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Promoter of chrysanthemum *actin* confers high-level constitutive gene expression in *Arabidopsis* and chrysanthemum

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ABSTRACT

A promoter that confers high-level constitutive expression of transgenes in crop plants, designated the *CmActin* promoter, was isolated from chrysanthemum (*Chrysanthemum morifolium* Ramat. 'White Wing'). We characterized *Arabidopsis* and chrysanthemum transformants bearing a *CmActin* promoter-β-glucuronidase (*GUS*) reporter construct. Analysis of GUS expression demonstrated that the promoter directed reporter gene expression during all developmental stages and tissues of transgenic *Arabidopsis* except in seeds. Higher expression of GUS was observed in organs and tissues of transgenic chrysanthemum compared to those of *35S* promoter-*GUS* transgenic plants. In addition, *BrSRS* expression driven by the *CmActin* promoter induced dwarf and compact plants with narrow upwardly curled leaves and short petioles. Transgene expression driven by the *CmActin* promoter was very similar to that of the *35S* promoter in *Arabidopsis* and was significantly stronger than that of the *35S* promoter in chrysanthemum, suggesting that the *CmActin* promoter can induce constitutive gene expression in various plants and that its function is presumably conserved in different species. The *CmActin* promoter may provide a practical choice for high-level constitutive expression of target genes and could be a useful tool in floriculture plant genetic engineering.

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1. Introduction

A promoter initiates and regulates transcription, which is the most important step in gene expression (Xiao et al., 2005). A promoter that strongly and constitutively expresses a foreign gene is required to generate useful transgenic plants with desirable phenotypes to genetically engineer plants (Shirasawa-Seo et al., 2002). A number of promoters confer constitutive expression of foreign genes in transgenic plants regardless of developmental and environmental cues. Among them, the *35S* promoter from cauliflower mosaic virus has been the most widely used in various configurations as a strong and constitutive promoter to introduce foreign genes into various plant species (Benfey and Chua, 1990; Samac et al., 2004; Shirasawa-Seo et al., 2002). The foreign gene under the *35S* promoter is expressed in all tissues and organs during plant growth and development. The *35S* promoter is a useful tool that functions as a promoter element for constitutive high-level expression of foreign genes in plants.

Several studies have shown that reporter genes driven by the *35S* promoter display unequal tissue and developmental expression patterns, and that transgene expression from the *35S* promoter in other plants causes loss of the transgenic phenotypic characteristics (Chen et al., 2013; Noda et al., 2013). In addition, it is less effective for monocots, such as cereals (Morita et al., 2012). The *35S* promoter gives rise to the gene-silencing phenomenon, which inactivates genes at the post-transcriptional level (Chen et al., 2013; Dong and von Arnim, 2003). Thus, many constitutive promoters isolated from various plants have an activity similar to that of the *35S* promoter in transgenic plants, including *Gmubi* from soybean (Hernandez-Garcia et al., 2009), *tCUP* from tobacco (Foster et al., 1999), *RUBQ1* and *OsAct2* from rice (He et al., 2009; Wang and Oard, 2003), *ubi4* and *ubi9* from sugarcane (Wei et al., 2003), *ubi7* from potato (Garbarino et al., 1995), and *ZmUbi1* from maize (Cornejo et al., 1993). These promoters are commonly used to drive ectopic gene expression and generate transgenic plants (Chen et al., 2013).

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Chrysanthemum is a member of the Asteraceae family and among the most popular plants in the global floriculture industry (Noda et al., 2013). Various promoters have been used to introduce useful agronomic traits into chrysanthemums. Several efficient promoters have been developed for high-level transgene expression in chrysanthemums using the β-glucuronidase (*GUS*) reporter gene, including *Lhca3.St.1* from potato; *UEP1*, *rbcS1*, and *cab* from chrysanthemum; and elongation factor 1 (*EF1*) from tobacco (Shinoyama et al., 2012; Takatsu et al., 2000). However, introduced transgenes have occasionally been expressed at lower levels or silenced in chrysanthemum transformants (Shinoyama et al., 2012). In a previous study, induction of the heterogeneous 3′, 5′-hydroxylase gene under control of the *Nicotiana tabacumm EF-1a* promoter failed to yield delphinidin in transgenic chrysanthemum (Noda et al., 2013). Furthermore, GUS activity under control of the *35S* promoter decreases in the next generation of nearly all transgenic plants (Takatsu et al., 2000). It appears that these constitutive promoters are not suitable for expressing transgenes in chrysanthemum, and that the expression of the transgenes and the resultant phenotypes may differ depending on the target species (Togami et al., 2006). Accordingly, novel plant sequences are needed that function as promoter elements suitable for the constitutive high-level expression of target genes in chrysanthemum (Xiao et al., 2005).

Here, we cloned and analyzed an *actin* gene promoter sequence from chrysanthemum (*Chrysanthemum morifolium* Ramat. 'White Wing') that functions as a promoter element for moderate constitutive expression. The *CmActin* gene promoter led to constitutive *GUS* reporter gene expression in tissues and organs during growth and development of transgenic *Arabidopsis* and chrysanthemum. In addition, constitutive expression of *Brassica rapa SHI*-related sequence *SRS* genes by the *CmActin* promoter induced identical phenotypic characteristics in transgenic *Arabidopsis*, such as dwarfism and upwardly curled leaves. These results suggest that the *CmActin* promoter is a useful and powerful constitutive promoter for baseline and practical studies in various plants including floral crops.

