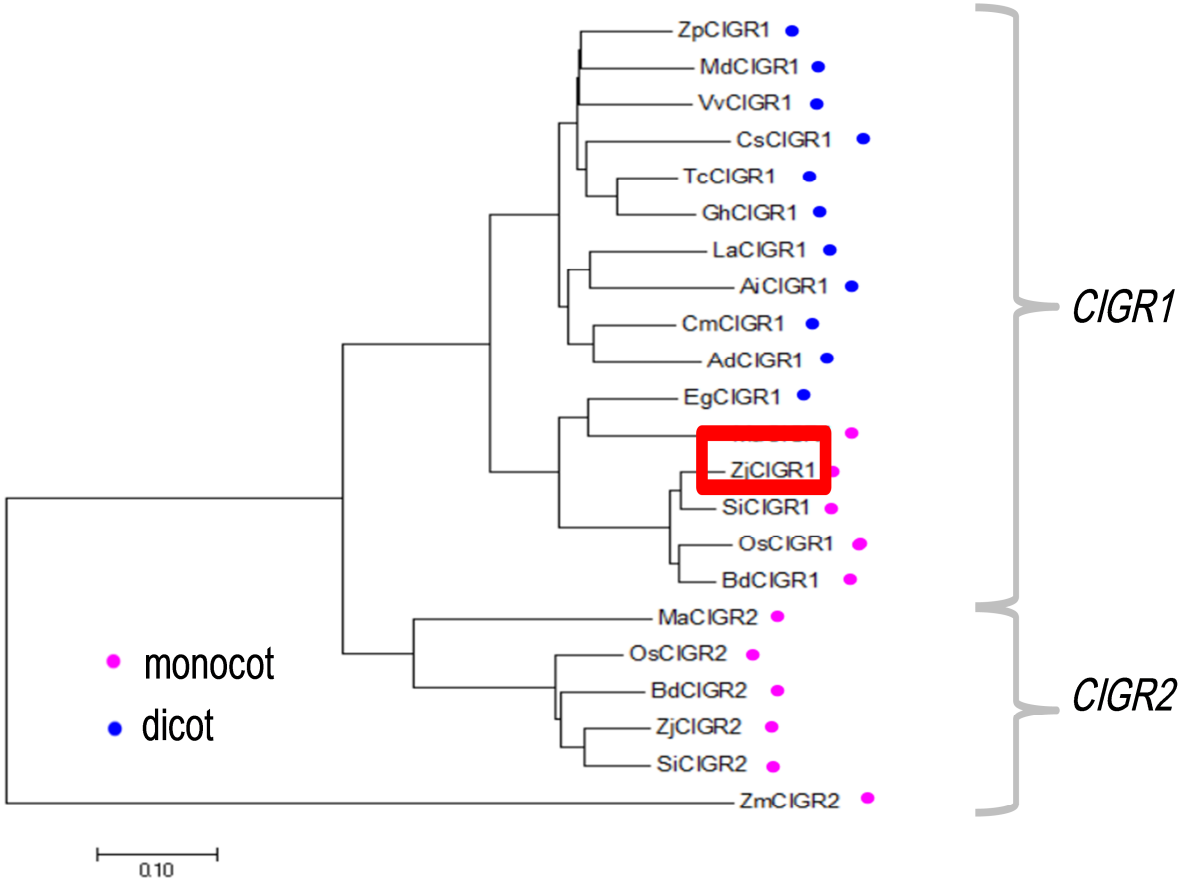


Fig. 4. Phylogenetic analysis of ZjCIGR1 with CIGR1, CIGR2 proteins of other plants. Zp, *Ziziphus jujuba* ; Md, *Malus domestica* ; Vv, *Vitis vinifera* ; Cs, *Citrus sinensis* ; Tc, *Theobroma cacao* ; Gh, *Gossypium hirsutum* ; La, *Lupinus angustifolius* ; Ai, *Arachis ipaensis* ; Gm, *Glycine max* ; Ad, *Arachis duranensis* ; Eg, *Elaeis guineensis* ; Ma : *Musa acuminata* ; Si, *Setaria italica* ; Os, *Oryza sativa Japonica* ; Bd, *Brachypodium distachyon* ; Zm, *Zea mays*

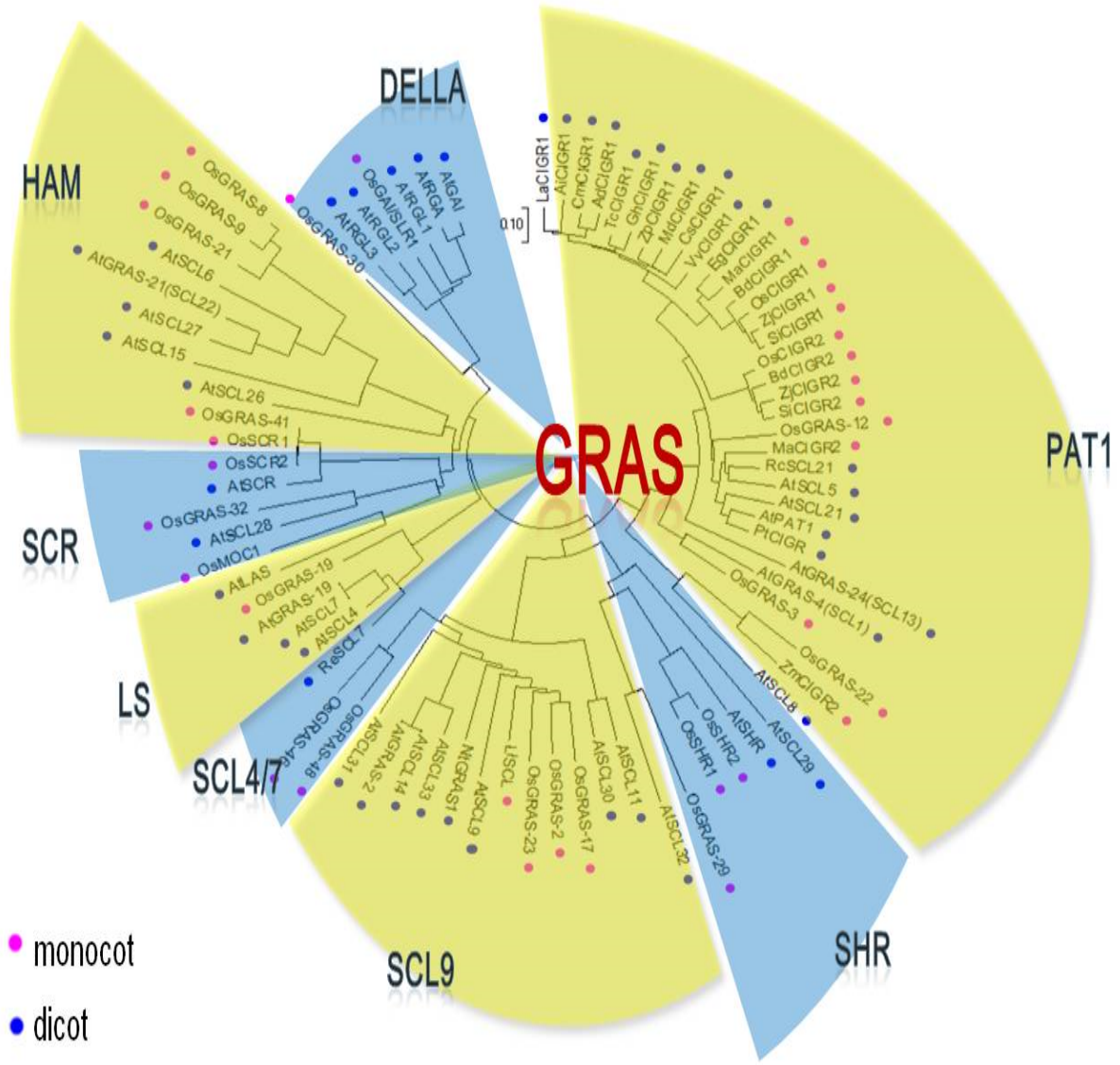


Fig. 5. Phylogenetic analysis of GRAS protein family. The proteins of *Arabidopsis thaliana* (At) and their GenBank accession numbers are as follows: At-GRAS4 (NP_173566), At-SCL4 (NP_201478), At-SCL6 (NP_191926), At-SCL7 (NP_190634), AT-SCL8 (NP_200064), At-SCL9 (NP_001323705), At-SCL11 (NP_200753), At-GRAS24 (NP_193456), At-SCL14 (NP_172233), At-SCL15 (NP_195389), At-SCL22 (NP_191622), At-SCL26 (NP_192565), At-SCL27 (NP_182041), At-SCL28 (NP_001319298), At-SCL29 (NP_188000), At-SCL30 (NP_001319695), At-SCL31 (NP_001322138), At-SCL32 (NP_190564), At-SCL33 (NP_001323611), At-GAI (NP_172945), At-RGL1 (NP_176809), At-RGA (NP_178266), At-SCR (NP_190990), At-RGL3 (NP_19725), At-PAT1

(NP_199626), At-LAS (NP_175954) and At-SHR (NP_195480). The proteins of *Oryza sativa* (Os) and their GenBank accession numbers are as follows: Os-GRAS2 (XP_015619294), Os-GRAS3 (XP_015610828), Os-GRAS8 (XP_01562707), Os-GRAS9 (XP_015627415), Os-GRAS17 (XP_015630941), Os-GAI/SLR1 (XP_015631543), Os-GRAS19 (XP_015628849), Os-GRAS22 (XP_015637100), Os-GRAS23 (XP_015637021), Os-GRAS29 (XP_015638716), Os-GRAS30 (XP_015638486), Os-GRAS32 (XP_015642886), Os-MOC1 (XP_015642672), Os-GRAS42 (XP_015615402), Os-GRAS46 (XP_015617900), Os-GRAS48 (XP_015616926), Os-PAT1 (XP_015626732), Os-SHR1 (A2YN56), Os-SHR2 (A2XIA8), Os-SCR1 (A2ZAX5) and Os-SCR2 (A2ZHL0). Other proteins of crops and their accession numbers are as follows: Pe-SCL7 (*Populus euphratica*, AHZ13509), Nt-GRAS1 (*Nicotiana tabacum*, ABE02823) and Ll-SCL (*Lilium longiflorum*, BAC77269).

