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A facile procedure for efficient plantlet regeneration from self-incompatible hybrids in *Antirrhinum*

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Abstract

A rapid and efficient regeneration system has been developed for interspecific self-incompatible hybrids between *Antirrhinum majus*×*A. hispanicum*. Multiple shoots were induced from hypocotyls explants on Murashige and Skoog medium or Loblolly pine medium supplemented with a low concentration of 3-indoleacetic acid (0.05 mg l^{-1}) and a high concentration of 6-benzyladenine (1 mg l^{-1}). Rooting took place in 1/2 Murashige and Skoog medium without growth regulators. By using this procedure, it takes about 5–6 weeks from seed to plantlet. The hypocotyls also had a potential to expand and form callus, providing a source for a continuous supply of multiple shoots from the calli at periodic intervals.

Abbreviations: 6-BA – 6-benzyladenine; 2,4-D – 2,4-dichlorophenoxyacetic acid; IAA – 3-indoleacetic acid; KT – kinetin; LM medium – loblolly pine medium; MS medium – Murashige and Skoog medium; NAA – 1-naphthaleneacetic acid; TDZ – thidiazuron

Antirrhinum, a member of the Scrophulariaceae, popularly known as snapdragon, is a commercially important ornamental plant. In recent years, *A. majus* has been used as a model plant because of the availability of molecularly characterized transposons, and several key genes controlling flower and leaf development have been isolated by transposon tagging (Coen, 1992; Bradley et al., 1993; Luo et al., 1999; Nath et al., 2003).

Nevertheless, gene function studies through transformation have been restricted because of the lack of a reproducible regeneration and transformation system in *Antirrhinum* (Heidmann et al., 1998; Cui et al., 2001). Previous studies on tissue culture of *Antirrhinum* focused on several lines of *A. majus*, and a few different tissue culture systems have been developed (Poirier-Hammon et al., 1974; Sangwan et al., 1975; Pfister et al., 1984; Atkinson et al., 1989), but with limited success. So far, plant regeneration by tissue culture in *A. hispanicum* has not been reported. *A. hispanicum* originated from Spain and is self-incompatible (Xue et al., 1996). In order to study genes encoded by the self-incompatibility (S) locus, as well as other genes, a transformation system is required. As a first step towards this goal, we report here a fast, simple and efficient plant regeneration system using self-incompatible interspecific hybrids. This system can directly induce multiple shoots from hypocotyls, and has a potential for repeated harvesting of more shoots from the calli originated from the hypocotyls.

Self-incompatible *Antirrhinum* lines were described previously (Xue et al., 1996). Seeds were collected from intercrossing SI lines. Seeds were surface-sterilized by a 10–12 min soaking in 5% (v/v) NaOCl (10–13% available chloride), then rinsed several times in sterile distilled water and sown on half strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose, and gelled with 0.7% (w/v) agar (BBI) or 0.32% (w/v) Gelrite (Sigma). The pH of the medium was 5.8–6.0 before autoclaving (120 °C, 15 min). They were cul-

tured in a growth chamber at 25 °C under a 16 h light/8 h dark period. These conditions were used throughout the experiment.

Ten to fourteen days after sowing, hypocotyls ca. 20 mm in length were cut and transplanted on a full-strength MS medium supplemented with different concentrations of 6-BA and IAA (Table 1). After 4–5 days, regenerated shoots were visible on the basal cut surface (Figure 1a). After 2 weeks, the numbers of shoots produced per explant were recorded. At least 30 explants were used per treatment, and the experiment was repeated three times. The effect of different treatments on multiple shoots regeneration was compared in a one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (α=0.05). When no hormone was used, there were also some regenerated shoots, but no callus was formed. When the concentrations of 6-BA and IAA were not adequate (6-BA 0.5 mg l^{-1} and IAA 2.0 mg l^{-1}; 6-BA 4.0 mg l^{-1} and IAA 0.0 mg l^{-1}; 6-BA 4.0 mg l^{-1} and IAA 0.05 mg l^{-1}), the regeneration capacity of hypocotyls was low, and the regenerated shoots appeared unhealthy. Two treatments provided the best results: 6-BA 1 mg l^{-1} with IAA 0.05 mg l^{-1}, or 6-BA 1 mg l^{-1} with IAA 0.5 mg l^{-1} (Table 1). There was no intervening callus phase in the regeneration process. Some basal calli were formed on the cut ends of hypocotyls near roots, which had the potential to expand. At the same time, multiple shoots were regenerated from the calli (Figure 1a, b). Whether or not we removed the old shoots, new shoots were continuously regenerated around the old shoots (Figure 1d). By callus selection, it has been possible to maintain the regeneration potential for at least 2 months.

