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Herbicide-resistant Acala and Coker cottons transformed with a native gene encoding mutant forms of acetohydroxyacid synthase

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Abstract

Herbicide-resistant transgenic cotton (*Gossypium hirsutum* L.) plants carrying mutant forms of a native acetohydroxyacid synthase (AHAS) gene have been obtained by *Agrobacterium* and biolistic transformation. The native gene, A19, was mutated *in vitro* to create amino acid substitutions at residue 563 or residue 642 of the precursor polypeptide. Transformation with the mutated forms of the A19 gene produced resistance to imidazolinone and sulfonylurea herbicides (563 substitution), or imidazolinones only (642 substitution). The herbicide-resistant phenotype of transformants was also manifested in their *in vitro* AHAS activity. Seedling explants of both Coker and Acala cotton varieties were transformed with the mutated forms of the A19 gene using *Agrobacterium*. In these experiments, hundreds of transformation events were obtained with the Coker varieties, while the Acala varieties were transformed with an efficiency about one-tenth that of Coker. Herbicide-resistant Coker and Acala plants were regenerated from a subset of transformation events. Embryonic cell suspension cultures of both Coker and Acala varieties were biolistically transformed at high frequencies using cloned cotton DNA fragments carrying the mutated forms of the A19 gene. In these transformation experiments the mutated A19 gene served as the selectable marker, and the efficiency of selection was comparable to that obtained with the NPT II gene marker of vector Bin 19. Using this method, transgenic Acala plants resistant to imidazolinone herbicides were obtained. Southern blot analyses indicated the presence of two copies of the mutated A19 transgene in one of the biolistically transformed R_0 plants, and a single copy in one of the R_0 plants transformed with *Agrobacterium*. As expected, progeny seedlings derived from outcrosses involving the R_0 plant transformed with *Agrobacterium* segregated in a 1:1 ratio with respect to herbicide resistance. The resistant progeny grew normally after irrigation with 175 μg/l of the imidazolinone herbicide imazaquin, which is five times the field application rate. In contrast, untransformed sibling plants were severely stunted.

Abbreviations: AHAS, acetohydroxyacid synthase; CaMV, cauliflower mosaic virus; ELISA, enzyme linked immunosorbent assay; FW, fresh weight; GUS, β-glucuronidase; IC_{50}, herbicide concentration that produces a 50% reduction in the fresh weight growth of cells; NAA, α-naphthaleneacetic acid; NPT II, neomycin phosphotransferase II; MS, Murashige and Skoog (1962).

Introduction

Imidazolinones and sulfonylureas are among several newer classes of herbicides which act by inhibiting acetohydroxyacid synthase (AHAS; EC 4.1.3.18), an enzyme found only in prokaryotes, fungi and plants [reviewed in 23]. AHAS is part of the synthetic pathway for the branched-chain amino acids leucine, iso-

leucine and valine [44], and its inhibition results in a toxic accumulation of an AHAS substrate, α-ketobutyrate, and the eventual termination of protein synthesis [23, 33].

Imidazolinones and sulfonylureas have many desirable qualities, including broad spectrum activity at low application rates and little or no known detrimental effects on animals. This latter trait is due in part to the fact that AHAS is absent in animals (thus, the need for the 'essential' amino acids in the diet). These herbicides also have little or no known toxic side effects in animals and exhibit no measurable mutagenic, carcinogenic or teratogenic properties [13, 21, 34, 39]. Thus, the development of crops which are resistant to these herbicides should be beneficial to growers and environmentally innocuous.

It has been shown that single amino acid substitutions in endogenous forms of AHAS, as well as forms derived from mutant genes introduced by gene transfer, can produce plants resistant to imidazolinones, sulfonylureas and other herbicides [14, 15, 20, 28, 38, 46]. We report here the *in vitro* mutagenesis of a cotton AHAS gene which is one of two encoding the main housekeeping forms of the enzyme, and its introduction into cotton cells by both *Agrobacterium* and biolistic transformation. In addition to producing the first transgenic Coker variety plants with resistance to imidazolinones and sulfonylureas, we have also succeeded in obtaining transgenic Acala variety plants with resistance to these herbicides. Acala varieties have been recalcitrant to *in vitro* manipulations that work well with Coker varieties [43].

