TRANSFORMATION OF CHRYSANTHEMUM (DENDRANTHEMA GRANDIFLORUM (RAMAT.) KITAMURA) VIA AGROBACTERIUM TUMEFACIENS

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Abstract

This study attempted transformation of chrysanthemum (Dendranthema grandiflorum (Ramat.) Kitamura) and studied several factors affecting stable transformation of chrysanthemum, including cultivars of chrysanthemum, strains of Agrobacterium tumefaciens, promoters, and conditions of the co-cultivation period. Four days of co-cultivation at 24°C were optimal using A. tumefaciens strain EHA101 and plasmid pIG121Hm. Integration of the transgenes was confirmed by Southern blot analyses about four transformation. The neomycin phosphotransferase gene (nptII) was integrated in all transformants which were selected by kanamycin resistance. One transformant lacked both the β-glucuronidase gene (uidA) and hygromycin B phosphotransferase gene (hpt), while the other transformants contained all three transgenes. RNA gel-blot analyses revealed the presence of nptII transcripts in two transformants that were resistant to kanamycin in the rooting test. Transcripts of the uidA and hpt genes were not detected in any of the transformants, suggesting that differences in promoters or introduced genes might significantly affect expression.

1. Introduction

Chrysanthemum is one of the most important flowers, economically and traditionally, and has been improved by conventional breeding methods. Recently, genetic engineering techniques have been used to improve traits such as flower color (Courtney-Gutterson et al., 1994), dwarf (Petty et al., 2000), resistance to viruses (Urban et al., 1994; Yepes et al., 1995; Sherman et al., 1996), mold (Takatsu et al., 1999) and insects (Mitouchkina et al., 1998). Although the susceptibility of chrysanthemum to Agrobacterium tumefaciens has been demonstrated, the stable production of transgenic chrysanthemum has not been definitively established. Transient expression of β-glucuronidase (GUS) in limited areas of leaves was reported (Ledger et al., 1991; De Jong et al., 1993); however, there are few examples of stable GUS expression after long-term culturing. It is important to distinguish between transient expression and stable integration, with expression of both β-glucuronidase gene (uidA) and neomycin phosphotransferase gene (nptII) genes, because accumulation of the transgene products occurs gradually and may affect phenotypic expression. Fukai et al. (1995) observed a rapid decrease in GUS activity after inoculation of chrysanthemum with A. tumefaciens. Recently, gene silencing was shown to occur in transgenic plants and to affect genes controlling virus resistance or flower color manipulation, for example (Johansen and Carrington, 2001). To eliminate the influence of accumulated products derived from non-integrated genes, we studied the transcriptional activity of the transgenes and observed whether gene silencing occurred during growth and development of transformed cells. In this study, we investigated factors affecting the efficiency of transient expression using the uidA gene and subsequently determined whether gene silencing occurs using several vectors, including various genes and promoters.
2. Materials and methods

2.1. Plant material

Chrysanthemum (Dendranthema grandiflorum (Ramat.) Kitamura) in vitro shoot cultures from four cultivars ‘Seiun’, ‘Shuhounotikara’, ‘Tenju’, and ‘Houkou’ were used as transformation material. Aseptically grown shoots were subcultured every three months and grown under continuous light. Young, mature leaves were used for transformation.

2.2. Bacterial strains and vectors

Four strains of A. tumefaciens were used: EHA101, LBA4404, AGL0, and C58C1. Plasmid vectors pIG121Hm, pMUBI, pNOS, pTOK233, and pKUBI were used (Fig. 1) in various combinations with the A. tumefaciens strains. The 35S promoter was used to control uidA gene expression in pIG121Hm and pTOK233. The vectors pMUBI and pKUBI contained the ubiquitin promoter from maize and chrysanthemum, respectively. The pNOS vector contained the nos promoter. The selectable marker in all the vectors was nos promoter-nptII.