Another medium, LM (Litvay et al., 1985; Heidmann et al., 1998), was also tested and modified in our work. LM medium differs from MS in having low Ca^{2+}, high Mg^{2+} and enrichment with PO_4^{3-} and minor inorganic salts (with respect to organic growth substances, LM contains the same concentration of myo-inositol, nicotinic acid with MS; LM also contains 0.1 mg l^{-1} thiamine-HCl, 0.1 mg l^{-1} pyridoxine-HCl, while MS contains 1 mg l^{-1} thiamine-HCl, 0.5 mg l^{-1} pyridoxine-HCl, and 2 mg l^{-1} glycine). We tested LM medium supplemented with 18 g l^{-1} lactose, 15 g l^{-1} sucrose, 6-BA 1 mg l^{-1} and IAA 0.05 mg l^{-1} for the capacity to regenerate multiple shoots from hypocotyls. The result showed that LM had a better capacity to induce callus formation than MS medium. During the shoot regeneration process, calli originated from the hypocotyls and spreaded as far as the regenerated shoots (Figure 1c). Then, we transferred the explants to MS medium with the same concentration of hormones; new shoots were continuously formed around the old shoots. After 2 weeks, a dense mass of profusely regenerating shoots was formed from the calli, which made it impossible to count the number of shoots per unit area (Figure 1e).

To investigate the difference between LM and MS, we compared the different contents of sucrose, lactose and other elements between these two media (Table 2). Based on MS medium, we used several treatments to test it. After 2 weeks culture, we found that when MS medium was altered to have the same concentration of sucrose and lactose as LM medium (designated as Medium A), there were more calli formed from the hypocotyls than before, and most of the regenerated shoots were normal. Carbohydrates had some effects on the regeneration process, but there were also other differences between LM and MS, which may have some effect on the process. We further analyzed the difference between major inorganic salts of these two media (see Table 2). LM medium differs from MS medium in having low Ca^{2+}, high Mg^{2+} and enrichment with PO_4^{3-}. When MS medium was altered to contain the same concentration of major inorganic salts, sucrose and lactose (designated Medium B), the growth of the explants was similar to those on LM. We also tried to change the minor inorganic salts of MS equivalent to LM, but found no obvious difference. These results suggested that the carbohydrates and major inorganic salts had a significant effect on the regeneration process of the hypocotyls.

In our experiments, we generally used young hypocotyls (from seedlings grown for 10–14 days). When the explants from seed grown for over one month were used, their regeneration capacity was reduced sharply. We also found that the original explants position on the hypocotyls influences direct regeneration as reported by Okubo et al. (1991). For the materials tested in this study, only the lower part of the hypocotyls had a strong capacity to regenerate multiple shoots around the root section end, and calli also originated from this position (see Figure 1a, b). But when we cut the young regenerated shoots (2–3 cm) and transferred their lower section to MS medium with 6-BA 1 mg l^{-1} and IAA 0.05 mg l^{-1}, they had lost the ability to regenerate multiple shoots. Usually, the lower hypocotyls section represents a 'transition region' where the vascular system of the stem gradually changes into the other tissues of the root (Okubo et al., 1991). Among other tested explants, such as stem internodes,

Table 1. Effect of growth regulator concentration on number of shoots, % of explants producing shoots after two weeks culture[1,2]

6-BA (mg l^{-1})	IAA (mg l^{-1})	% of explants producing shoots	Mean number of shoots per regenerating explant[3]	Mean number of shoots per explant
0.0	0.0	90.3^{ab}	3.00^{cd}	2.73^{efg}
0.5	0.0	84.7^{abc}	4.09^{bc}	3.49^{def}
0.5	0.05	92.8^{a}	4.79^{b}	4.44^{cd}
0.5	0.5	84.3^{abc}	4.32^{bc}	3.74^{cde}
0.5	2.0	73.0^{d}	2.66^{d}	1.95^{gh}
1.0	0.0	76.4^{cd}	4.60^{b}	3.51^{def}
1.0	0.05	90.7^{ab}	6.81^{a}	6.18^{a}
1.0	0.5	88.8^{ab}	6.43^{a}	5.69^{ab}
1.0	2.0	91.3^{ab}	5.29^{ab}	4.84^{bc}
4.0	0.0	35.8^{f}	2.83^{cd}	0.99^{h}
4.0	0.05	45.9^{e}	4.89^{b}	2.27^{fg}
4.0	0.5	82.1^{bc}	4.87^{b}	3.99^{cde}
4.0	2.0	72.3^{d}	3.75^{bcd}	2.72^{efg}

