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Original Paper

Suitability of small and branching sunflower varieties for molecular genetic experiments and their transformation by *Agrobacterium* infection

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Abstract We examined the applicability of small and branching varieties of sunflower (*Helianthus annuus* L. cv. Sonja, Valentine, and Pacino) for plant regeneration and gene introduction. Some small and branching sunflower varieties have advantages as transformation materials. The frequency of shoot regeneration in MS+0.1 mg l^{-1} BA medium was high in Sonja and Valentine and many shoots formed on each explant. Roots formed easily from regenerated shoots in MS+0.1 mg l^{-1} NAA medium and the regenerated plants grew normally and formed flowers and seeds in Pacino. In addition, the introduction of genes into branching sunflowers was shown to be straightforward. About 20–50% of regenerated shoots displayed GUS expression over wide areas of tissue in all three varieties. In Pacino, some uniformly transformed shoots were observed after simple infection with *Agrobacterium*. All of the steps required to obtain transformants of branching sunflowers are simple and straightforward. These small and easily transformed sunflower varieties are therefore useful subjects for molecular genetic experiments.

Key words: *Agrobacterium*, branching sunflowers, shoot regeneration.

The sunflower, *Helianthus annuus* L., is one of the world's major oilseed crops. Sunflower oil contains a higher proportion of unsaturated fatty acids than other vegetable oils and is therefore useful as a raw material in the production of biodiesel oil. The technology for processing sunflower oil into biodiesel oil has recently been developed; consequently, the importance of the sunflower is increasing.

Despite the importance of this plant, effective methods for sunflower transformation have not yet been established. Although many attempts have been made to establish plant regeneration and gene transformation systems for the sunflower, only a limited number of reports have described successful transformation in this species. The transient expression of foreign genes, such as the GUS reporter gene, has been accomplished following the direct transfer of genes into sunflower protoplasts (Kirches et al. 1991) and particle bombardment into tissues (Schenk et al. 1999, 2001). In addition, transgenic calli and tumors containing the GUS gene have been obtained by direct gene introduction into protoplasts (Moyne et al. 1989) and infection of tissues with *Agrobacterium tumefaciens* (Czarnecka et al. 1992). These methods, however, have not been used to successfully generate transgenic sunflower plants.

Shoot regeneration systems have been used to produce transgenic sunflower plants. Specifically, transgenic shoots have been regenerated from mature, immature or germinated seeds infected with *A. tumefaciens* (Schrammeijer et al. 1990; Knittel et al. 1994; Malone-Schoneberg et al. 1994; Rao and Rohini 1999; Muller et al. 2001). However, these systems are not efficient enough for the production of sunflower transformants, because the frequency of shoot regeneration and the efficiency of gene introduction into the regenerated shoots are low in order to use practically. Moreover, the introduced genes are confined to small limited areas of tissue. Several attempts have been made to overcome these problems using wounded explants derived from immature seeds (Lucas et al. 2000; Hewezi et al. 2002, 2003). Others have attempted to improve shoot regeneration systems from seedling shoot apical meristem explants by using sonication, macerating enzymes and/or wounding methods (Grayburn et al. 1995; Scott and Vick 1995; Alibert et al. 1999; Weber et al. 2003). Weber et al. (2000) reported successful shoot regeneration in some interspecific hybrid progenies. Despite these improvements, however, no highly efficient

Abbreviations: GUS, β-glucuronidase; BAP, Benzylaminopurine; NAA, Naphthalene acetic acid.

method for obtaining T1 seeds from transformed sunflowers is currently available. The sunflower transformants regenerated using the above methods have been small and inefficient at seed formation.

In recent years, new sunflower varieties with branching habits have become popular for use in gardens. Sunflowers generally exhibit strong apical dominance and form only one large flower, but the branching sunflower varieties form numerous well-developed axillary buds and many small flowers. The plant body, flowers and seeds of these varieties are smaller than those of general (non-branching) sunflowers. Branching sunflower varieties may regenerate a large number of shoots and therefore display more efficient plant regeneration and higher rates of seed formation than non-branching sunflowers varieties. In this study, we attempted to establish practical plant regeneration and gene introduction systems for small and branching sunflower varieties.