Materials and methods

Plant materials

Acid delinted, fungicide-treated seed of cotton (*Gossypium hirsutum* L.) Acala varieties (GC510, B3991, B1654, CSC28 and Royale) and Coker 315 were obtained from Dr H.B. Cooper, J.G. Boswell Cotton Seed Company, Corcoran, CA. Seeds of C4 and B2 (Coker type varieties) were obtained from Chembred Cotton Seed Company, Maricopa, AZ.

Herbicides

An analytical grade sample of the sulfonylurea compound chlorsulfuron was purchased from Chem-Service (West Chester, PA). Analytical grades of imidazolinone herbicides imazethapyr (Pursuit) and imazaquin (Scepter) were provided by Dr Mark Dahmer, American Cyanamid Company, Princeton, NJ. Herbicides were stored at 20 °C as stock solutions with DMSO as the solvent. All stock solutions were filter-sterilized using Acrodisc CR (0.22 μm, Gelman Sciences, Ann Arbor, MI) and were added to pre-cooled (ca. 50 °C) medium after autoclaving.

Initiation and maintenance of embryogenic callus and cell suspension cultures

Seeds were surface sterilized by first treating with 70% ethanol for 3 min, followed by a 20 min treatment with a 20% Clorox solution (1% available chlorine) containing 0.01% of the surfactant Tween-20. Seedlings were grown under 16 h light (40–60 μE m^{-2} s^{-1}) and 8 h dark at 26 ± 2 °C on agar-solidified (TC Agar, Hazleton Biologics, Lenexa, KS) White's medium [41] containing 1 mg/l kinetin. Embryogenic callus cultures were first established from seedling explants according to the procedures of Rangan [31]. Briefly, cotyledon and hypocotyl explants from 7- to 10-day old seedlings were placed on a callus induction medium (MS [26]) supplemented with 0.4 mg/l thiamine HCl, 30 g/l glucose, 2.0 mg/l α-naphthaleneacetic acid (NAA), 1.0 mg/l kinetin, 100 mg/l myo-inositol and 0.8% (w/v) agar. The cultures were incubated at 27 ± 2 °C under conditions of 16 h light and 8 h dark, light intensity at 60 μE m^{-2} s^{-1}, in an environmentally controlled incubator (Percival, Boone, IA). Callus formed on these explants within three to four weeks. Callus pieces were selectively subcultured to enrich for friable, yellowish-green callus every three to four weeks on the same medium, except the carbon source was sucrose (20 g/l) instead of glucose. Depending on the variety, embryogenic callus capable of forming small globular somatic embryos appeared one to four subcultures after initiation. This regeneration procedure was successfully used in a broad range of cotton species and varieties, including Acala varieties [31], most of which failed to produce embryogenic callus on media used for regenerating Coker varieties [9, 43, 45]. Embryogenic callus was maintained and multiplied by routine subculture every three to four weeks on MS medium containing 100 mg/l myo-inositol, 20 g/l sucrose, 2.0 mg/l NAA and 0.8% (w/v) agar (maintenance medium).

Cell suspension cultures were initiated from finely dispersed embryogenic callus cultures in liquid maintenance medium agitated (120 rpm, 27 ± 2 °C) on a

gyratory shaker (New Brunswick G-10, Edison, NJ). The suspension cultures were enriched for small, isodiametric, densely cytoplasmic and highly embryogenic cells by periodically discarding free floating cells and large aggregates ($\geq$ 840 μm) every week. Two days before use, these cultures were subcultured in 250 ml Erlenmeyer flasks containing 40 ml of maintenance medium. The cell suspension cultures used in our experiments were rapid growing embryogenic cells that exhibited a doubling of fresh weight in four to six days (the logarithmic phase of growth begins two days after subculture). In the biolistic transformation experiments, cell suspension cultures initiated from cryopreserved cultures [32] were used. All cell suspension cultures used for biolistic transformation experiments had a cumulative age of three to four months.