[1]Each experiment repeated three times, and at least 30 explants were used in each treatment.
[2]Numbers followed by different letters are significantly different at 5% confidence level according to Duncan's Multiple Range Test.
[3]Only the explants which can produce multiple shoots were calculated.

Table 2. Difference in carbohydrates and major inorganic salts between MS, LM and medium A and B*

Compound (g l^{-1})	Sucrose	Lactose	KNO_3	NH_4NO_3	$KH2PO_4$	$MgSO_4$ $7H_2O$	$CaCl_2$ $2H_2O$
In MS	30	0	1.9	1.65	0.17	0.37	0.44
In A	15	18	1.9	1.65	0.17	0.37	0.44
In B*	15	18	1.9	1.65	0.34	1.85	0.022
In LM	15	18	1.9	1.65	0.34	1.85	0.022

*Note: Medium B had the same carbohydrates and major inorganic salts as LM medium, but had minor inorganic salts and organic growth substances different from LM medium.

cotyledons, petioles, and leaves, only internodes were capable of regenerated shoots with a low frequency.

We also tested several other hormone combinations. 2,4-D (with KT or 6-BA) had a good capacity to induce calli, just as reported (Heidmann et al., 1998). But, when the calli were transferred to the regeneration medium with other hormone combinations (such as Zea [or TDZ] 0–0.5 mg l^{-1} with NAA 0.2 mg l^{-1}, Zea 1 mg l^{-1}, TDZ 2 mg l^{-1}), no regenerated shoots were observed. Explants on the medium with NAA and 6-BA can only produce some limited whitish calli around both cut ends of the hypocotyls, with occasionally regenerated shoots. For the materials used in this study, the auxin concentration in the medium had a marked influence on shoot regeneration. Thus, high level of auxin can promote callus formation, but had a detrimental effect on the regeneration. Direct multiple shoots regeneration was observed in the hypocotyls of *Antirrhinum* with a low concentration of auxin (0.05 mg l^{-1} was enough), and a high concentration of 6-BA.

Rooting was easily achieved when the shoots were very young (about 2–3 cm) and cut from the explants, then transferred to 1/2 MS medium, after about one week (Figure 1f), some root tips were originated from the basal end of regenerated shoots. From a total number of 241 regenerated shoots, 87% of the shoots produced roots after 2 weeks.

The multiple plantlet regeneration procedure described in this study took about 4–5 weeks from seed to plantlet. Gonzalez-Benito et al. (1996) also reported a direct multiple shoot regeneration from seeding