Materials and methods

Plant materials

Seeds of various sunflower (*Helianthus annuus*) varieties were surface-sterilized using one of two methods. In the first method, the seed coats of each sunflower variety were removed and the embryos were treated with 70% ethanol for 5 min and then 0.2% antiformin for 7 min, after which the embryos were washed five times in sterile water. Alternatively, the seeds were treated with 1% antiformin solution for 15 min and then washed five times in sterile water. The sterilized embryos and seeds were sown on 0.8% agar-solidified half-strength MS (1/2 MS) medium containing 1% sucrose. Seeds of the sunflower varieties Sonja and Valentine were cultured at 4°C for four days and then transferred into conditions of 25°C and a light cycle of 18 hr light (14 μM photons $m^{-2} s^{-1}$)/6 hr dark. Seeds of the varieties Pacino and Hybrid Sunflower were cultured continuously at 25°C under conditions of 25°C and a light cycle of 18 hr light (14 μM photons $m^{-2} s^{-1}$)/6 hr dark.

Induction of shoots

Shoot formation was induced from shoot apical meristem explants as described by Weber et al. (2003).

Bacterial strain and vectors

The *Agrobacterium tumefaciens* strain C58C1Rif^R, which harbors the binary plasmid pIG121-Hm, was used for transformation experiments (Ezura et al. 2000).

Sunflower transformation

Shoot apical meristem explants derived from axenic sunflower seedlings were incubated for 5 min in a culture of *Agrobacterium tumefaciens* containing the pIG121-Hm plasmid. Next, the explants were transferred onto 0.4% Gelrite-solidified MS medium containing 3% sucrose and 0.1% BA and cultured for two days at 25°C in an 18 hr light (14 μM photons $m^{-2} s^{-1}$)/6 hr dark photoperiod. The explants were then transferred to 0.4% Gelrite-solidified MS medium containing 3% sucrose, 0.1% BA, and two tablets l^{-1} augmentin (250 mg l^{-1} potassium clavulanate and 500 mg l^{-1} amoxicillini, Glaxo Smithkline Co., Tokyo, Japan), and cultured under the same conditions for four days. Finally, the explants were transferred to 0.4% Gelrite-solidified MS medium containing 3% sucrose, 0.1% BA, two tabletsl^{-1} augmentin, and 20 mg l^{-1} kanamycin and cultured under the same conditions for 18 days.

Histochemical GUS assay

Histochemical GUS assays were carried out according to Jefferson (1987). *Agrobacterium-tumefaciens*-infected sunflower explants with regenerated shoots were dipped in a staining solution containing 1 mM X-gluc (5-bromo-4-chloro-3-indolyl-β-glucuronide), 50 mM NaH_2PO_4 and 0.1% Tween 20, and incubated overnight at 37°C.

Results

Characterization of sunflower varieties

To determine the appropriate sunflower varieties for use in the experiments, several characteristics were examined in seven sunflower varieties: the non-branching-type sunflower varieties (Sunlight, Russia and Hybrid Sunflower) and the branching-type varieties (Pacino, Valentine, Prado Red and Sonja). The cultivation characteristics of each variety were examined in a culture room.

The branching-type sunflowers were shorter than the non-branching-type (Table 1). Pacino and Sonja were particularly small (Figures 1B, D). This characteristic of Pacino and Sonja is advantageous for indoor cultivation and experimentation. The seeds of all varieties except Prado Red were produced by self-fertilization and Valentine produced a particularly large number of seeds (Table 1). Outgrowth of axillary buds was observed in both Valentine and Sonja (Table 1, Figures 1B, C, F, G) before the apical flowers bloomed. In Pacino and Prado Red, growth of the axillary buds was observed after the first flower had bloomed (Table 1, Figures 1D, H).

To examine the tissue culture responses of these varieties, cotyledon and hypocotyl explants derived from sterilized seedlings were cultured on phytohormone-free solid half-strength Murashige and Skoog (1/2 MS) medium. In Russia and Hybrid Sunflower, adventitious roots formed on all of the examined tissues (Table 1). In addition, adventitious root formation from the lower region of the stem was also observed in the non-branching-type sunflowers cultivated in soil (Table 1).