4.2. *ZjCIGR1* expression profile

The expression patterns of *ZjCIGR1* mRNA were analyzed by RT-PCR from roots, meristems, leaves and flowers (Fig. 6A). *ZjCIGR1* was expressed in all organs, especially highly in flowers. Expression of the *18s* rRNA in leaves was low, but the expression of *ZjCIGR1* was similar those of other organs, suggesting that *ZjCIGR1* in leaves is also highly expressed. Real-time PCR was also performed to quantify the relative *ZjCIGR1* expressions compared with a control gene (Fig. 6B). As with RT-PCR, *ZjCIGR1* was expressed in all organs and showed high expression in flowers and leaves. *Oryza sativa GAI*, a GRAS family gene, was also expressed in all organs, but expression profile of each gene was in different organs in rice (Ogawa *et al*. 2000). However, *Arabidopsis RGA*, a GRAS family gene, was ubiquitously expressed in all organs (Silverstone *et al*. 1998). These results suggest that the zoysiagrass is more homologous to monocot rice than to dicot *Arabidopsis*.

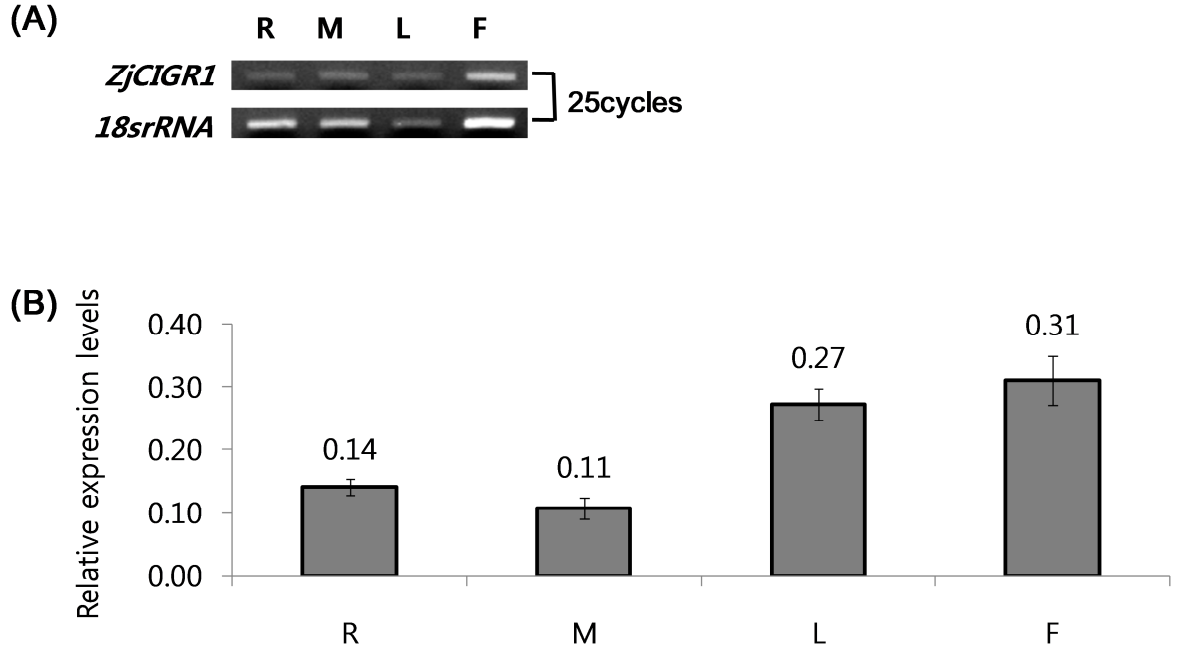
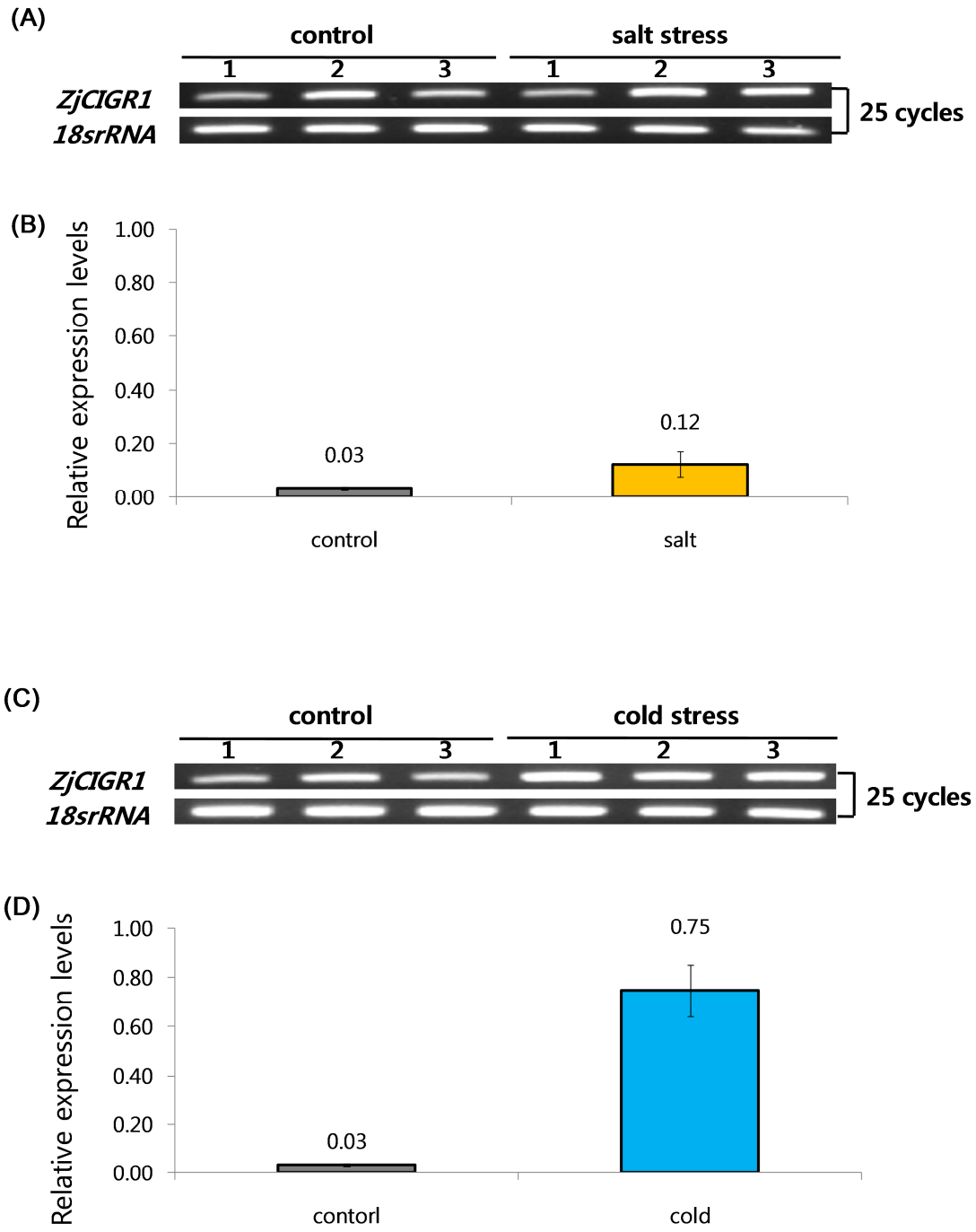


Fig. 6. *ZjCIGR1* transcript expression and relative expression in different organs. (A) *ZjCIGR1* transcript expression in different organs; R, root; M, meristems; L, leaves; F, flowers. Amplification of *18s* rRNA gene was used to ensure that equal amounts of template were used in each PCR reaction. Twenty cycles of PCR were performed. (B) The relative expression of *ZjCIGR1* transcripts in zoysia grass was quantified with real-time PCR. Vertical scales show the relative amounts of *ZjCIGR1* transcripts compared to the internal standard (*18s* rRNA).

To determine whether *ZjCIGR1* was involved in environmental stress in wild-type *Zoysia japonica*, expression patterns of the gene were analyzed by RT-PCR (Fig. 7A, 7C and 7E) and real-time PCR (Fig. 7B, 7E and 7F) under salt, cold and dark conditions. They were analyzed after each stress treatment for 3 hours. As shown in Fig. 7A-F, the expression was higher under salt and cold stresses compared to control (4.0-fold and 25.0-fold, respectively), although there was no significant difference from the dark kept control. With increasing number of days under stress treatments, the expression level of *ZjCIGR1* increased relative to the wild-type (Fig. 8A-C), especially on 1st and 11th day (about 4.6-fold and 5.1-fold, respectively) (Fig. 8A). For light deficiency treatment, there was no significant difference from the wild-type as a whole, but on the 1st day, the expression increased by more than 2 fold compared with the wild-type (Fig. 8B). GRAS family genes, *Vitis amurensis PAT1* and *Oryza sativa GRAS23*, were also induced to a higher level than the wild-type plants under cold and salt stresses (Yuan *et al*. 2015; Xu *et al*. 2015). Analysis of transcripts of Miscanthus at low temperature also showed the expression of GRAS transcription factors (Chung *et al*. 2013). These observations suggest that the expression of *ZjCIGR1* in response to stress treatments of the wild-type is similar to the previously reported results. It appears that the *ZjCIGR1*, GRAS family gene, responds to salt, cold and dark stresses in *Zoysia japonica*.