Mutagenesis of the A19 AHAS gene

A previously characterized cotton AHAS gene, A19, which is one of two encoding the main housekeeping forms of AHAS in the cotton AD allotetraploid *G. hirsutum* L. [12], was selected for *in vitro* mutagenesis. A 2.6 kb *Kpn*I/*Pst*I fragment containing 1.6 kb of the coding region and 1.0 kb of 3′-flanking DNA (Figure 1) was subcloned into the replicative form of phage vector M13. Mutagenesis was carried out using the method of Kunkel [19] and two oligonucleotides: 5′-TATGGTTGTTCAATCCGAGGACCGGTTTTA-3′ and 5′-ATGATCCCCAATGGAGGCGCTTTC-3′. These oligonucleotides were selected to create different mutations in the codons for amino acids 563 and 642 of the precursor polypeptide. In the A19 gene, these codons correspond to those encoding amino acids 574 and 653 of the *Arabidopsis thaliana* precursor polypeptide. Normally these codons encode a tryptophan and a serine in both *G. hirsutum* and *A. thaliana* [12]. They were chosen for mutagenesis on the basis of previous studies which have shown that substitutions at the serine residue confer resistance to imidazolinones [38], while substitutions at the tryptophan residue produce multiple resistance to imidazolinones, sulfonylureas, and other herbicides [for reviews, see 13, 14, 34]. Figure 2 shows the wild-type DNA sequence of the A19 gene in the regions containing the 563 and 642 codons, and the introduced mutations.

Constructions used for transformation experiments

The mutant forms of the A19 gene were reassembled in vector pUC19, and restriction site analysis and DNA sequencing [27, 37] were performed to verify the integrity of the gene and the presence of the desired mutations. For transformation experiments employing the biolistic device [36], gel-purified 8.2 kb *Bam*HI/*Pst*I genomic clone fragments containing the A19 gene with either the 563 or the 642 mutation were utilized (Figures 1 and 2). In biolistic transformation experiments which included the GUS and/or NPT II genes, entire plasmid DNAs were utilized. In the case of the GUS gene, the plasmid utilized was a pUC19 subclone of the 3 kb *Eco*RI fragment from plasmid pAGUS1-TN2 [42]. The NPT II gene plasmid was a pUC19 subclone of pPHY1 [47], in which case the CaMV 35S promoter runs the NPT II gene.

For transformation experiments with *Agrobacterium*, 10.0 kb *Bam*HI/*Sal*I fragments containing the A19 gene with either the 563 or the 642 mutation (Figures 1 and 2) were subcloned into the *Bam*HI and *Sal*I sites in the polylinker of binary vector Bin 19 [3, 10]. These constructions were subsequently introduced [1] into *Agrobacterium tumefaciens* strain A136/542 [16].

Agrobacterium *cultures*

Agrobacterium cultures for transformation experiments were initiated in 50 ml of YEB liquid medium using frozen glycerol stocks (500 μl) as inoculum. These cultures were grown overnight for about 18 h at 26 $\pm$ 2 °C on a gyratory shaker. The optical density (A_{600}) values were adjusted to 0.6–0.8 in liquid MS medium [26] prior to use.

Agrobacterium*-mediated transformation of cotton seedling explants*

The cotyledon (1 cm^2) and hypocotyl (1 cm long) explants used for *Agrobacterium* transformations were prepared from 5- to 7-day old seedlings. The transformation procedures followed those of Firoozabady *et al.* [9], except that regeneration was achieved by a different method [31] and selection for transformed colonies utilized the antibiotic G418 (Gibco BRL, Life Technologies, Gaithersburg, MD) at 10 mg/l or the herbicides (3 μM imazethapyr or 1 μM imazaquin). G418 was utilized instead of its analogue, kanamycin, because we have observed that kanamycin has a detrimental effect on cotton embryogenesis (Rajasekaran *et al.*, unpublished).