Table 1. Characterization of sunflower cultivars.

Varieties	Cultivation characteristics in the culture room				Tissue culture response	
	Branching	Plant height	Seed formation	Adventive roots	Hypocotyl explants	Cotyledon explants
Hybrid Sunflower	No	100 cm	+	+++	Shoot and root	Root
Russia	No	120 cm	+	+++	Root	Root
Sunlight	No	80 cm	++	++	N.D.	N.D.
Pacino	Late	25 cm	+++	+	Shoot and root	Callus
Prado Red	Late	70 cm	−	−	Callus and root	Died
Sonja	Early	30 cm	+	+	Root	No response
Valentine	Early	80 cm	+++	++	Shoot	No response

Sunflower varieties were grown under conditions of 25°C and a photoperiod of 16 h light/8 h dark, and the characteristics of each were examined. Hypocotyls and cotyledon explants derived from 9-day-old axenic seedlings were cultured on phytohormone-free solid MS medium at 25°C in a photoperiod of 16 h light/8 h dark. "Seed formation" indicates that seeds were formed by self-pollination. N.D., not determined.

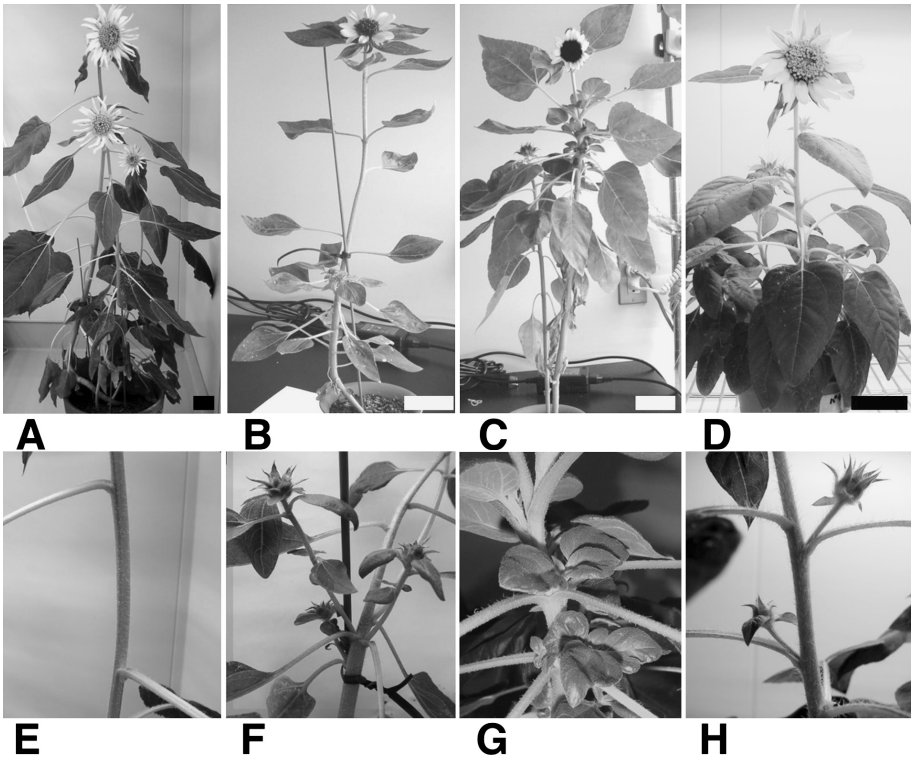

Figure 1. Sunflower varieties used. A and E, Hybrid sunflower after 3 months of growth. B and F, Sonja after 2.5 months of growth. C and G, Valentine after 2.5 months of growth. D and H, Pacino after 3 months of growth. A, B, C and D show whole sunflower plants. Bar = 10 cm. E, F, G and H show axillary buds from each variety.

These results indicate that the root-formation potential of non-branching-type sunflowers is high. In contrast, the cotyledon explants derived from the branching-type sunflower seedlings did not form roots (Table 1) and some explants derived from hypocotyls did form shoots at a very low frequency (Table 1). In addition, relatively few adventitious roots developed from the stems of soil-cultivated branching-type sunflowers (Table 1). This suggests that the root regeneration ability of these varieties may be comparatively low and that shoot regeneration may be more efficient in the branching-type sunflowers.

