Mutant acetolactate synthase gene conferring resistance to the herbicide 'imazethapyr' is an efficient *in vitro* selection marker for genetic transformation of cotton

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In this communication, we report a transformation protocol for cotton using the herbicide molecule imazethapyr as a selection agent. This protocol using a double-mutant acetolactate synthase gene is based on *Agrobacterium*-mediated transformation of cotyledonary leaf explants, followed by regeneration via somatic embryogenesis. The protocol simplifies and shortens the time taken to recover transgenic lines compared to our earlier protocol that uses kanamycin as a selection agent. Transformation efficiency based on percentage of explants giving rise to transgenic lines, confirmed by Southern hybridization, ranged from approximately 3 to 14% in different experiments. We also report a method for successful maintenance and propagation of cotton shoot cultures *in vitro*.

Keywords: *Acetolactate synthase*, genetic transformation, *Gossypium hirsutum*, imazethapyr, shoot cultures.

TRANSGENICS in cotton have been developed either by particle bombardment or via *Agrobacterium*-mediated transformation, wherein the neomycin phosphotransferase (*nptII*) gene conferring resistance to the antibiotic kanamycin is the most commonly used *in vitro* selection marker. *Agrobacterium*-mediated transformation protocols in cotton mostly involve regeneration by somatic embryogenesis. The use of kanamycin as a selection agent has been observed to have a detrimental effect on the growth and proliferation of embryogenic callus as well as on the induction and maturation of the somatic embryos. As a consequence, in all the protocols lowering the concentration of kanamycin is recommended for the embryogenic callus stage. This often leads to transgenics, which either has poor expression or no expression of the transgene[1]. We report here the use of a mutant acetolactate synthase gene (ALS^{dm}) which confers resistance to imidazolinone, as an efficient marker for *in vitro* selection of transgenics in cotton.

We have earlier reported a protocol for *Agrobacterium*-mediated genetic transformation of cotton (*Gossypium hirsutum* L. cv. Coker 310 FR)[2], wherein regeneration occurred via somatic embryogenesis[3]. In this protocol, formation of calli from cotyledonary explants was initiated in a medium containing hormones 2,4-D (0.1 mg/l) and kinetin (0.5 mg/l) in the presence of kanamycin (50 mg/l) acting as a selection agent. For the induction of somatic embryogenesis from embryogenic calli, a basal MS medium containing reduced levels of kanamycin (25 mg/l) had to be used. The germination of somatic embryos was induced by extended desiccation on a MS basal medium, supplemented with KNO_3 (1.9 g/l) in the absence of kanamycin. Shoots obtained from such embryos were then grafted on wild-type rootstocks, hardened in culture and subsequently transferred to soil. We, however, observed that the induction and maturation of somatic embryos even on a medium containing lower levels of kanamycin led to the development of a large number of abnormal embryos, thus leading to an overall decline in the frequency of the transgenics recovered.

In this communication, we report a transformation protocol for cotton using the herbicide molecule imazethapyr as a selection agent. This protocol based on *Agrobacterium*-mediated transformation of cotton using a double mutant acetolactate synthase gene as an *in vitro* selection marker, is short and simple. We also report a method for the successful maintenance and propagation of cotton shoot cultures *in vitro*, which will help in maintaining stocks of transgenics.

A double-mutant acetolactate synthase gene (ALS^{dm}) from *Arabidopsis thaliana* has been reported by Ray *et al.*[4,5] to be an efficient *in vitro* as well as field-level selection marker for the development of transgenic plants in *Brassica juncea*. The *ALS* gene with two amino acid changes (from Pro to Ser at position 197 and Ser to Asn at position 653), confers resistance to both imidazolinones and sulfonylureas. There is an earlier report on the use of a mutant version of a native acetolactate synthase gene (*A19*) from cotton for developing herbicide-resistant cotton[6]. Although the paper reports the successful use of this gene in *Agrobacterium*-mediated transformation of cotton seedling explants and biolistic transformation of embryogenic cell suspension cultures, it does not discuss the detailed protocol, including the appropriate concentration of the herbicide for the recovery of transgenics following the initial transformation events. Further, no details of the efficiencies of the regeneration protocol as well as the transformation frequencies observed are available. Recently, Aragao *et al.*[7] reported the use of a mutant *ALS* gene from *A. thaliana* for cotton transformation by particle bombardment of the apical meristem. The average frequency of cotton transformation in these experiments was 1.17%, whereas the frequency of germ line transformation was even lower at 0.55%.