The explants were treated with *Agrobacterium* suspension for 15 to 30 min, blotted dry, and then plated on 12 cm diameter filter paper (Whatman No. 1) placed

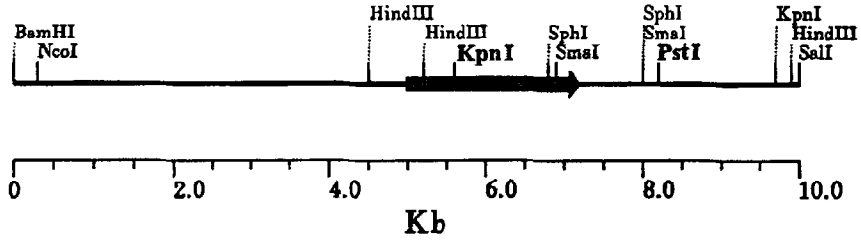

Figure 1. Restriction site map of the 10 kb *Bam*HI/*Sal*I cotton DNA fragment containing the A19 AHAS gene (arrow). The *Kpn*I and *Pst*I sites used to subclone part of the gene coding region into the replicative form of phage vector M13 are shown in bold.

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                563
                Trp
 w.t.  TATGGTTGTTCAATGGGAGGACCGGTTTTA
mutant ----------------CC--------------
                Ser

                642
                Ser
 w.t.       ATGATCCCCAGTGGAGGCGCTTTC
mutant      ----------A-------------
                Asn
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Figure 2. Mutations introduced into the A19 AHAS gene by *in vitro* mutagenesis. The wild type (w.t.) DNA sequences in the regions encoding amino acids 563 (tryptophan) and 642 (serine) of the polypeptide precursor [12] are indicated. The sequences of two oligonucleotides that were used to create two mutant forms of A19 are indicated below each wild type sequence. In one case the mutations result in the substitution of a serine for the tryptophan at residue 563, and in the other case an aspargine for the serine at residue 642. Base differences between the wild-type sequences and the oligonucleotides are shown, while dashes indicate sequence agreement.

on freshly made, agar-solidified callus induction medium [31] in 15 cm diameter Petri dishes containing 60 ml of medium. Cocultivation was carried out for 48 h in a Percival incubator maintained at 26 ± 2 °C, 16 h light, 60–90 μE m^{-2} s^{-1}. Following cocultivation, the explants were thoroughly washed in MS [26] liquid medium containing 200 mg/l cefotaxime (Cal-Biochem) and 200 mg/l carbenicillin (Sigma), blotted dry and placed on freshly prepared callus induction medium containing the selection agent and the same concentrations of cefotaxime and carbenicillin to control bacterial growth.

Cotyledon segments were plated at seven and hypocotyls at 10 per Petri dish (9 cm diameter) containing 25 ml callus induction medium. Hypocotyl segments were plated with the longitudinal cut end facing the medium. The cultures were kept in Percival incubators for three to four weeks. After the first subculture the explants were transferred to freshly made callus induction medium to encourage more callus production in the presence of selection pressure. Further details on selection are given in Results. NPT II ELISAs were carried out according to the procedures of Firoozabady *et al.* [9] to identify transformed callus colonies.

Biolistic transformation of embryogenic cell suspension cultures

The DNA(s) used in the biolistic experiments were coated onto 1.0 μm gold particles, using $CaCl_2$ and spermidine as described previously [36]. The particles were accelerated using the PDS 1000/helium biolistic device (BioRad, Richmond, CA).