We decided to use three branching-type varieties (Pacino, Sonja and Valentine) and one non-branching-type variety (Hybrid Sunflower) as a material for further experiments.

Shoot regeneration ability

We examined the frequency of shoot regeneration from the shoot apical meristem explants. To determine the optimum sterilization conditions, the seeds were sterilized either with or without the seed coat (see Materials and methods). To examine the effect of seedling age on shoot regeneration, 1- to 4-day-old seedlings were used. The shoot apical meristem explants were cultured on Gelrite (0.4%)-solidified MS medium containing 0.1 mg l^{-1} BA (6-benzylaminopurine).

In all of the varieties tested, shoot regeneration was initially observed after 4 to 13 days of culture (Figure 2A–D). Subsequently, the number of regenerated shoots

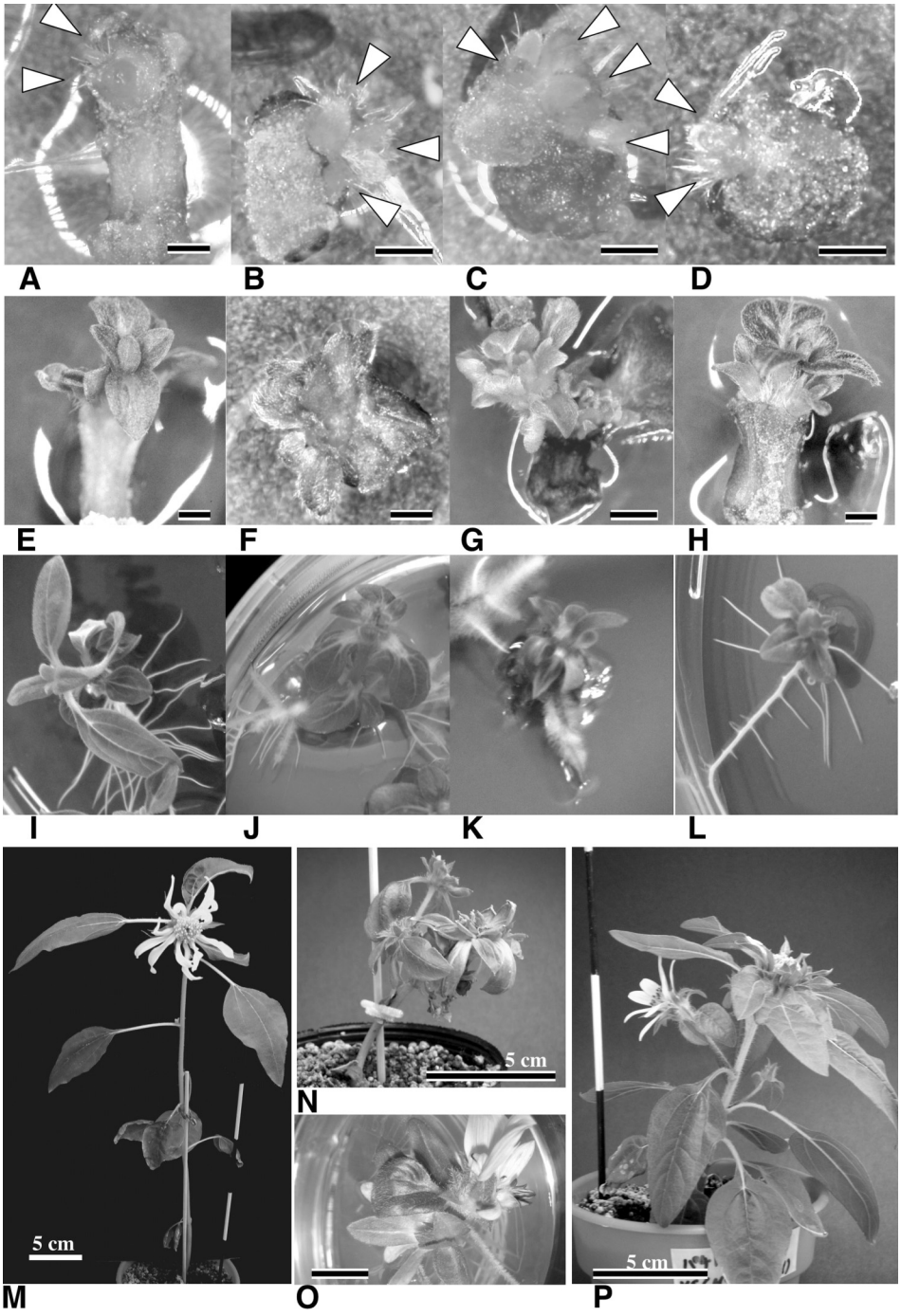

Figure 2. Plant regeneration from shoot apical meristem explants. A, B, C and D show regenerated shoots from shoot apical meristem explants in the young stage after seven days of culture (Bar=1 mm). Arrowheads indicate the formation of regenerated shoots. E, F, G and H show regenerated shoots cultured for different time periods. E and G, 21 days of culture; F, 41 days of culture; H, 19 days of culture. (Bar=1 mm). I, J, K and L show rooted plantlets cultured on MS medium with 0.1 mg l^{-1} NAA for 14 days. M, N, O and P show regenerated plants (Bar=5 cm or 1 cm). A, E, I and M, Hybrid sunflower; B, F, J and N, Sonja; C, G, K and O, Valentine; D, H, L and P, Pacino.

increased and the shoots continued to grow (Figure 2E–H). The frequency of shoot regeneration and the number of regenerated shoots were determined after 24 days of culture.