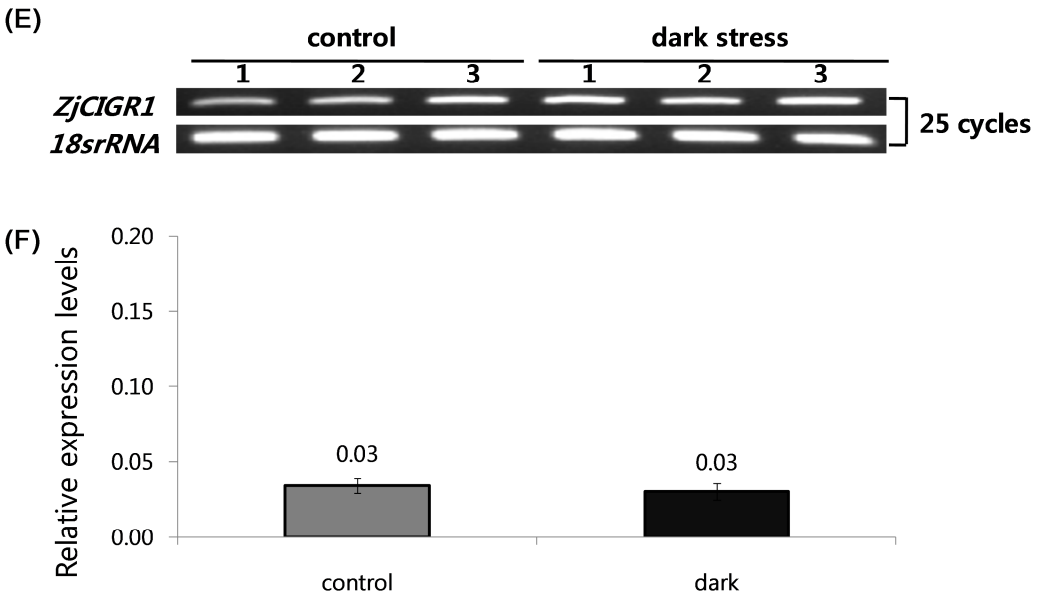
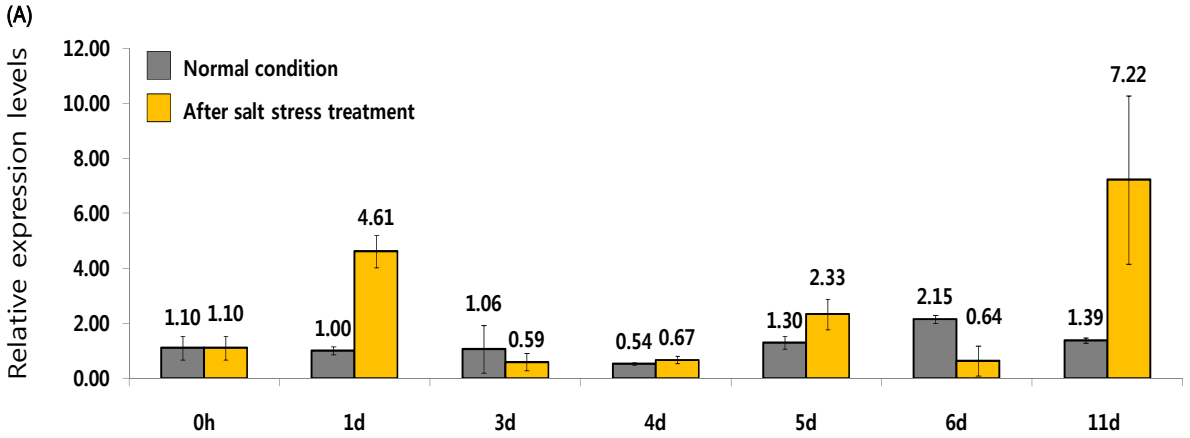
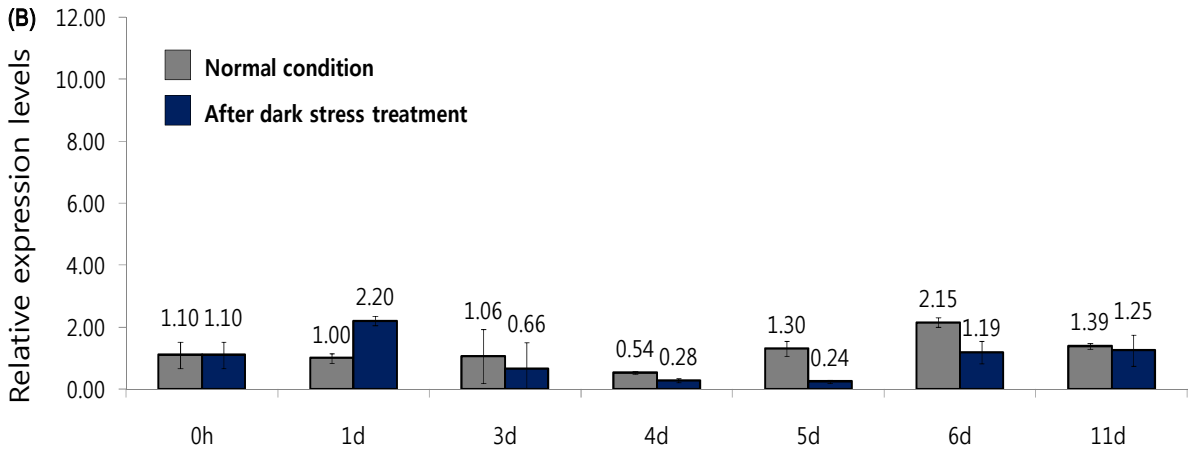


Fig. 7. Expression patterns of *ZjCIGR1* in response to salt (200mM NaCl; A and B), cold (4℃; C and D) and dark (light deficiency; E and F) in wild type *Zoysia japonica* plants. (A), (C) and (E) *ZjCIGR1* transcript expression. Amplification of 18s rRNA was used to ensure that equal amounts of the template were used in each PCR reaction. Thirty cycles of PCR were performed. (B), (D) and (F) The relative expression of *ZjCIGR1* transcripts in zoysiagrass was quantified with real-time PCR. Vertical scales show the relative amounts of *ZjCIGR1* transcripts compared to the internal standard (*18s* rRNA).