In the present study, cotton, *Gossypium hirsutum* L. Coker 310 FR line[2] was used for genetic transformation. The herbicide imazethapyr, an imidazolinone (active

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component of the commercial preparation 'Pursuit', BASF India Ltd), was used as a selection agent. The optimal concentration of imazethapyr that could be used for *in vitro* selection was determined by placing untransformed cotyledonary explants from 7-day-old seedlings on MST1 medium containing different concentrations of imazethapyr (0, 1, 2, 3, 4 and 5 μM) and observing inhibition in callus initiation after 30 days of culture. Imazethapyr at the low concentration of 1 μM inhibited callus formation, although the cotyledonary explants remained green. At 2 μM there was onset of chlorosis and at 3 μM and beyond, all the explants turned brown in one month. Thus, 3 μM imazethapyr was used for *in vitro* selection in transformation experiments.

For genetic transformation, the binary vector pPZP200 : *nptII* :: ALS^{dm} :: *gus-int* (Figure 1 *a*) (developed in an earlier study by Ray *et al.*[4]) was transformed into a disarmed *Agrobacterium tumefaciens* strain GV3101 by electroporation. The genetic transformation of cotyledonary explants from 7-day-old seedlings was carried out according to the protocol described below and summarized in Figure 2. Following co-cultivation with *Agrobacterium* for 48 h, the cotyledonary explants were placed on MST 1 medium containing 3 μM imazethapyr. After 30–35 days of culture, calli were observed at the cut ends of almost all the explants. On an average, 22.2% of these calli converted into white and purplish granular embryogenic calli on MSO T2 medium containing 3 μM imazethapyr (Table 1). These embryogenic calli were transferred to MSO T3 medium with 3 μM imazethapyr and after about 30–40 days of culture, approximately 53.6% of the embryogenic calli (around 11.9% of the initial explants) gave rise to somatic embryos (Table 1). The number of embryos obtained from each embryogenic callus varied. As the binary vector also contained a *gus–intron* gene, calli at the cut ends of the explants, embryogenic calli and somatic embryos were analysed for the expression of *β*-glucuronidase (*gus*) by histochemical staining[8]. These were found to stain blue, confirming their transgenic status. The transformed embryos obtained on MSO T3 medium were subsequently placed on the same medium without imazethapyr and allowed to desiccate for 30–35 days. Following desiccation, elongated embryos with either differentiated radicle and cotyledonary ends or only with differentiated cotyledonary ends were transferred to 1/2 MSD medium. After about 15 days of culture, on average 31.8% of the embryos transferred to 1/2 MSD medium converted into plantlets with a well-developed root and shoot system (Figure 3 *a*; Table 1). On 1/2 MSD medium embryos obtained from callus of only some of the initial explants developed into plantlets. Thus, the percentage of explants giving rise to plantlets ranged from 2.8 to 13.8%. These plantlets were transferred to soil after another 10–15 days of culture.