2.3. Leaf explant transformation and regeneration

Leaf explants (ca. 0.5 cm$^2$) were cut from sterile cultured shoots and inoculated for 30 min with A. tumefaciens strains harboring the appropriate Ti plasmid. Co-cultivation with A. tumefaciens was carried out on basal MS medium (1× MS, 1 mg/l BAP, 2 mg/l NAA, 1 g/l casamino acid, 0.3% Gelrite, pH 5.8) for four days at 24°C in darkness as standard conditions. After three washes with sterile water containing 200 mg/l Timentin, leaf discs were cultured on solid MS plus Timentin for seven days to remove A. tumefaciens and were transferred to selection medium (1× MS, 50 mg/l Km, 200 mg/l Timentin) for three months. Timentin was used to kill A. tumefaciens growing on the surface of the plant (Nauerby et al., 1997; Ling et al., 1998). Small green calluses appeared one month after inoculation. Thereafter, leaf discs were transferred to shoot-inducing medium (1× MS, 1 mg/l BAP, 2 mg/l NAA, 200 mg/l Timentin) for two months and transferred to rooting medium (1× MS). Regenerated plantlets were acclimatized, transferred to pots containing soil and vermiculite, and grown in a greenhouse.

2.4. GUS activity

GUS activity of transformed cells was determined by staining with X-gluc (Kosugi et al., 1990). The area of leaf stained blue was measured photometrically and calculated as a percentage of the total area.

2.5. Integration of transgenes

Genomic DNA was isolated from leaves of transgenic plants (ca. 5 cm in height) following a combination of the procedures of Wagner et al. (1987) and Bousquet et al. (1990). Primary screening of putative transformants was carried out by PCR analysis to confirm the presence of transgenes. Subsequently, Southern blot analysis was carried out to confirm transgene integration in positive transformants.

2.6. Transcription of transgene

Levels of transgene transcripts were measured by RNA gel blot to determine the level of transgene expression.

3. Results

3.1. Screening of A. tumefaciens strains, Ti plasmids, and chrysanthemum cultivars for
transformation efficiency

Several groups have reported the influence of host plant cultivar, *A. tumefaciens* strain, and vector used for transformation. Our first objective was to identify the best combination of *A. tumefaciens* strain and chrysanthemum cultivar for transformation. Four *A. tumefaciens* strains were used: EHA101, LBA4404, AGL0, and C58C1. Leaves were inoculated with each strain harboring the vector pIG121Hm. After co-cultivation, GUS activity was measured using a transient assay (Fig. 2a). Of the strains tested, EHA101 resulted in the highest GUS activity and, therefore, was primarily used in subsequent transformations.

Next, we tested the effectiveness of four vectors with various promoters controlling the expression of the *uidA* gene (Fig. 2b). GUS activity was highest when leaf discs were inoculated with pIG121Hm, which contained the 35S promoter; however, GUS activity rapidly decreased to 2.84% of the initial activity by day 14 in culture (Fig. 3). GUS activity also decreased to 14.5% in the same time span when the *nos* promoter (pNOS) was used. When the maize ubiquitin promoter (pMUBI) was used, GUS activity decreased to 0.37% after 7 days in culture. In contrast, the pKUBI vectors, which contained the chrysanthemum ubiquitin promoter, showed no GUS activity initially; however, after 14 days in culture, showed slight GUS activity. The combination of *A. tumefaciens* and vectors also affected transient GUS activity. When *A. tumefaciens* strain LBA4404 was used, pNOS showed the highest levels of transient GUS activity among the different vectors tested. When strain EHA101 was used, pIG121Hm showed higher GUS activity than pMUBI.

Next, four chrysanthemum cultivars were tested as transformation hosts: ‘Seiun’, ‘Shuhounotikara’, ‘Tenju’, and ‘Houkou’ (Fig. 2c). When *A. tumefaciens* strain EHA101, harboring pIG121Hm, was inoculated to the different cultivars, ‘Shuhounotikara’ showed the highest levels of GUS activity, followed by ‘Houkou’.