The cell suspensions (< 840 μm), subcultured two days earlier, were vacuum-deposited as a thin layer onto moist filter paper (Whatman No. 1; 3.5 cm diameter) in sterile Petri dishes (5.5 cm diameter). One ml of suspension cells (1 × 10^6 cells) was transferred to each dish. A 400 mesh nylon screen was placed over the surface of the suspensions to serve as a baffle. In our hands, the optimal bombardment conditions included the use of 10 MPa rupture disks, a distance between the stopping screen and the cell suspensions of 7.5 cm and a macrocarrier travel distance of 10 mm. During the bombardment, the vacuum in the sample chamber was 95 kPa. Bombardment of the cells was repeated three to five times at two-day intervals to maximize the transformation frequency. Cell suspensions were bombarded with the mutated forms of the A19 AHAS gene and selected with the herbicides imazethapyr or imazaquin (see below). In parallel experiments, cells were transformed with the NPT II gene and/or GUS gene to monitor the transformation efficiency by selection with the antibiotic G418 or by histochemical staining for GUS expression [17].

Following particle bombardment, the cell suspension cultures were grown for a week without any selec-

tion in maintenance medium. Selection with the antibiotic G418 or the herbicides imazethapyr and imazaquin was applied by gradually increasing the concentration each week. Selection with G418 was initiated at 10 μg/ml and increased by 10 μg/ml increments at five to seven day intervals to achieve a final concentration of 50 μg/ml after three to four weeks. Imazethapyr selection was started at 1 μM and increased at weekly intervals to 3, 10, 30, 100 and 300 μM over a period of 6–8 weeks. Similarly, selection with imazaquin was started at 0.3 μM and increased to 100 μM over a period of six to eight weeks. In some experiments, cells were directly exposed to only one high level of antibiotic (G418 at 50 μg/ml) or herbicides (imazethapyr at 100 to 300 μM; imazaquin at 100 μM) at the beginning of the selection process. The number of independent transformation events was determined by plating a known number of cells after biolistic bombardment at a low density (2×10^4 cells per plate) on solidified maintenance medium containing the selection agent. This method of counting helped us avoid overestimating the number of independent transformation events due to clonal multiplication of transformed cells in liquid cultures.

Growth of transformed cell lines in the presence of herbicides

The herbicide resistance conferred by the A19 transgene with mutations at codons 563 or 642 was evaluated in the presence of toxic levels of imazethapyr (300 μM) and chlorsulfuron (2.8 μM). These concentrations are 100 times higher than the IC_{50} value of each herbicide. The growth studies were conducted with transformed embryogenic and control (non-transformed) suspension cultures to determine the inhibitory effects of the herbicides. An equal amount of cells (200 mg FW) was used to inoculate 40 ml of liquid maintenance medium with or without herbicide. After 14 days of incubation, the cells were harvested by vacuum filtration onto a membrane filter and their fresh weight determined.

AHAS enzyme activity assay

AHAS enzyme assays were performed as described previously [40]. Protein concentrations were measured with a protein assay reagent (BioRad) using bovine serum albumin as a standard [4]. Uninhibited AHAS enzyme activities in the presence of toxic herbicide levels (100 μM of imazethapyr or 2.8 μM of chlorsulfuron) are presented as a percentage relative to activity in the absence of herbicide. The values from at least three independent determinations were averaged to derive each data point.

Evaluation of herbicide resistance: recallussing assay and greenhouse experiments

Regenerated plants from transformed cell suspension and callus cultures were evaluated *in vitro* and *in planta* for herbicide resistance. The *in vitro* recallussing assay measured the ability of leaf and petiole explants from potentially transformed plantlets to produce callus in presence of imazethapyr (3, 10, 25 and 50 μM). An agar solidified MS basal medium containing 5 mg/l 2-isopentenyladenine (2iP) and 0.1 mg/l NAA was used for this purpose. The cultures were evaluated after four weeks of incubation. Explants with a proliferation of greenish callus were scored as positives (herbicide-tolerant).

In planta assays were performed in a greenhouse. Progeny derived from outcrosses involving transformed R_0 plants were germinated in an artificial soil mix (6:4 perlite/peat) in the presence of 175 μg/l (560 nM) imazaquin. This dosage is five-fold higher than the field application (pre-emergence) rate. After three weeks of treatment, the plants were evaluated for visible injury to height, internode length, meristems and foliage.