More than 20% of the Hybrid Sunflower explants derived from 1- to 2-day-old seedlings from seeds sterilized without the seed coat formed shoots (Figure 3). Most of the explants formed less than three shoots.

The frequency of shoot regeneration was relatively low in Pacino. About 20% of the explants from 4-day-old seedlings derived from seeds sterilized without the seed coat formed shoots (Figure 3). Only one or two shoots regenerated from each explant (Figure 3), but many of the regenerated shoots had a normal morphology (Figure

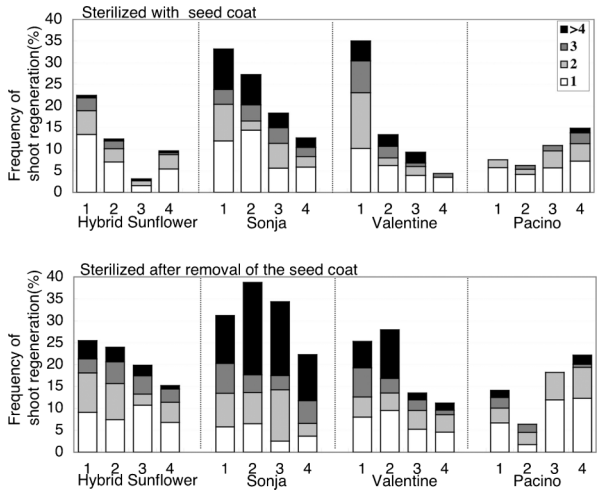

Figure 3. Frequency of shoot regeneration and the number of the shoots formed from each sunflower variety. Dry seeds of each sunflower variety were sterilized either without removing the seed coat (upper panel) or after removal of the seed coat (lower panel), after which the seeds were germinated on 1/2 MS medium with 1% sucrose. Apical meristem explants derived from axenic seedlings were cultured on MS medium with 0.1 mg l^{-1} BA, and the numbers of explants that formed shoots were counted after 24 days of culture. Data are averages of three independent experiments. The numeral under each bar shows the day after sowing of material axenic seedlings. The color of each bar reflects the number of explants that formed different numbers of shoots.

2H).

Sonja exhibited the highest shoot regeneration potential. About 30–40% of the explants derived from 1- to 3-day-old seedlings from seeds sterilized without the seed coat formed shoots (Figure 3). Moreover, about half of the explants regenerated at least four shoots.