3.2. Effects of temperature and length of co-cultivation

Significant factors affecting transformation efficiency include the length of co-cultivation and temperature (Trick and Finer, 1998; Dillen et al., 1997). Using strain EHA101 and vector pIG121Hm, we studied the co-cultivation period at various temperatures. Dillen et al. (1997) reported that the optimum temperature is 22°C, irrespective of the type of helper plasmid. The effect of temperature differed with the period of co-cultivation. In our case, four days of co-cultivation at 24°C was optimal for transient GUS activity in chrysanthemum (data not shown). Although two days co-culture is commonly used for transformation of a number of plants (Trick and Finer, 1998), a one to two day co-culture was insufficient for chrysanthemum transformation. Periods of co-cultivation longer than four days resulted in a decrease in GUS activity, presumably due to an overgrowth of *A. tumefaciens*, which damages leaf tissues. Temperature was also examined. After four days of co-cultivation, growth at 22°C and 20°C resulted in GUS activity levels comparable to those at 24°C. However, GUS activity after 3 days of co-cultivation was significantly lower at both 20°C and 22°C compared to 24°C. Therefore, we carried out co-cultivation at 24°C as the standard condition.

3.3. Selection of putative transformed calluses and plant regeneration from kanamycin-resistant calluses

In the presence of more than 10 mg/l kanamycin, both callus formation and shoot formation from untransformed leaf sections were not induced. We used 50 mg/l kanamycin to select transformants. Kanamycin-resistant calluses were selected and were used to regenerate plantlets. To eliminate false positives, two tests based on kanamycin resistance were carried out. In the rooting test, escapes were unable to root under the stress of a high concentration of kanamycin (100 mg/l), although genuine transformants formed roots and developed further. In the second test, the leaf test, putative transformed leaves were placed on selection medium containing 50 mg/l kanamycin and cultured for several days. False positives began to lose color in the leaves and eventually died within 30 days. In contrast, the leaves of transformants
remained green and formed adventitious shoots.

3.4. Integration of transgenes

To confirm stable integration of transgenes in putative transgenic plants selected on kanamycin, PCR analysis was carried out to detect the presence of the transgene and to screen a number of samples (Fig. 4). Of four independent lines of regenerated plantlets, three lines resulted in the amplification of 412-, 657-, and 453-bp bands, which were derived from the nptII, uidA, and hpt genes, respectively. Since PCR amplified non-integrated transgenes as well as integrated genes and did not exclude the possibility of contamination by A. tumefaciens, Southern blot analyses were subsequently carried out in the PCR-positive lines. Three probes were used, consisting of the nptII, uidA, and hpt genes (Fig. 5). In three transformants, the nptII, uidA, and hpt probes all detected bands, while one line lacked the uidA and hpt bands.

One putative transgenic plant lost the uidA gene, which resulted in the loss of GUS activity. Due to its location near the right border of the T-DNA, next to the nptII gene, the uidA gene tended to be deleted during integration. In addition, other plantlets that had integrated the uidA gene lost GUS activity during prolonged culture of leaf discs inoculated with A. tumefaciens strains harboring the uidA gene. These phenomena demonstrated the suppression of uidA expression in transgenic chrysanthemum.

3.5. RNA-gel blot analyses

NA-gel blot analyses was used to confirm the transcriptional activity of transgenes in the plants (Fig. 6). A band was only detected with the nptII probe, while no bands were detected using uidA or hpt. This implies that only the nptII gene was transcribed, while uidA and hpt were not, although they had integrated. This phenomenon did not depend on the promoter used, since GUS activity disappeared early during culture after transformation regardless of the promoter used to drive the uidA gene.

4. Discussion

Most selectable markers are located closer to the left border of the T-DNA, while the desired transgene is located at the right border, since the T-DNA is thought to transfer from right to left border (Gleave, 1992). In this study, we constructed vectors containing two selectable markers: nptII and hpt. With one exception (Renou et al., 1993), kanamycin was previously used as a selectable marker for chrysanthemum transformation. We also preferred kanamycin, since it is easy to use and we used kanamycin successfully in preliminary experiments. Leaves of chrysanthemum are highly sensitive to kanamycin; 10 mg/l inhibits growth and the redifferentiation of adventitious shoots (De Jong et al., 1994). After culturing inoculated leaf discs on selection medium containing 50 mg/l kanamycin, we used two methods to exclude escapes from among the regenerants. The rooting test in the presence of 50 mg/l kanamycin was effective for selecting genuine transformants and resulted in no escapes. However, escapes occasionally survived in the leaf test.