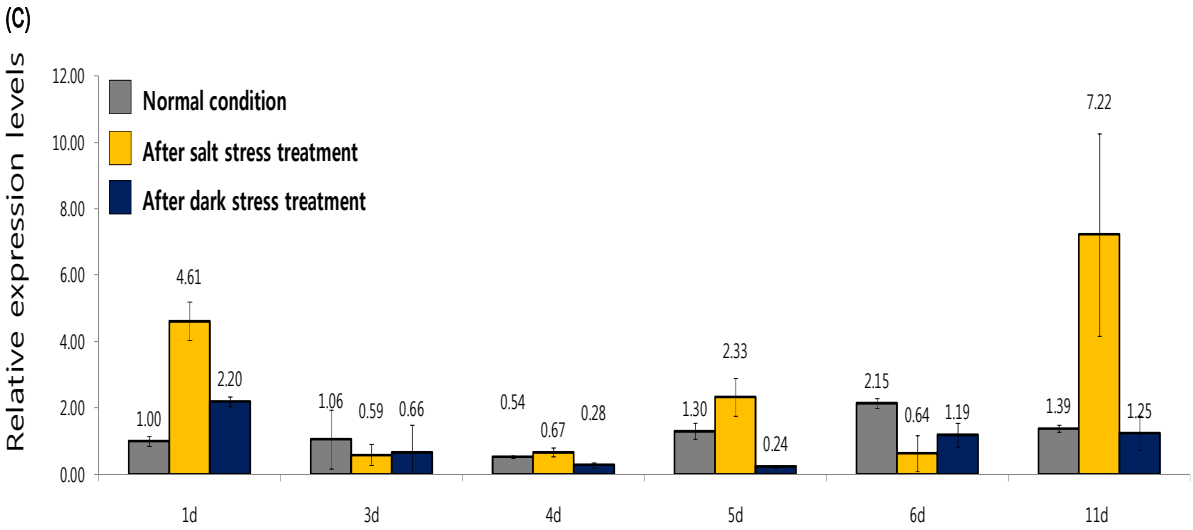
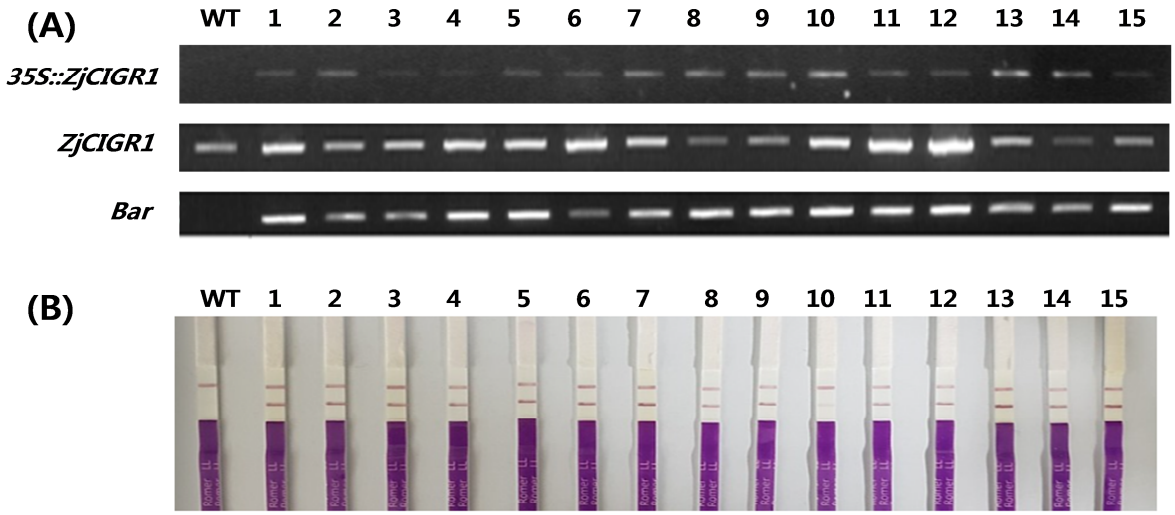
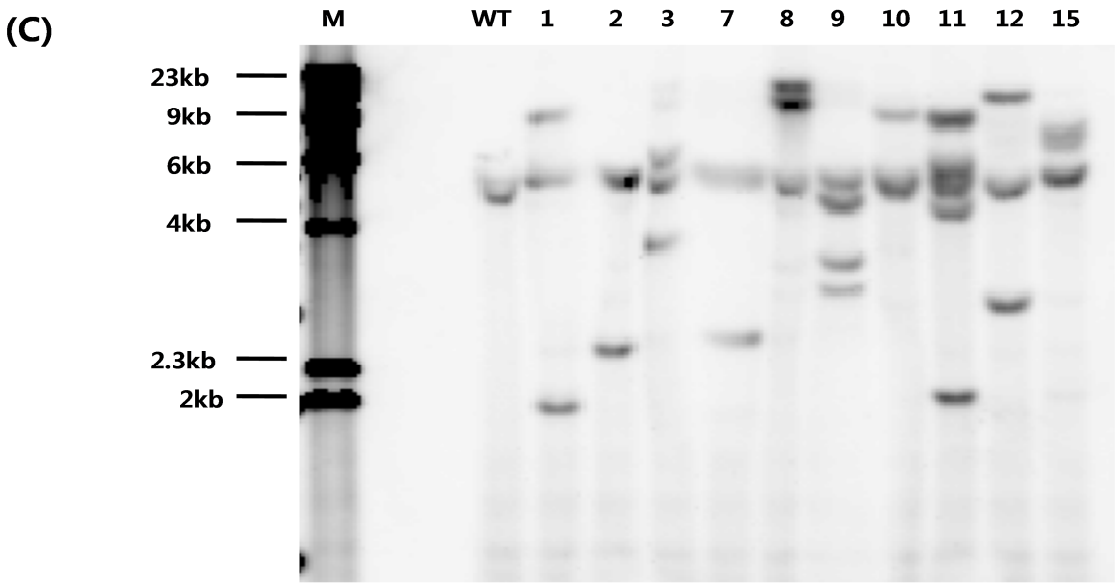


Fig. 8. Expression patterns of *ZjCIGR1* in response to salt (200mM NaCl; A) and dark (light deficiency; B) in wild type *Zoysia japonica* plants; d, day. Relative expression of *ZjCIGR1* transcripts in zoysiagrass was quantified with real-time PCR. Vertical scales show the relative amounts of *ZjCIGR1* transcripts compared to the internal standard (*18s* rRNA). (C) Comparison of the expression patterns of *ZjCIGR1* in salt and dark stress treatments.

4.3. Molecular and phenotypic analyses of *ZjCIGR1* transgenic zoysiagrass plants

In order to confirm the insertion of the *ZjCIGR1* gene into the transgenic zoysiagrass, genomic DNA PCR was carried out by using the 35S promoter, *ZjCIGR1* and *bar* primers (Fig. 9A) and phosphinothricin-acetyl-transferase (PAT) strip test was performed to identify the transformants (Fig. 9B). Also, insertion of the *ZjCIGR1* and *bar* genes was confirmed by Southern blot analysis (Fig. 9C and 9D).





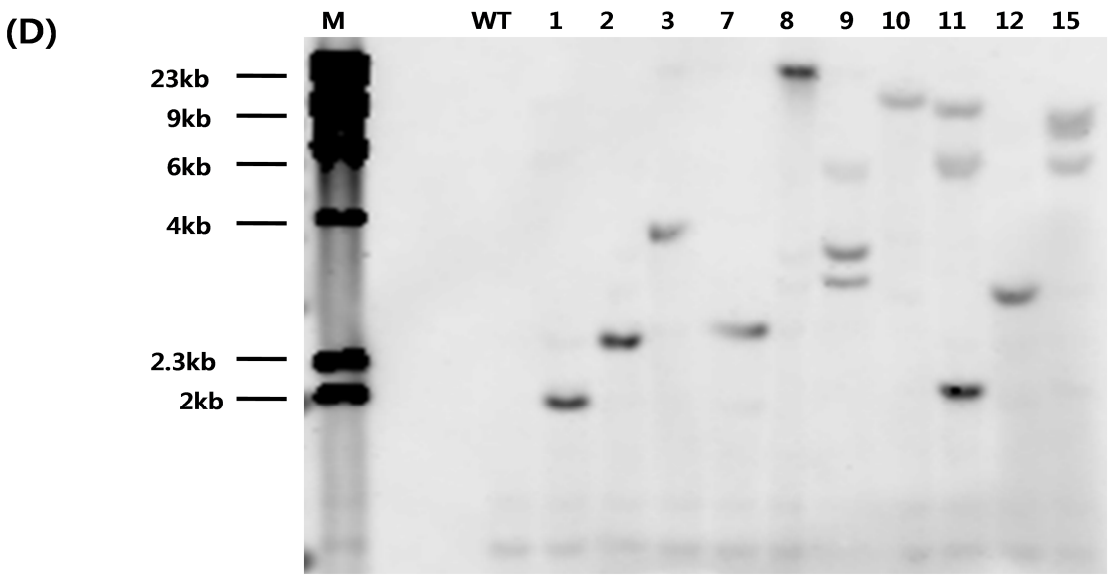


Fig. 9. Identification of *35S::ZjCIGR1* transgenic zoysiagrass. (A) PCR of 35S promoter, *ZjCIGR1* and *bar* genes using genomic DNA; WT, wild-type plant; 1-15, *35S::ZjCIGR1* transgenic plants. (B) phosphinothricin-acetyl-transferase (PAT) strip test. WT, wild-type plant; 1-15, *35S::ZjCIGR1* transgenic plants. (C) Southern blot analysis of wild-type and *35S::ZjCIGR1* transgenic plants; M, Dig size marker; WT, wild-type plant; 1-15, *35S::ZjCIGR1* transgenic plants. Insertion of the *ZjCIGR1* gene of transgenic zoysiagrass was confirmed with Southern blot analysis using single disgestion with *Xba* Ⅰ, followed by hybridization with the *ZjCIGR1* probe. (D) Southern blot analysis of wild-type and *35S::ZjCIGR1* transgenic plants; M, Dig size marker; WT, wild-type plant; 1-15, *35S::ZjCIGR1* transgenic plants. Insertion of the *ZjCIGR1* gene of transgenic zoysiagrass was confirmed with Southern blot analysis using single disgestion with *Xba* Ⅰ, followed by hybridization with the *bar* probe.

Because overexpression of *PAT1* was resistant to salt and cold stress in *Vitis amurensis* (Yuan *et al*. 2015), stress test was performed to test whether each *ZjCIGR1*-overexpressing zoysiagrass was resistant to stress. For salt stress (Fig. 10A-F), the wild-type began aging on the 6th day (Fig. 10C and 10D), and the leaves became completely yellow on the 8th day (Fig. 10E and 10F), while the transformants maintained the green leaves (Fig. 10E and 10F). For dark stress (Fig. 11A-11F), on the 2nd day, the leaves of wild-type began aging (Fig. 11C and 11D) and they all aged by the 9th day (Fig. 11E and 11F). On the contrary, the leaves of the transformants stayed green (Fig. 11E and 11F). In tomato (*Solanum lycopersicum*), the overexpression of *GRAS40* has resistance to salt stress (Liu *et al*. 2017), overexpression of *SCL21* and *PAT1* in *Arabidopsis* were responded to light signaling (Bolle *et al*. 2000; Toress-Galea *et al*. 2013). These results suggest that *ZjCIGR1*-overexpressing lines are resistant to stress and display delayed senescence, as the transformants sustained the green leaves under salt or dark stress conditions compared to the wild-type.