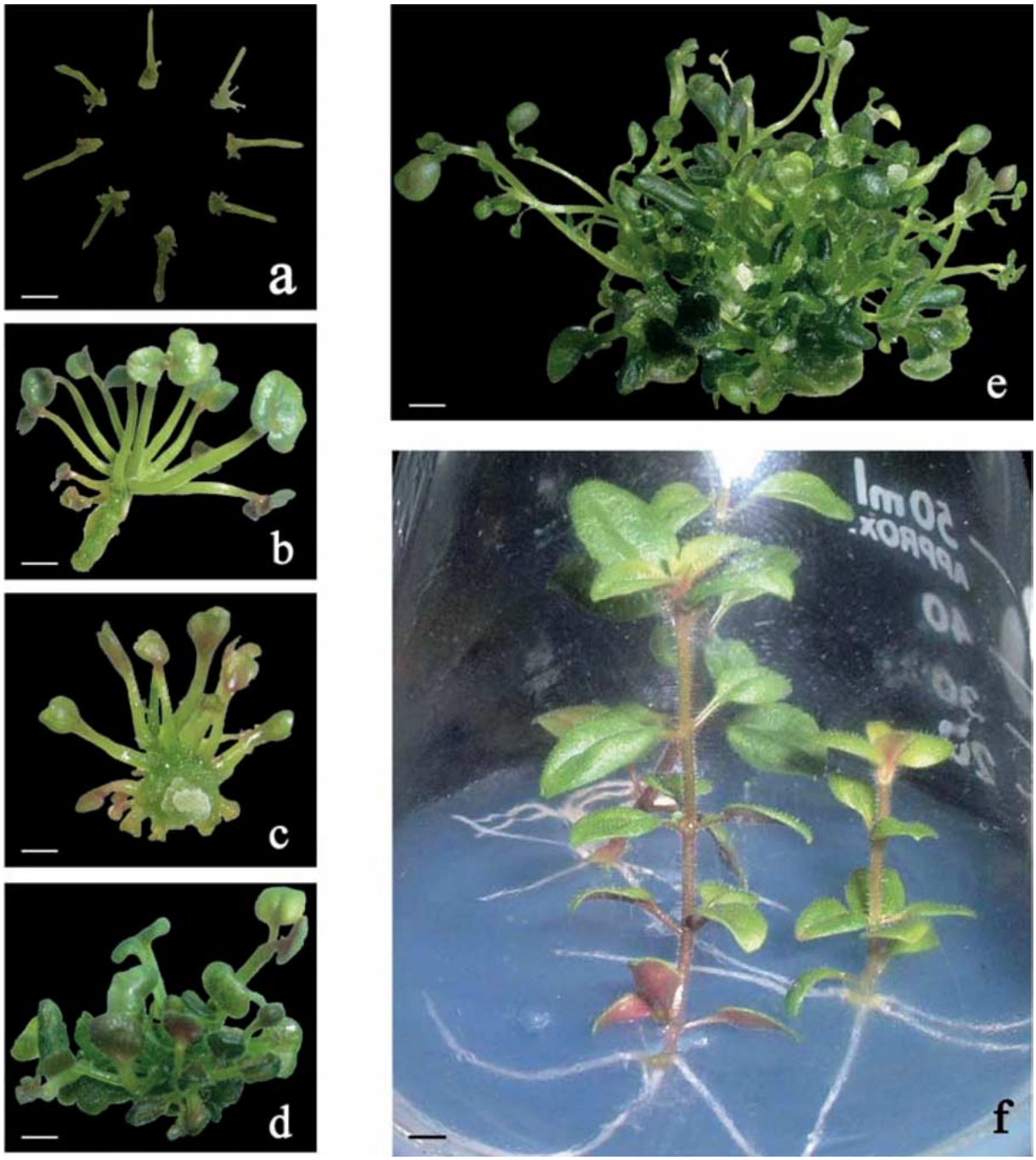

Figure 1. Plantlet regeneration from hypocotyls of *Antirrhinum*. (a) Shoot regenerated and basal calli formed from hypocotyls after 6 days culture on MS medium with 6-BA 1 mg l^{-1} and IAA 0.05 mg l^{-1} (bar 5 mm). (b) Shoot regenerated from hypocotyls after 2-week culture on MS medium with 6-BA1 mg l^{-1} and IAA 0.05 mg l^{-1} (bar 5 mm). (c) Shoot regenerated and calli formed from hypocotyls after 2-week culture on LM medium with 6-BA 1 mg l^{-1} and IAA 0.05 mg l^{-1} (bar 5 mm). (d) After 2-week culture and MS medium with 6-BA 1 mg l^{-1} and IAA 0.05 mg l^{-1}, regenerated shoots were removed and the explants subcultured to new medium for another 2 weeks (bar 5 mm). (e) After 2-week culture on LM medium with 6-BA 1 mg l^{-1} and IAA 0.05 mg l^{-1}, the explants transferred to MS medium with the same hormone combination and cultured for another two weeks (bar 3 mm). (f) Rooted shoots after 3-week culture on MS medium (bar 3 mm).

nodes of *Antirrhinum* on MS medium supplemented with 5 μM BAP, but this procedure needed at least 9–10 weeks from seed to multiple shoots without rooting. In addition, the explants used in our procedure also had a capacity for continually regenerating more shoots from calli originated from the hypocotyls through a periodical transfer onto fresh medium every 2 weeks. We are currently using this regeneration method to develop a transformation system in order to study the function of the *S* locus-encoded gene and other genes by various transgenic approaches in *Antirrhinum*.

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