Although the integration of the uidA gene was confirmed by Southern blot analyses, GUS transcriptional activity and expression disappeared after long periods in culture (Fig.6). Transcription of nptII was maintained long after co-cultivation, even in regenerated plants, likely due to the continuous stress of kanamycin selection. Similar phenomena have been reported in chrysanthemum and other plants (De Jong et al., 1994). Transient expression of nptII was observed in other plants and calluses resistant to kanamycin during the expression of a non-integrated nptII gene (Ledger et al., 1991). In petunia, Janssen and Gardner (1989) observed that GUS activity increased up to four days after co-cultivation and then declined; however, a second peak was seen later. The authors proposed that the first peak was due to transient expression by non-integrated transgenes and the second peak was derived from a stably integrated transgene. During A. tumefaciens-mediated transient expression in higher plants, silencing of reporter genes, such as green fluorescent protein (GFP) or GUS, is well-
documented (Johansen and Carrington, 2001). Post-transcriptional gene silencing often occurs when foreign genes are highly expressed or when aberrant transgenes are produced (Meins, 2000). One proposed trigger of gene silencing is double-stranded (ds) RNA (Bass, 2000). In our case, the rate of the decline of GUS activity differed among vectors with different promoters (Fig.3). This difference seems to support these past findings, since the strong 35S-promoter is a known inducer of gene silencing. Further study is required to examine the process of gene silencing in this case.

References

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Figure 1. Schematic representation of the T-DNA regions of vectors used in this study. RB, T-DNA right border; LB, T-DNA left border; NOS-pro, promoter of the nopaline synthase gene; 35S-pro, 35S promoter of cauliflower mosaic virus; MUbi-pro, promoter of the maize ubiquitin gene; KUbi-pro, promoter of the chrysanthemum ubiquitin gene; NOS-ter, terminator of nopaline synthase gene; 35S-ter, terminator of cauliflower mosaic virus; NPTII, neomycin phosphotransferase II structural gene; GUS, ß-glucuronidase gene (uidA); HPT, hygromycin B phosphotransferase gene.

Figure 2. GUS activity associated with A. tumefaciens strains, vectors, and cultivars of Dendranthema grandiflorum (Ramat.) Kitamura. (a) Four strains of A. tumefaciens containing pIG121Hm were used for transformation of Dendranthema grandiflorum (Ramat.) Kitamura ‘Shuhounotikara’. The frequency of GUS expression was measured after co-cultivation. (b) The frequency of GUS expression was measured using EHA101 containing pIG121Hm or pMUBI, and LBA4404 containing pIG121Hm, pNOS, or pTOK233. (c) The frequency of GUS expression in four cultivars of chrysanthemum was measured using EHA101 containing pIG121Hm.
Figure 3. Differences in GUS activity among various promoters linked to the *uidA* gene. 35S, NOS, MUBI, and KUBI represent transient assays using vector containing each promoter. GUS activity was measured at given intervals after co-cultivation.

Figure 4. Detection of transgenes by PCR. Upper panel: Sites of primers used and corresponding lengths of DNA fragments are indicated in the vector scheme. Lower panel: M, molecular marker; N, non-transgenic; UBI, putative transgenic plants inoculated with EHA101 (pMUBI); 35S, putative transgenic plants inoculated with EHA101 (35S); P, pMUBI vector.
Figure 5. Southern blot analyses of transgenes.
Upper panel: The same as in Fig. 4. Each DNA fragment, represented by facing arrows, was used as a probe. Lower panel: Southern blot analyses using the probes described below. N, non-transgenic; UBI, putative transgenic plants inoculated with EHA101 (pMUBI); 35S, putative transgenic plants inoculated with EHA101 (35S); Each is an independent transgenic line; H, genomic DNA cut with HindIII; E, genomic DNA cut with EcoRI.

Figure 6. RNA-gel blot analyses of transgene expression.
Upper panel: The same as in Fig. 4. Each underlined DNA fragment was used as a probe. Lower panel: Gel-blot analyses using the probes described below and ethidium-bromide-stained gels. N, non-transgenic plant; UBI-1, transgenic line inoculated with EHA101 (pMUBI).