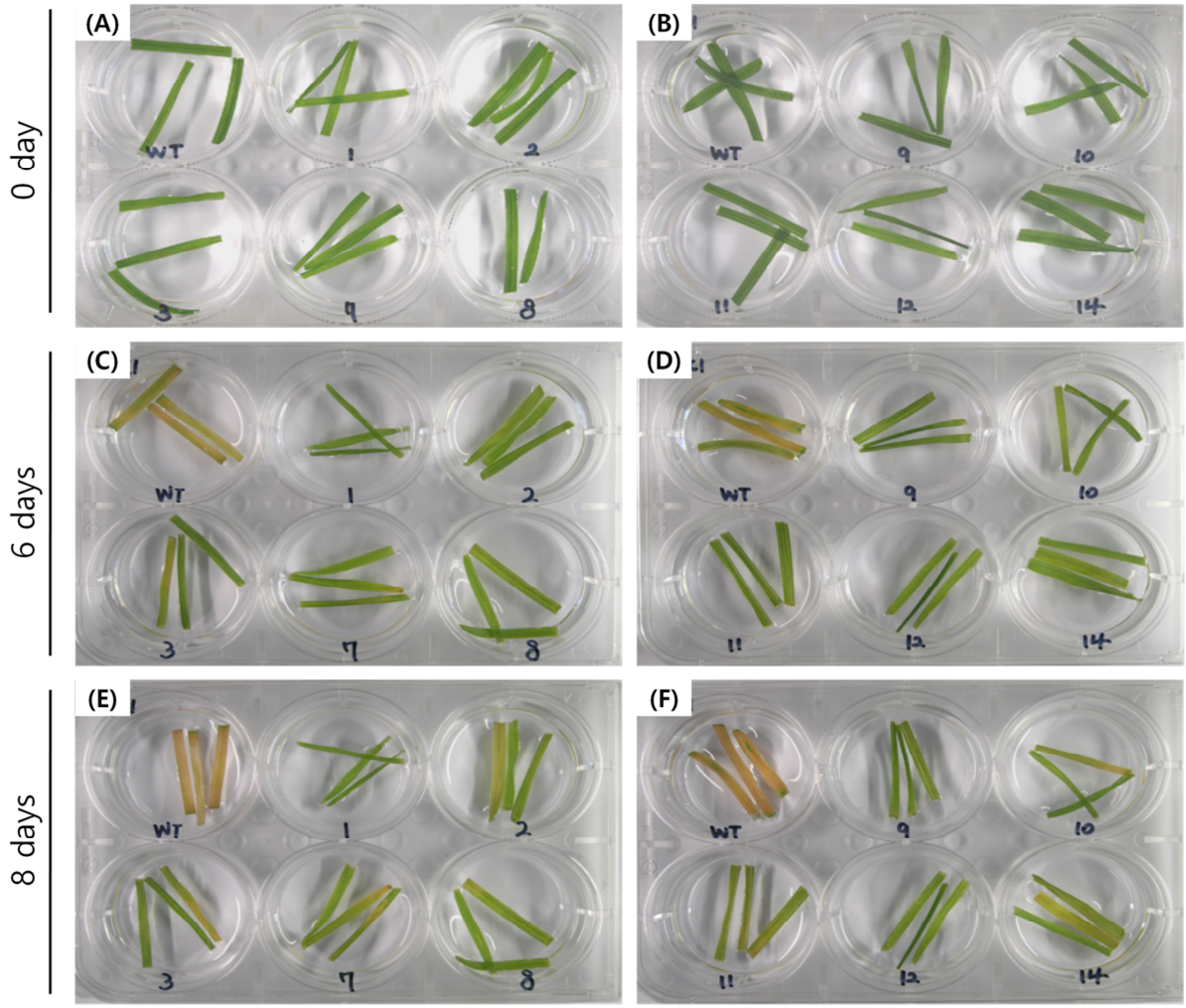


Fig. 10. Phenotype of wild-type (WT) and transgenic *Zoysia japonica* plants under salt stress treatment (NaCl 250mM; A-F). (A) 0 day after salt stress treatment (wild type and transgenic line 1, 2, 3, 7 and 8). (B) 0 day after salt stress treatment (wild type and transgenic lines 9, 10, 11, 14). (C) 6 days after salt stress treatment (wild type and transgenic lines 1, 2, 3, 7 and 8). (D) 6 days after salt stress treatment (wild type and transgenic lines 9, 10, 11, 14). (E) 8 days after salt stress treatment (wild type and transgenic lines 1, 2, 3, 7 and 8). (F) 6 days after salt stress treatment (wild type and transgenic lines 9, 10, 11, 14).

Fig. 11. Phenotypes of wild-type (WT) and transgenic *Zoysia japonica* plants under dark stress (light deficiency; A-F). (A) 0 day after dark stress treatment (wild type and transgenic lines 1, 2, 3, 7 and 8). (B) 0 day after salt stress treatment (wild type and transgenic lines 9, 10, 11, 14). (C) 5 days after dark stress treatment (wild type and transgenic lines 1, 2, 3, 7 and 8). (D) 5 days after salt stress treatment (wild type and transgenic lines 9, 10, 11, 14). (E) 9 days after dark stress treatment (wild type and transgenic lines 1, 2, 3, 7 and 8). (F) 9 days after salt stress treatment (wild type and transgenic lines 9, 10, 11, 14).

In order to test whether expression of *ZjCIGR1* increased when the *ZjCIGR1*-overexpressing lines were stressed compared to the wild-type, we analyzed *ZjCIGR1* expression in transgenic zoysiagrass and wild-type plants under stress conditions (Fig. 12A and 12B). Three hours after the salt stress treatment, the expression level in the transformants was about 30-fold higher than that of the wild-type (Fig. 12A). The expression in the transformants decreased by 4th day, but still increased by more than 2- fold over the wild-type (Fig. 12A). In particular, the expression markedly leveled up at 5th day by about 24-fold relative to that of the wild-type (Fig. 12A). For dark stress, after 3 hours of stress treatment, the expression level was about 80-fold higher than that of the wild-type (Fig. 12B). By 6th day, the gene expression leveled off, but still at least 3-fold higher than that in the wild-type plant (Fig. 12B). Strikingly, the expression level of transformants increased sharply by more than 79-fold by 11th day (Fig. 12B). Expression of *Oryza sativa GRAS23* and tomato (*Solanum lycopersicum) GRAS40* went up in the overexpressing lines under stress conditions, as the genes were reported to be involved in stress-related processes (Xu *et al*. 2015; Liu *et al*. 2017). We suggest that the *ZjCIGR1* gene also plays a regulatory role in responding to the abiotic stress.

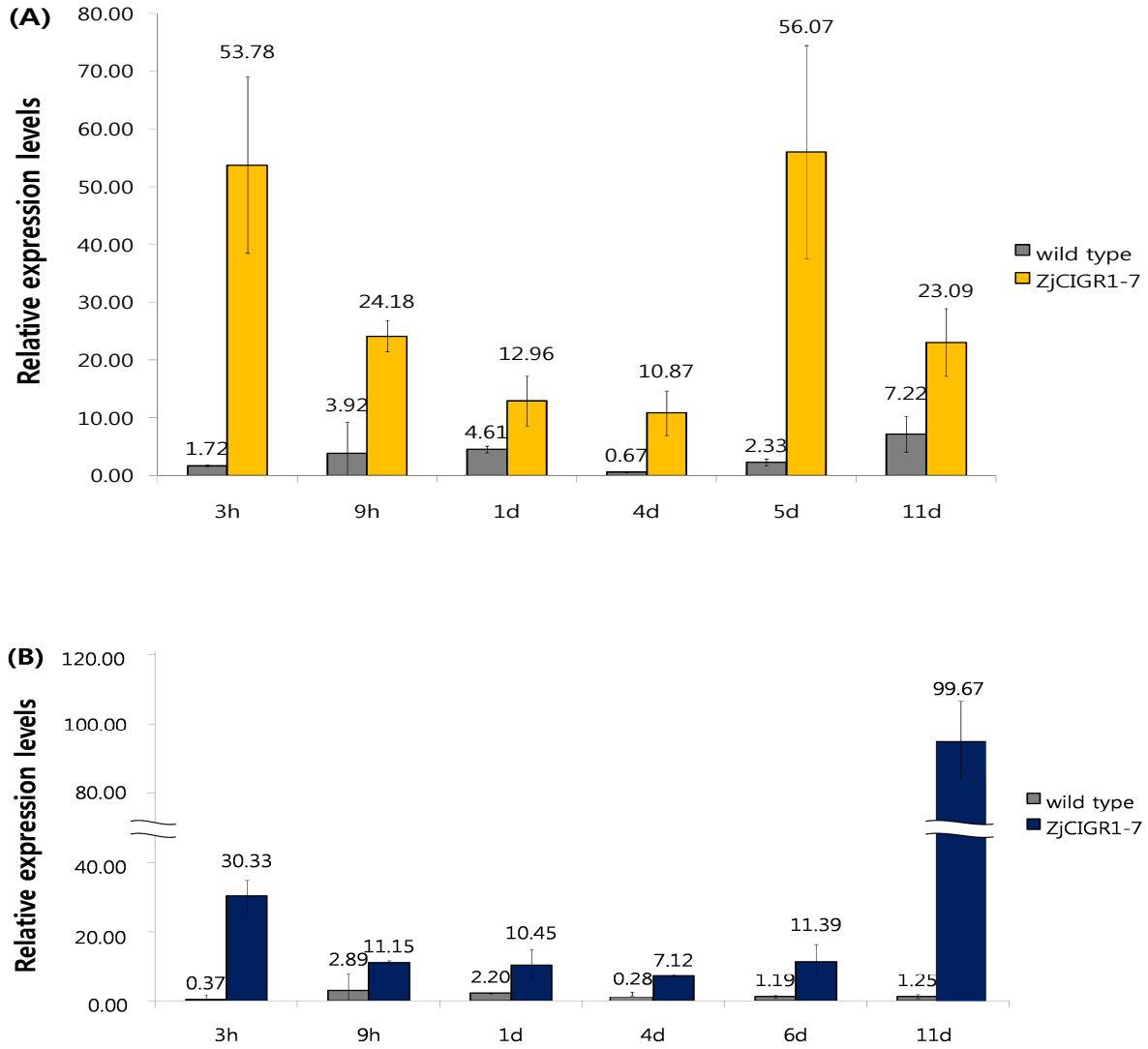
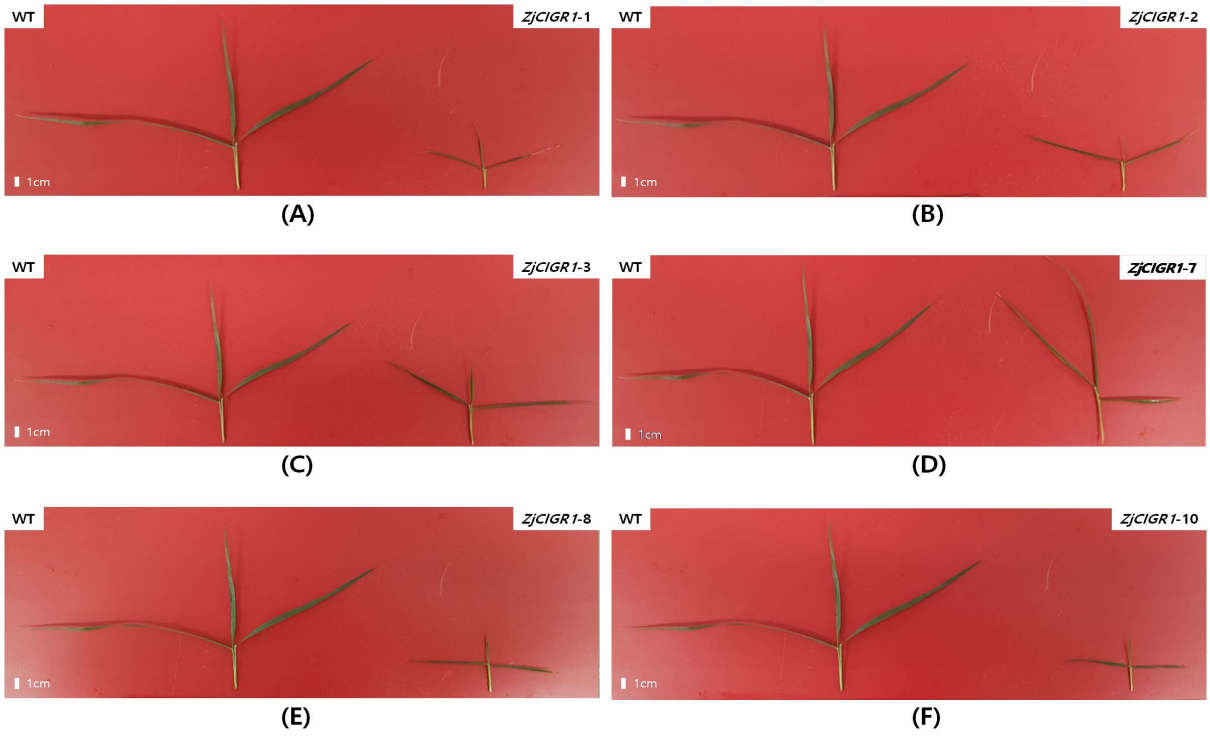