2. Materials and methods

2.1. Plant materials and growth conditions

Chrysanthemum plants (*Chrysanthemum morifolium* Ramat. 'White Wing') were grown in a growth chamber under long-day conditions (16 h light/8 h dark photoperiod) at 25 °C (Suh et al., 2015). Young leaves from 4-week-old plants with identical growth status were collected and prepared for cloning the *CmActin* promoter.

Surface-sterilized *Arabidopsis thaliana* Columbia (Col-0) seeds were placed on MS (Murashige and Skoog) medium supplemented with vitamins, 1% sucrose, and 0.3% phytagel (Sigma, St. Louis, MO, USA) and stratified at 4 °C for 3 days in the dark to induce synchronous germination. The stratified seeds were germinated under long-day conditions. The plants were transferred to horticultural substrate and grown at 23 °C under long-day conditions for transformation. Samples were collected throughout the growth period.

2.2. Cloning and characterization of the CmActin promoter

The chrysanthemum *actin* promoter was isolated using the genome-walking method according to the manufacturer's instructions (Siebert et al., 1995; GenomeWalker Universal kit; Clontech, Palo Alto, CA, USA). Genomic DNA was isolated from chrysanthemum leaves using the cetyltrimethylammonium bromide (CTAB) method. Genomic DNA was digested with *Dra*I, *Eco*RV, *Pvu*II, and *Stu*I blunt-end restriction enzymes and ligated to GenomeWalker adaptors. The desired genomic region was amplified via polymerase chain reaction (PCR) using the specific AP1 primer and *actin*-specific primer1 (GSP1; Supplementary Table 1). The PCR products were diluted 1:5000 and 1 μL was used as template for the second PCR amplification (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min) in a 25 μL reaction volume using nested GSP2 and AP2 primers. PCR reactions contained 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.5 μM each primer, 0.2 mM each dNTP, and 2.5 units Taq DNA polymerase (Takara, Shiga, Japan). The PCR amplicon was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced to confirm the fidelity of amplification. Exon-intron splice sites were analyzed and confirmed by comparing the *Chrysanthemum lavandulifolium actin* (*ClActin*) partial coding sequence (accession no. JN638568) and *Chrysanthemum seticuspe actin* (*CsActin*) coding sequence (accession no. AB679277) using GENSCAN software (http://genes.mit.edu/GENSCAN.html; Burge and Karlin, 1998) and GeneMark software (http://opal.biology.gatech.edu/GeneMark/; Lukashin and Borodovsky, 1998). The *cis*-acting elements of the promoter sequence were analyzed using plantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (http://www.dna.affrc.go.jp/PLACE/) databases (Higo et al., 1999; Rombauts et al., 1999). Signal peptide analysis was performed with the SignalP Version 4.1 (http://www.cbs.dtu.dk/services/SignalP/) program.

The upstream sequence containing the first translation site (*CmActin* promoter, −1372 to +60) was retrieved from the isolated 1.8 kb *CmActin* gene fragment for analysis in transgenic plants. The *CmActin* gene promoter region was amplified by PCR from the isolated clone using the specific primer set (Supplementary Table 1). The *CmActin* promoter was digested with *Bam*HI and *Spe*I and subcloned into the same restriction sites of the pCAMBIA1381 vector. The pCAMBIA2300 vector containing kanamycin-resistance gene (*nptII*) as a selectable marker was digested with *Xho*I to select transgenic chrysanthemum plants. Subsequently, the digested *nptII* fragment was subcloned into the *CmActin* promoter-GUS vector, which had previously been digested with the same enzymes. We also generated *CmActin* promoter-*BrSRS* constructs. The *CmActin* promoter was digested with *Pst*I and *Bam*HI and subcloned into the same restriction sites of the pCAMBIA1390 vector. The *Spe*I and *Eco*RI fragments of three *BrSRS* cDNAs were inserted into the sense orientations between the *CmActin* promoter and the nopaline synthase terminator. Correct insertion of the promoter region was confirmed by full vector sequencing. The constructs were transformed into *Agrobacterium tumefaciens* strains GV3101 and LBA4404 using the freeze-thaw shock method (Holsters et al., 1978).

2.3. Generation of transgenic Arabidopsis and chrysanthemum plants

The binary vectors containing the *CmActin* promoter-*GUS* or *CmActin* promoter-*BrSRS* expression constructs were introduced stably into *Arabidopsis* plants using the *Agrobacterium tumefaciens*-mediated spray method (Hong et al., 2012; Kim et al., 2007). *Arabidopsis* flowers were sprayed with *A. tumefaciens* GV3101 containing the binary vectors suspended in 5% sucrose and 0.05% Silwet-L77, the plants were incubated in a growth chamber at 23 °C and 100% humidity for 1 day, and then they were grown in a growth chamber under a 16 h light/8 h dark photoperiod at 23 °C. T_1 seeds and progeny were germinated on MS medium containing 30 mg L^{-1} hygromycin or 50 mg L^{-1} kanamycin to select the transformants. T_3 generation transgenic plants were selected for analysis.

The chrysanthemum transformation method was similar to that of Aida et al. (2004) and Suh et al. (2015). Leaf explants (5 mm) for *Agrobacterium* inoculation were pre-cultured on MSIN pre-

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