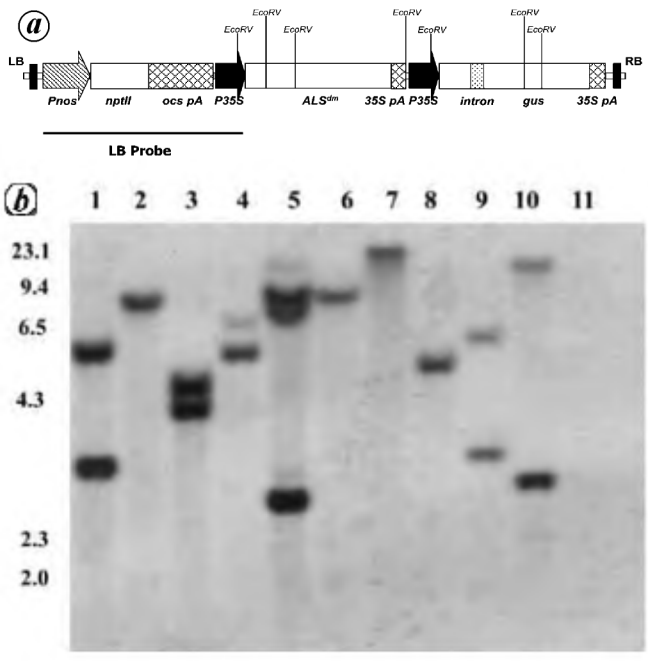

Figure 1. ***a***, T-DNA region of the binary vector pPZP200 : *nptII* :: ALS^{dm} :: *gus–int* construct. Location of *Eco*RV sites used for digestion of genomic DNA is marked. The *nos–nptII–ocspA* cassette was used as a probe in southern analysis. ***b***, Lanes 1–10, Southern hybridization of genomic DNA from leaves of putative transgenics and lane 11, Untransformed control. Genomic DNA from all transgenic and control lines were digested with *Eco*RV and probed with the *nptII* gene for analysis of junction fragment towards the left border of the T-DNA. Lambda DNA digested with *Hin*dIII was used as a size marker, the sizes (in Kb) of which are given on the left of the figure.

In cases where only shoot formation occurred and proper root formation was absent, the shoots (4–5 cm long) were excised and transferred to a glass bottle (5.5 cm diameter) containing approximately 40 g of autoclaved mixture of soil and soilrite in the ratio 1 : 4. This mixture was supplemented with 200 μl of 1 mg/ml IBA and provided enough water to keep it moist (IBA was added to the soil–soilrite after autoclaving). Cultures were maintained under controlled environmental conditions (28 ± 2°C; 750 lux light intensity; 16/8 h (light/dark) period). Efficient root differentiation was observed at the cut ends of these shoots in around 20–25 days.

Integration of the transgene was confirmed in the putative transgenics by Southern hybridization (Figure 1 *b*). All the analysed plantlets were found to carry the transgene cassettes, which showed that no escapees were obtained on imazethapyr. To analyse the resistance conferred by the T0 transgenics against the herbicide, leaf explants (1 cm^2 surface area) from 30-day-old plantlets from *in vitro* cultures were placed on MST 1 medium containing different concentrations of imazethapyr (3, 60, 120, 180, 240, 300, 600 and 900 μM). All cultures were maintained under controlled environmental conditions (28 ± 2°C; 750 lux light intensity; 16/8 h (light/dark) period). Initiation of callus formation was observed after 30 days. Most of the T0 transgenics analysed were found to be resistant to 120 μM imazethapyr based on leaf-disc assays, while