Fig. 12. Expression patterns of *ZjCIGR1* in response to salt (200mM NaCl; A) and dark (light deficiency; B) in wild type and *35S::ZjCIGR1* transgenic *Zoysia japonica* plants; h, hour; d, day. Relative expression of *ZjCIGR1* transcripts was quantified with real-time PCR. Vertical scales show the relative amount of *ZjCIGR1* transcripts compared to the internal standard (*18s* rRNA).

In order to follow the growth in the soil, the *ZjCIGR1*-overexpressing and wild-type *Zoysia japonica* plants were transferred to a plant cultivating pot. Their growth was observed by focusing on leaves. As shown in Fig. 13A-L, the leaves of the transgenic plants were smaller than those of the wild-type. The *ZjCIGR1*-7 plant was slightly smaller in height and leaf length (1.3-fold smaller in leaf width than the wild-type). Other transformants displayed a 1.4-fold reduction in plant height, 1.3-fold in leaf size relative to the wild type (Fig. 14A-C).

Gibberellin is known to play a key role in plant height (Itoh *et al*. 2002; Spielmeyer *et al*. 2002). For example, gibberellins-responsive gene *CIGR1* is involved in regulating the developmental and pathogen defense signaling (Bolle 2004; Day *et al*. 2004; Itoh *et al*. 2005; Richards *et al*. 2000; Tian *et al*. 2004), and has been reported as a useful semi-dwarf gene (Kovi *et al*. 2011). Also, overexpression of *PAT1* belonging to the same group as *CIGR1* exhibited shortening of the hypocotyls and plant height (Torres-Galea *et al*. 2013). Consistent with these findings previously reported, the *ZjCIGR1*-overexpressing lines exhibit similar phenotypic traits.



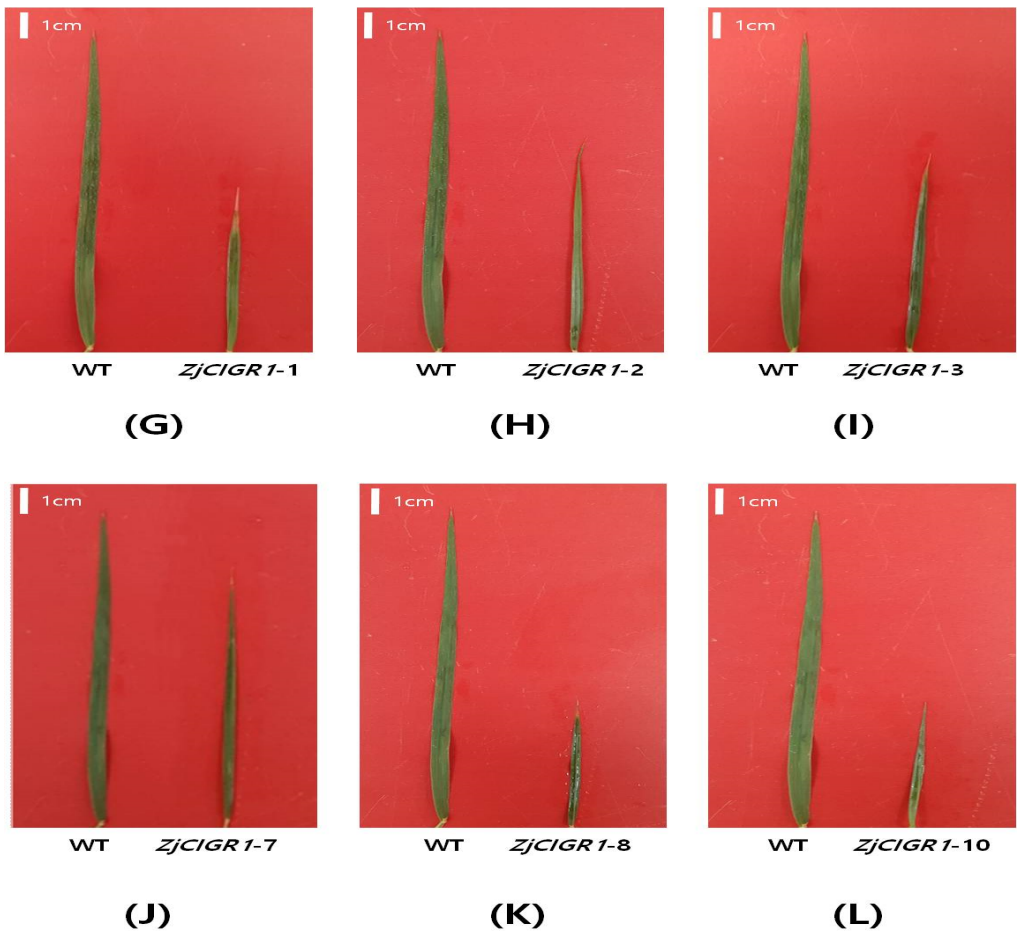


Fig. 13. Phenotype analysis of *35S::ZjCIGR1* transgenic *Zoysia japonica* plants in soil. (A)-(F) Comparison between wild-type (WT) and transgenic plants in whole leaves; (A) Comparison between WT and *ZjCIGR1*-1; (B) Comparison between WT and *ZjCIGR1*-2; (C) Comparison between WT and *ZjCIGR1*-3; (D) Comparison between WT and *ZjCIGR1*-7; (E) Comparison between WT and *ZjCIGR1*-8; (F) Comparison between WT and *ZjCIGR1*-10. (G)-(L) Comparison between wild-type (WT) and transgenic plants in third leaf; (G) Comparison between WT and *ZjCIGR1*-1; (H) Comparison between WT and *ZjCIGR1*-2; (I) Comparison between WT and *ZjCIGR1*-3; (J) Comparison between WT and *ZjCIGR1*-7; (K) Comparison between WT and *ZjCIGR1*-8; (L) Comparison between WT and *ZjCIGR1*-10.