In Valentine, about 25% of the explants formed shoots when 1- to 2-day-old seedlings from seeds sterilized without the seed coat were used as the starting material (Figure 3). The explants derived from these seedlings regenerated large numbers of shoots (Figure 3).

These results indicate that the frequency of shoot regeneration was greatest in Valentine and Sonja, and that these varieties may therefore be most suitable for the establishment of sunflower transformation systems.

Root induction and plant regeneration

To obtain whole sunflower plants, we determined the optimum culture conditions for the induction of roots from regenerated shoots. Regenerated shoots were isolated and cultured on 0.4% Gelrite-solidified 1/2 MS with 1% sucrose, MS with 3% sucrose, MS with 3% sucrose + 0.1 mg l^{-1} NAA (a-naphthylacetic acid), and MS with 3% sucrose + 1 mg l^{-1} NAA. The frequency of root formation was determined after 28 days of culture.

In all four varieties, root formation occurred at a greater frequency in the presence of NAA than under phytohormone-free conditions (Table 2). The root formation rates were 40–50% in Pacino, about 65% in Valentine, 74% in Hybrid Sunflower, and about 80% in Sonja. In the presence of NAA, root formation was observed early in the culture period, from as early as the sixth day (data not shown, Figure 2I–L). However, when shoots were cultured on solid MS + 1 mg l^{-1} NAA medium, the stems of the shoots formed calli (data not shown). Therefore, the MS + 0.1 mg l^{-1} NAA medium was the best medium to use for root induction.

Table 2. Frequencies of root formation.

	1/2 MS +1% suc	MS +3% suc	MS +3% suc +NAA (0.1)	MS +3% suc +NAA (1.0)
Hybrid Sunflower	18%	27%	74%	73%
Pacino	13%	4%	43%	50%
Sonja	61%	38%	81%	76%
Valentine	0%	0%	65%	63%

Regenerated shoots were isolated and cultured on 1/2 MS + 1% sucrose, MS + 3% sucrose, MS + 3% sucrose + 0.1 mg l^{-1} NAA, or MS + 3% sucrose + 1 mg l^{-1} NAA media at 25°C in a photoperiod of 16 h light/8 h dark. The shoots were examined for root formation after 28 days of culture. The data are combined results from two independent experiments.

Following root regeneration, the sunflower plants were transferred onto solidified phytohormone-free MS medium and cultured for 1 or 2 months, after which the plants that had grown sufficiently were planted in pots containing soil. Plant regeneration, flowering and seed formation proceeded successfully in Hybrid Sunflower and Pacino (Figure 2M, P). Moreover, Pacino formed comparably large numbers of mature seeds. In Sonja, plant regeneration and flowering were achieved (Figure 2N). In Valentine, although small regenerated plants were obtained, the plants did not grow sufficiently on phytohormone-free MS medium. The regenerated Valentine plants developed abnormal flowers with only two or three petals (Figure 2O) and did not form seeds. In summary, plant regeneration was most efficient in the Hybrid Sunflower and Pacino varieties.

Introduction of genes

The introduction of genes into the branching sunflower varieties was examined using our previously established culture method. The shoot apical meristem explants derived from 2-day-old seedlings of Sonja and Valentine, 3-day-old seedlings of Hybrid Sunflower, and 4-day-old seedlings of Pacino were infected with *A. tumefaciens* strain C58C1Rif^R harboring the binary plasmid pIG121-Hm (see Materials and methods). The frequency of shoot regeneration was slightly decreased by infection with *Agrobacterium* (Figure 4). The explants that formed shoots were used in histochemical GUS assays.

The frequency of gene introduction was high in the branching varieties. In Hybrid Sunflower, GUS spots were observed in only 10% of the regenerated shoots and 50% of the original explants; only small areas displayed

blue staining (Figure 5A, E). In contrast, over 80% of the original explants of the branching varieties exhibited GUS staining, and 20–50% of the regenerated shoots contained transformed cells (Figure 4). The blue-stained area was larger in the branching varieties (Figure 5), in both the regenerated shoots (Figure 5F–H) and the original explant regions (Figure 5B–D). These results indicate that transformed cells were present in wide areas of tissue in the branching sunflowers. The region in which the foreign gene had been introduced in each shoot was particularly large in Pacino and Sonja (Figure 5F, D, H). Uniformly transformed shoots were obtained with Pacino (Figure 5H). The number of GUS spots per explant did not vary widely among the different varieties (Table 3).