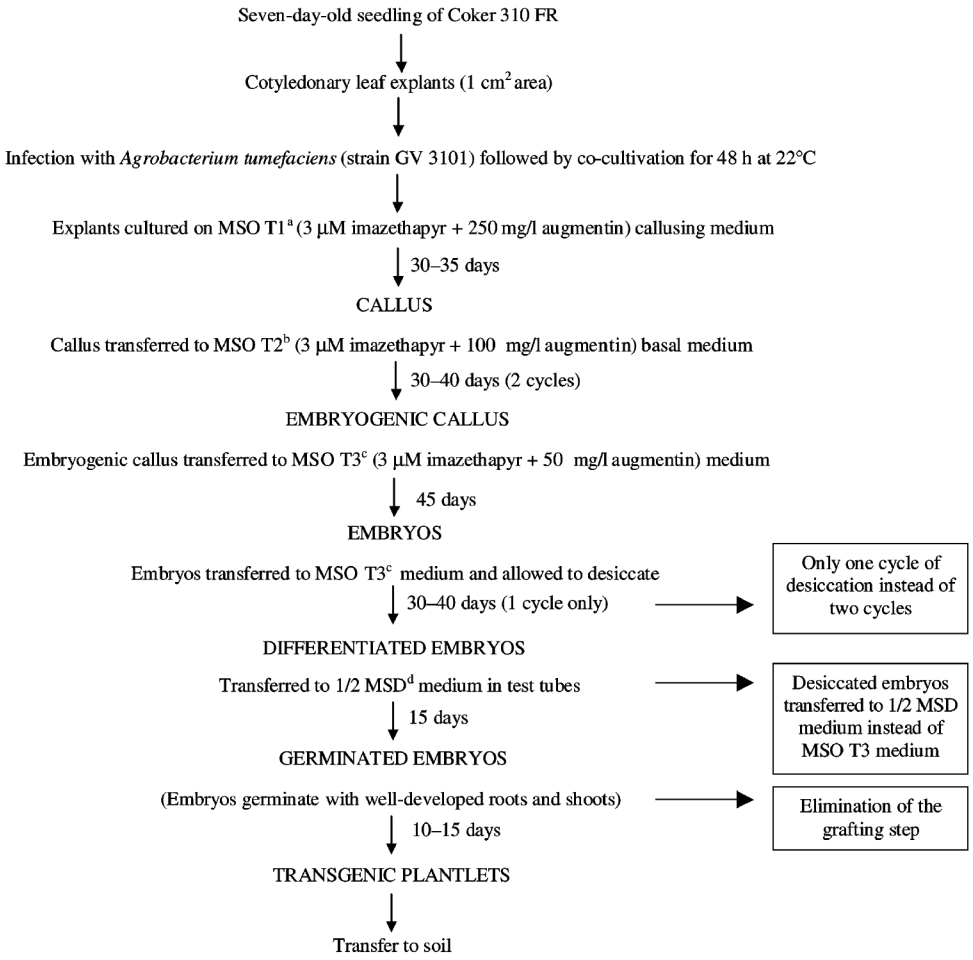

Figure 2. Flow chart showing the steps involved in the protocol developed in the present study based on the earlier protocol of Chaudhary *et al.*[3]. Information in the boxes highlights the major improvements from the earlier protocol, apart from the use of imazethapyr as a selection agent. All cultures were grown in 9 cm diameter disposable petri dishes (Tarson, India) under controlled environmental conditions [28 ± 2°C; 750 lux light intensity at the culture level; 16/8 h (light/dark) photoperiod]. Composition of the media used are as follows: [a]MS salts (1x) + B5 vitamins (1x) + 3% glucose + 100 µg/l 2,4-D + 500 µg/l kinetin + 0.2% phytagel (pH = 5.8). [b]MS salts (1x) + B5 vitamins (1x) + 3% glucose + 0.2% phytagel (pH = 5.8). [c]MS salts (1x) + B5 vitamins (1x) + 3% glucose + 1.9 g/l KNO_3 + 0.2% phytagel (pH = 5.8). [d]MS salts (0.5x) + B5 vitamins (0.5x) + 1.5% glucose + 0.2 mg/l IBA + 0.4% phytagel (pH = 5.8).

Table 1. Frequency of embryogenic callus, somatic embryogenesis and plantlet formation in genetic transformation experiments

Experiment no.	Number of explants	Percentage explants giving rise to embryogenic callus	Percentage explants giving rise to embryos	Percentage embryo germination on 1/2 MSD medium*	Percentage explants giving rise to plantlets
I	58	32.8	22.4	37.9	13.8
II	52	13.5	9.6	29.3	5.8
III	74	18.9	9.4	17	4.1
IV	31	16.1	10	75	6.5
V	36	30.6	11.1	50	2.8
Weighted average (%)		22.2	11.9	31.8	6.8

*Percentage is based on the number of embryos (more than one from each explant) transferred to 1/2 MSD medium.

some were found to be resistant to even 240 µM imazethapyr (Table 2).

The present protocol is a marked improvement over the earlier protocol of Chaudhary *et al.*[3], where elongated embryos had to be desiccated on MSO T3 medium for 2–3 cycles (each cycle ranging from 30 to 40 days) before single or multiple shoots emerged from the cotyledonary ends. Further, these regenerated shoots had to be excised

Figure 3. Various stages of plantlet formation and shoot culture of transformed cotton lines. ***a***, Embryos germinated on 1/2 MSD medium. ***b***, Transgenic shoot cultures maintained *in vitro* on soil : soilrite mixture. ***c***, Plantlets transferred to pots after sub-culture.