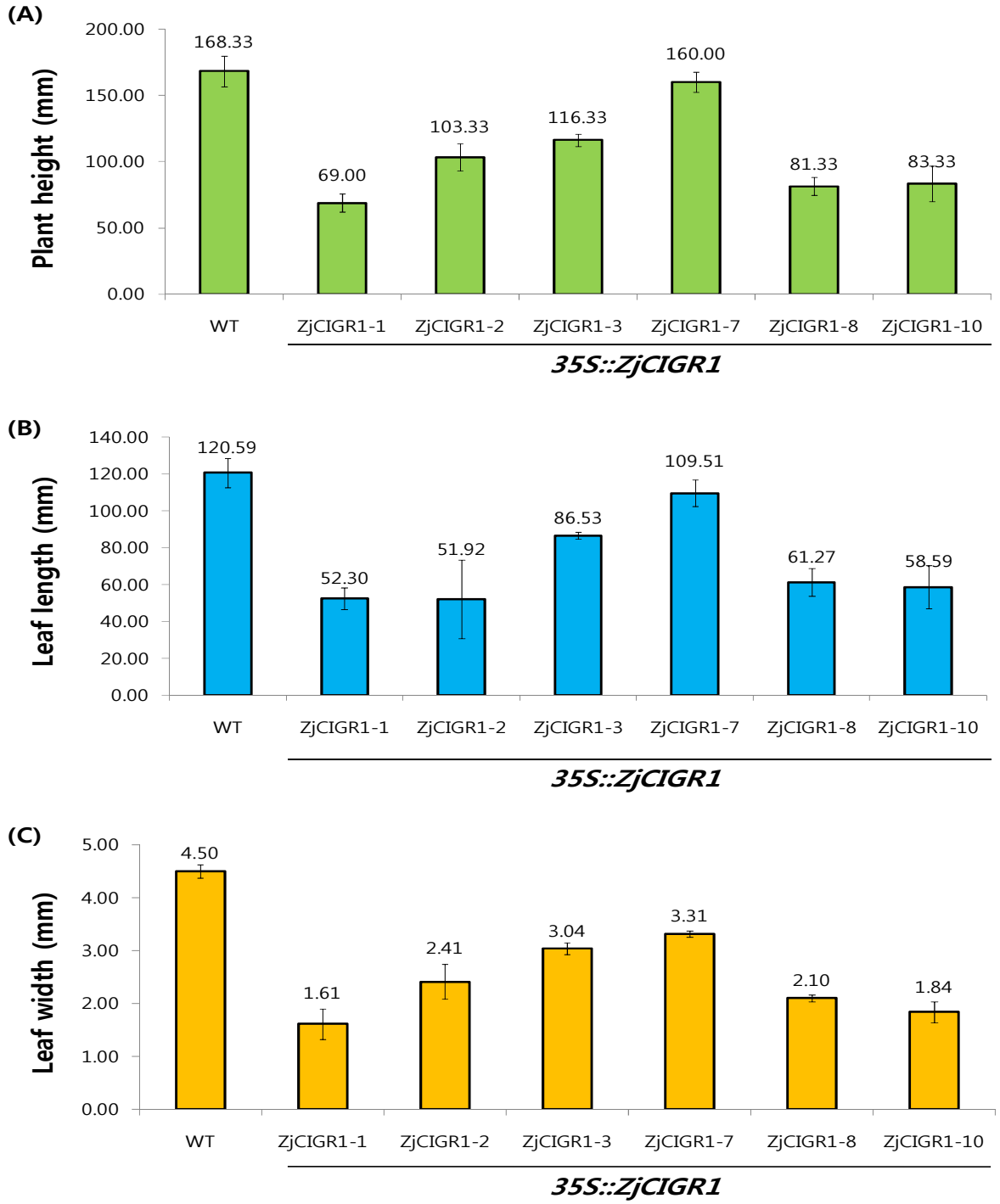


Fig. 14. Growth analysis of *35S::ZjCIGR1* transgenic zoysiagrass. (A) Plant height. (B) Leaf length. (C) Leaf width; WT, wild-type *Zoysia japonica* plants; *ZjCIGR1*-1, 2, 3, 7, 8 and 10, *35S::ZjCIGR1* transgenic plants.

4.4. Molecular and phenotypic analyses of *ZjCIGR1* transgenic *Arabidopsis* plants

To identify the *ZjCIGR1* gene in wild-type *Arabidopsis*, Southern blot analysis was conducted by using genomic DNA of wild-type *Arabidopsis* (Fig. 15A). Results showed that there was no *ZjCIGR1* gene in wild-type *Arabidopsis* (Fig. 15A). Then, in order to confirm the insertion of the *ZjCIGR1* gene into the transgenic *Arabidopsis* plants, genomic DNA PCR was carried out by using *ZjCIGR1* and *bar* primers (Fig. 15B).

To test whether the *ZjCIGR1* gene confers resistance to senescence and stress in its transgenic *Arabidopsis*, Arabidopsis T_3 plants were analyzed. 9 of the total 14 transgenic lines obtained late-flowered compared to the wild-type *Arabidopsis* (Fig. 16A). According to Lim *et al*. (2007) and Woo *et al*. (2004), flowering was delayed in senescent *Arabidopsis* transformants. Thus, it is likely that the late flowering we observed reflects a delayed aging in the transgenic *Arabidopsis* lines.

In order to check whether the transgenic *Arabidopsis* was resistant to abiotic stresses, plants were treated to with cold stress at 4℃ for 1 day, followed by further temperature decline at a rate of 2℃/hr from 0℃ to -8℃, kept for 1 hour each. 4 of the total 14 transgenic plants survived (Fig. 16B) and total RNA was extracted from the survived, and expression of *ZjCIGR1* and other stress-related genes including *AtCOR15A*, *AtCBF3*, *AtRD29A*, *AtHKT1* and *AtProDH1* were probed.

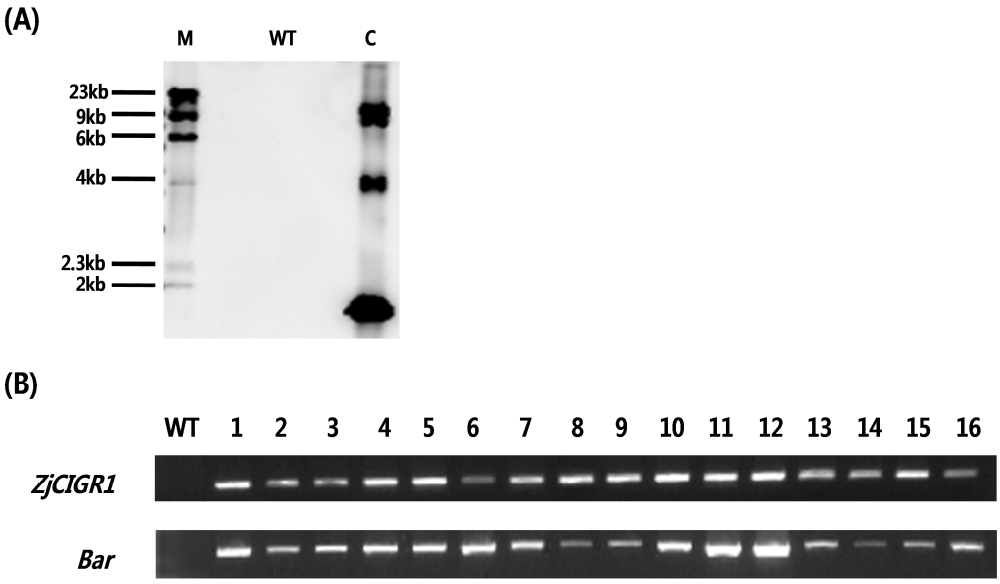


Fig. 15. Identification of *35S::ZjCIGR1* transgenic *Arabidopsis.* (A) Southern blot analysis of wild-type *Arabidopsis*; M, Dig size marker; WT, wild-type *Arabidopsis*; C, plasmid DNA of *ZjCIGR1* as positive control. Insertion of the *ZjCIGR1* gene in wild-type *Arabidopsis* was confirmed with Southern blot analysis using single digestion with *Xba* Ⅰ, followed by hybridization with the *ZjCIGR1* probe. (B) PCR of *ZjCIGR1* and *bar* genes using genomic DNA; WT, wild-type *Arabidopsis*; 1-16, *35S::ZjCIGR1* transgenic *Arabidopsis*.

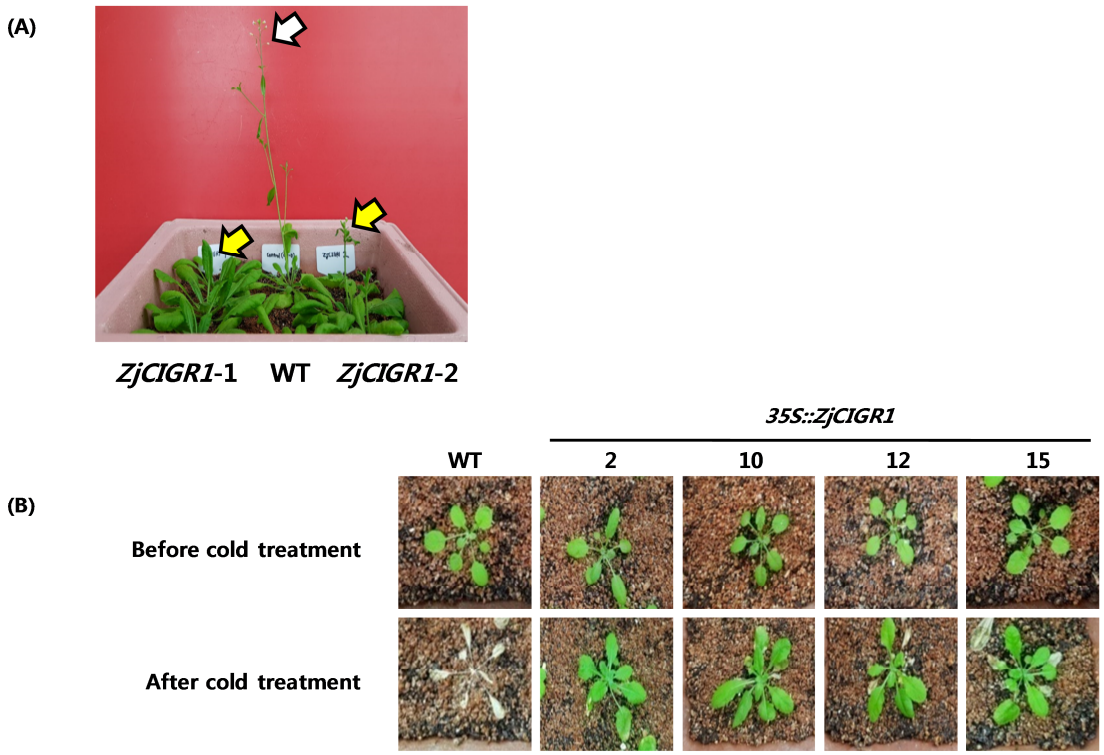


Fig. 16. Phenotype analysis of *35S::ZjCIGR1* transgenic *Arabidopsis*. (A) Under normal condition; WT, wild-type *Arabidopsis*; *ZjCIGR1*-16, *35S::ZjCIGR1* transgenic plant. (B) Under cold stress treatment; WT, wild-type *Arabidopsis*; 2, 10, 12, 15, *35S::ZjCIGR1* transgenic plant.