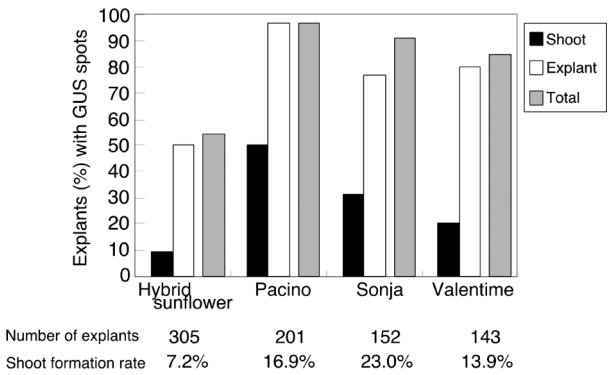

Figure 4. Frequency of GUS-positive explants and shoots. Apical meristem explants were infected with *Agrobacterium* (see Materials and methods), cultured for 24 days, and then subjected to a histochemical GUS assay. The number of GUS-positive samples was counted whether the GUS-positive spot was present at the original explant region, in the regenerated shoot, or both. Black bars indicate samples, with blue staining indicating GUS activity in the regenerated shoots, white bars indicating samples with blue staining in the original explant and shaded bars indicating the total. The values represent the percent of responding explants. The data are the sum of results of two independent experiments.

Discussion

In this study, we attempted to establish a plant regeneration and gene introduction system in branching varieties of the sunflower and showed that some of these varieties could provide practical transformation materials.

The branching varieties tested were smaller than the non-branching sunflower varieties (Table 1, Figure 1), making it possible to easily handle large numbers of plants for tissue culture, transformation and molecular genetic experiments. For example, the number of Sonja

Table. 3. Numbers of GUS spots per explant.

	Explant region	Shoot region
Hybrid Sunflower	5.8	1.5
Pacino	6.8	2.1
Sonja	3.4	1.5
Valentine	4.5	1.25

The numbers of GUS spots par explant were counted after 24 days of culture. The data are combined results from two independent experiments.