Table 2. Response of leaf discs from representative T0 transgenic events when placed in a medium with different concentrations of imazethapyr

	Copy no.		Concentration of imazethapyr (μM)							
Event no.	LB	RB	3	60	120	180	240	300	600	900
2.3	2	2	++++	+++	+++	+	+	+	–	–
2.2	1	1	++++	++++	+++	+	+	–	–	–
2.1	5	5	++++	++++	++++	+++	+++	++	+	–
3.7	1	3	++++	+++	+++	++	+	+	–	–
3.0	1	1	++++	++++	–	–	–	–	–	–
1.7	2	2	++++	++++	++++	+++	++	+	–	–
1.2	1	1	++++	++++	++++	++	++	+	–	–
3.2	1	2	++++	++++	++++	++++	++++	+	–	–

++++, Normal callusing; +++, Reduced callusing; ++, Explants remain green, no callus; +, Explants unhealthy; –, All explants dead.

and grafted on wild-type rootstock[3], which is a cumbersome process. The present protocol thus leads to a reduction in time taken for recovering transgenics and obviates the need for grafting.

In vitro expansion of cotton shoot cultures has always been a problem due to the inefficiency of root induction in cultured shoots. Several rooting media consisting of full or half-strength MS salts with activated charcoal or growth regulators like NAA, kinetin and IBA in different concentrations and combinations have been proposed by different groups[9–12]. However, in our studies (not reported here) none of these protocols could assure 100% rooting efficiency and long-term growth of shoot cultures. We observed that cotton shoots, transgenic or non-transgenic, could be rooted in a soil–soilrite (ratio 1 : 4) mixture supplemented with IBA with approximately 100% efficiency within 20–25 days under controlled environmental conditions (28 ± 2°C; 750 lux light intensity; 16/8 h (light/dark) photoperiod) and the plantlets could be maintained on the same mixture for approximately 2 months. Further, a second sub-culture was carried out in which the apical region of the shoots maintained *in vitro* was cut and transferred to a fresh mixture of soil and soilrite. Plants of approximately 15 cm height from the first and second sub-cultures were transferred to soil in pots and grown in a greenhouse with 95% success rate (Figure 3 *c*). Successful rooting and proper growth of roots allowed the long-term maintenance of cotton transgenics through regular sub-culture.

In conclusion, we have demonstrated the use of imazethapyr as an efficient *in vitro* selection agent for *Agrobacterium*-mediated genetic transformation of cotton. An additional advantage of the ALS^{dm} gene is that it can confer resistance to the herbicide even under field conditions. The current protocol is significantly simplified and also takes a shorter time for the development of transgenics in cotton. The method for shoot culture described here could be used for *in vitro* propagation of elite germplasm and transgenic lines.

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Orthorectification of IRS-P6 LISS IV data using Landsat ETM$^+$ and SRTM datasets in the Himalayas of Chamoli District, Uttarakhand

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The availability of high-resolution data from IRS LISS IV, IRS Cartosat, QuickBird and IKONOS has added new dimensions to the use of satellite data for cartographic and natural resources management. High-resolution data have high relief displacement in hilly terrains. Therefore, to be able to integrate the dataset generated from coarse and medium-resolution satellite data with that of high-resolution satellite data, high-resolution images need to be orthorectified before they can be used further for long-term bio-resources monitoring. Although the use of stereo images for ortho image generation has been well documented, use of single-frame orthorectification is less known. The acquiring of stereo images is a costly affair. Orthorectification is important for proper geometric registration of LISS IV layers with other ancillary and GIS layers generated from coarse-resolution satellite data. The available orthorectified reference dataset of Landsat ETM$^+$ in conjunction with Digital Elevation Model derived from Shuttle Radar Topography Mission (SRTM) have been used to orthorectify high-resolution LISS IV image. This communication discusses the methodology for rectifying high-resolution images using Landsat ETM$^+$ and SRTM data available on the internet. The methodology can be applied in highly complex terrain with high to very high elevation regions like the Himalayas, where contour data are either lacking or not available. The root mean square error is 0.0415 m.

Keywords: High-resolution satellite data, hilly terrain, orthorectification, relief displacement.

IN satellite images the vertical perspective projection differs from that of the orthographic projection of the ground, which causes relief displacement. As a result, any erect elevated object leans away from the principal point of the photograph radially[1]. It may be explained by formula $D = rh/H$, where D is the relief displacement, r the radial distance on the photograph from the principal point to the displaced image point, h the height above the datum of the object point and H is the flying height above the same datum. Therefore, the relief displacement depends

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