AtCOR15A protected the plant from cold stress with a concomitant rise in its expression, while inhibiting the condensation of stromal proteins (Nakayama *et al*. 2007; Lin *et al* 1992). In *Vitis amurensis*, overexpression of *PAT1* conferring tolerance to cold, drought and salt stresses was accompanied by an increased expression of the *AtCOR15* gene compared to the wild-type (Yuan *et al*. 2015). *AtCBF1* is an upstream transcriptional regulator in the ICE-CBF-COR pathway under cold stress conditions (Novillo *et al*. 2007) and *AtRD29A* is also low temperature-responsive gene (Msanne *et al*. 2011). Furthermore, *AtRD29A* is rapidly induced by drought and salt stress (Lee *et al*. 2016; Msanne *et al*. 2011). *AtCOR15* and *AtRD29A* are downstream genes involved in various stress responses and regulated by various transcription factors (Thalhammer *et al*. 2014). The *AtHKT1* gene is known for its regulatory function in responding to salt stress, conferring salt tolerance to the plants. Also, expression of *AtHKT1* induces cold stress-related genes including *AtCBF3* and *AtRD29A* (Wang *et al*. 2017).

The aforementioned findings prompted us to perform real-time PCR to test the expression of the same genes in the transgenic *Arabidopsis* plant (Fig. 17A-F). Expression of *ZjCIGR1* increased by about 6- to 17-fold in all the four survival transgenic plants compared to the wild-type (Fig. 17A). Except for *AtProDH1*, expression of the stress-related genes was also higher than wild-type (Fig. 17B - E). In contrast, expression of *AtProDH1* in transgenic *Arabidopsis* plants declined by about 1.2- to 3-fold compared to the wild-type (Fig. 17F). *AtProDH1* encodes for proline dehydrogenase and down-regulated in stress tolerant plants in response to abiotic stress (Cabassa-Hourton *et al*. 2016; Yuan *et al*. 2015). Proline is an osmolyte that accumulates to protect the cell membrane system from abiotic stresses and is a physiological indicator of stress tolerance and cellular damage (Qamar *et al*. 2015). A low expression of *AtProDH1* marks less cellular damage due to the stress than in the wild-type plants. Taken together, we suggest that *ZjCIGR1* induced expression of several stress-related genes and enhanced resistance to cold stress in its transgenic plants. It appears that *ZjCIGR1* is involved directly or indirectly in the stress response. However, several transgenic *Arabidopsis* plants did not show a cold-resistance phenotype. As pointed out previously (Tester *et al*. 2005), the lack of

the expected cold resistance phenotype may be attributable to the fact that the monocot gene of zoysiagrass was suppressed and silenced to make it functional in the dicot *Arabidopsis thaliana*.

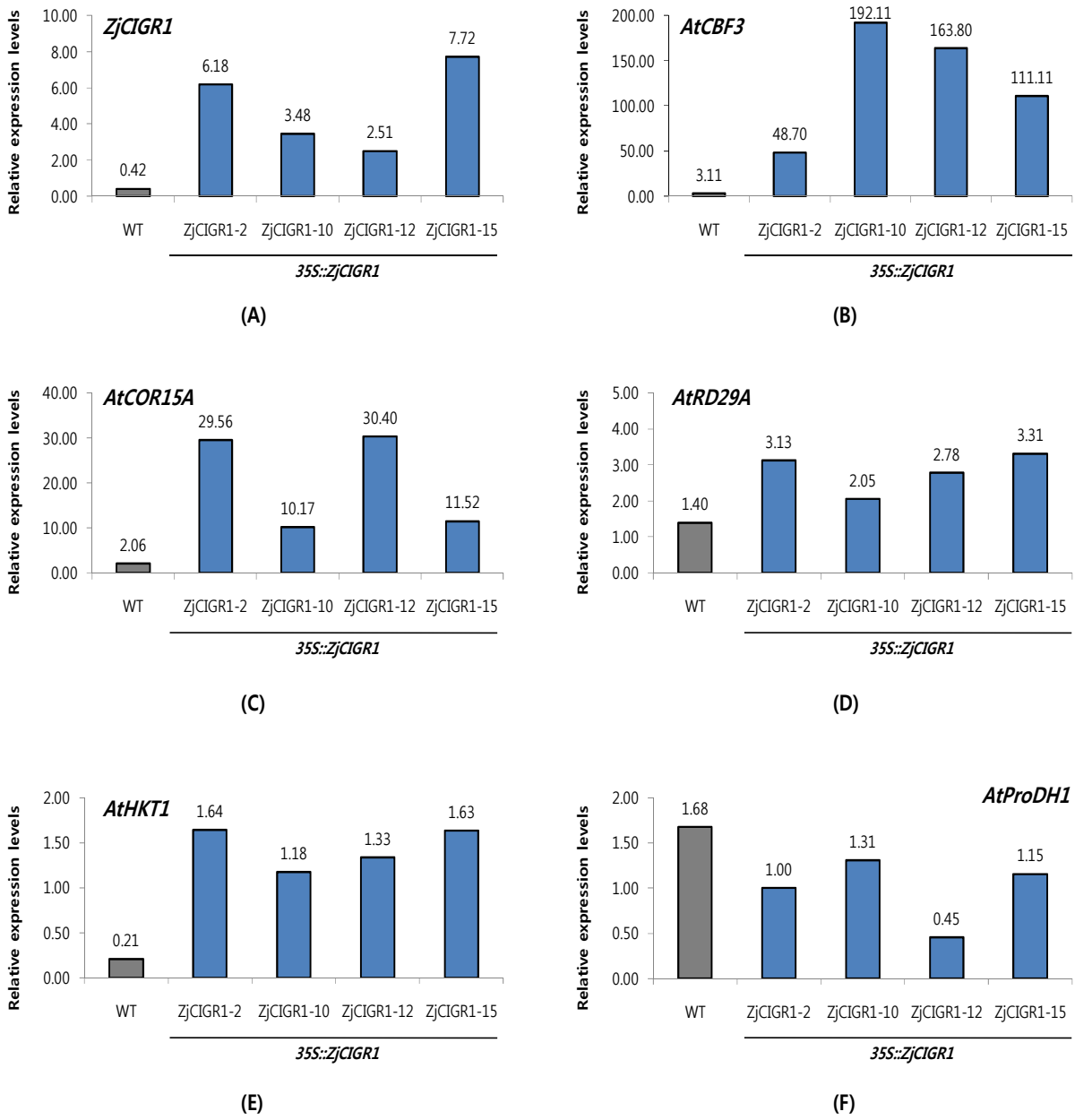


Fig. 17. Expression of *ZjCIGR1* and stress-related genes in WT and *35S::ZjCIGR1* transgenic *Arabidopsis* plants under cold treatment. (A) *ZjCIGR1*. (B) *AtCBF3*. (C) *AtCOR15A*. (D) *AtRD29A*. (E) *AtHKT1*. (F) *AtProDH1*; WT, wild-type plant; *ZjCIGR1*-2, 10, 12 and 15, 35S::*ZjCIGR1* transgenic plants.

5. CONCLUSIONS

Zoyia japonica Steud. is a typical warm-season Korean lawn grass, which is more resilient to high temperature conditions than cold-season lawn grass. Because the grass grows fast with disease resistance trait, maintenance cost of the lawn is relatively low (Song *et al*. 2006). In Korea, the zoysiagrass is cultivated in many places such as river banks, roadside and fields. Recently, it has also been used for landscape gardening in newly developed towns, home and school yards and the Saemangeum reclaimed land all of which contribute to a reduced water pollution (Bae *et al*. 2013; Bae *et al*. 2016). However, there still is a disadvantage of frequent mowing, and the grass grows poorly under shade and low temperature conditions. This study aims at securing the useful gene(s) to develop stress tolerant and dwarf plants.

The c*hitin-inducible gibberellins-responsive 1* (*CIGR1*) gene belonging to the PAT1 group of GRAS protein family (Day *et al*. 2003; Bolle *et al*. 2004; Tian *et al*. 2004). Recently, the PAT1 group have been implicated in affecting environmental stress (Yuan *et al*. 2015). In rice, the *CIGR1* gene is a candidate for a major locus affecting plant height (Kovi *et al*. 2011). Therefore, we studied the function of *ZjCIGR1* in abiotic stress tolerance and plant height using the transgenic plants.

Open reading frame (ORF) of *ZjCIGR1* was 1710bp long and the ZjCIGR1 protein is a member of the PAT1-group of GRAS protein family. The *ZjCIGR1*-overexpressing lines confered tolerance to salt and light deficiency (darkness) and displayed reduction in plant height and leaf size relative to the wild-type. The transgenic *Arabidopsis* showed a delayed senescence as well as resistance to cold stress. Whether or not the transgenic zoysiagrass is also cold-tolerant remains to be elucidated in a further study.

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