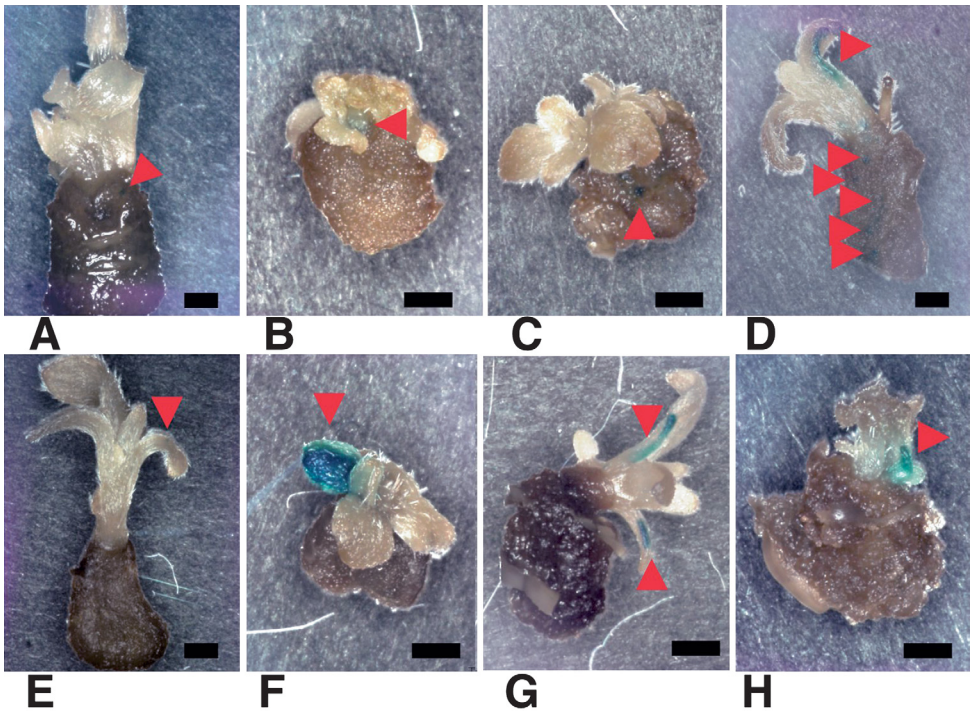

Figure 5. Histochemical GUS assay of CaMV 35S-promoter-derived intron-GUS transgenic sunflowers. A and E, Hybrid sunflower; B and F, Sonja; C and G, Valentine; D and H, Pacino. Red arrowheads indicate GUS staining. Bar=1 mm.

seeds that can be cultivated in one plate is 1.5–2 times greater than the number of Hybrid Sunflower seeds (data not shown) and more branching sunflowers can be handled in cultivation rooms and growth chambers. Although the branching-type sunflowers are smaller than non-brancing sunflower varieties, they produce more or equal quantities of seeds under culture room conditions (Table 1). These characteristics suggest that branching sunflower varieties may be more suitable than general (non-branching) varieties for some molecular genetic experiments.

To be considered practical material for molecular genetic experiments, a plant must also have a simple transformation system. As there were no published data about the tissue culture systems of small and branching-type sunflowers, the present research began with investigations of their regeneration abilities. In branching sunflowers, root formation ability is less and shoot regeneration ability is slightly greater than in non-branching sunflowers (Table 1).

The frequency of shoot regeneration was high in Sonja and Valentine (Figures 2, 3), and each explant of these varieties formed many regenerated shoots (Figures 2, 3). Weber et al. (2000) reported that many shoots regenerate from explants of certain interspecific hybrid lines. Equal or greater numbers of shoots formed from the Sonja and Valentine explants. During the initiation of adventitious shoots in some plant species, the initiation of the second and third shoots is inhibited by the apical dominance of the first shoot. On the other hand, the branching morphology of Sonja and Valentine indicates that these varieties may have weak apical dominance compared to non-branching-type sunflowers. Therefore, the formation of the second and third shoots may not be inhibited in Sonja and Valentine. However, in Pacino, which also has a branching morphology, only one or two shoots formed per explant (Figure 3). These results indicate that the number of regenerated shoots differs among the different varieties of branching sunflowers.

Although the root-formation ability of the branching sunflowers was less than that of non-branching sunflowers (Table 1), the isolated shoots regenerated from shoot apical meristem explants of branching-type sunflower varieties could be induced to form roots in medium containing NAA (Table 2). With all of the tested varieties, we were able to obtain small plantlets of normal morphology, including shoots and roots (Figure 2I–L). While Weber et al. (2003) used grafting to obtain seeds from regenerated sunflower plants, no grafting was necessary for the sunflower varieties tested in our study. The regenerated plantlets of Hybrid Sunflower (Figure 2M), Sonja (Figure 2N), and Pacino (Figure 2P) formed normal plants and developed flowers. The most important factor in the healthy growth of the regenerated sunflower plantlets was cultivation in a pot of sufficient size. The plantlets cultured in small pots eventually ceased to grow and formed incomplete flowers. Unexpectedly, further growth of the regenerated Valentine plants was difficult to achieve (Figure 2O). The Valentine stems were often hypertrophied when grown under phytohormone-free conditions. It is possible that another medium is required for optimal growth of Valentine plants.

The possibility of gene introduction in the small and branching sunflower varieties was also investigated. The introduction of genes was more efficient in the branching varieties than in the non-branching varieties. In the branching varieties, the frequency of transformed shoot regeneration was higher than in Hybrid Sunflower (Figure 4). In addition, the proportion of transformed cells was greater in the branching varieties (Figure 5). In particular, uniformly transformed shoots were obtained for Pacino. These results indicate that certain branching sunflower varieties are suitable as transformation materials.

Sunflowers are one of the major oilseed crops in the world, and more molecular genetic analyses will be required in the future. In this study, we showed that some small and branching sunflower varieties have advantages as genetic experimental material. These varieties may be useful as model sunflowers for molecular genetic experiments.

Acknowledgments

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