Southern blot analyses

Southern blots were carried out according to the methods of Klessig and Berry [18]. The probe for each blot was the gel-isolated insert of cDNA clone pAHAS2 [12], labeled by nick-translation [35].

Results

Transformation of seedling explants by Agrobacterium

Table 1 provides a summary of our transformation experiments using *Agrobacterium* with the Bin 19-derived vectors. In experiments utilizing cotyledon explants, the emergence of separate colonies from the cut surface provided a reliable method of identifying independent transformation events. On the other hand, it was difficult to identify independent transformation events from the large mass of callus produced by

hypocotyl explants. Therefore, we focused on cotyledon explants to measure the transformation frequency. However, we were able to obtain transformed plants from hypocotyls as well as cotyledons.

Both cotyledon and hypocotyl explants produce few transformed colonies when subjected to high selection pressure (G418 at 50 μg/ml). Only one transformed colony was obtained per 20 to 30 explants, regardless of the variety. However, we appeared to obtain much better transformation frequencies using a low level of antibiotic (G418 at 10 μg/ml). This result is consistent with the antibiotic selection regime of Firoozabady *et al.* [9]. Notably, there was a marked difference in transformation frequency between Coker and Acala varieties. For example, about six to eight antibiotic-resistant colonies were obtained after six to eight weeks of selection from each Coker cotyledon explant. In contrast, only two to three colonies were obtained from each Acala cotyledon explant over the same selection period.

While the low level selection regime greatly increased the number of apparent transformants, we found a substantial number of these were 'escapes'. Again, we observed a significant difference between Coker and Acala varieties. For example, up to 60% of Coker callus colonies continued to exhibit vigorous growth after an additional six to eight weeks on antibiotic selection (the rest were considered escapes). However, only 15–20% of Acala callus colonies continued to grow vigorously after the same period of additional antibiotic selection. At this stage, 85–100% of the vigorously growing colonies of both varieties were NPT II ELISA positive. Using this selection strategy, we obtained an average of 450 independently transformed colonies in Coker varieties C4 and B2, and an average of 40 independently transformed colonies in each of the Acala varieties (GC510, B3991, CSC28 and Royale), for every 100 cotyledon explants infected with *Agrobacterium*.

Another major difference between Coker and Acala cotton varieties is the frequency with which transformed callus colonies become embryogenic. For Coker varieties, an average of 60 to 75% of the transformed colonies turned embryogenic six to eight weeks after the initiation of antibiotic selection. On the other hand, only 20% of transformed Acala colonies turned embryogenic. To obtain embryogenic callus from transformed, non-embryogenic Acala callus colonies, it was usually necessary to remove the antibiotic selection pressure (which was not necessary for transformed Coker colonies). Acala colonies also took longer (8 to 24 weeks) to become embryogenic.

The results we obtained using imidazolinone herbicides to select for transformation of seedling explants by *Agrobacterium* essentially paralleled those we obtained with G418 (Table 1).

Biolistic transformation of embryogenic cell suspension cultures

In contrast to our experience with *Agrobacterium* transformation, we did not observe any significant difference in the biolistic transformability of cell suspension cultures derived from Coker and Acala cotton varieties (Table 1). In preliminary experiments with the GUS marker gene, which were conducted to optimize our biolistic transformation protocol, transient expression was readily apparent in cells two to three days after bombardment. At least 5000 cells transiently expressed the GUS gene in every plate of cells (1×10^6) that was subjected to one bombardment. Multiple bombardment during the logarithmic phase of the cell growth increased the frequency of transient GUS expression at least three-fold. Thus, we employed multiple bombardment in all subsequent experiments (see Materials and methods).

Further optimization of our biolistic transformation protocol was achieved by adjusting the selection regime. We observed that selection at high concentrations of herbicides (imazethapyr at 100 to 300 μM; imazaquin at 100 μM) resulted in less than 50 stable and independently transformed cell lines in each sample of 1×10^6 cells. When we cotransformed cell suspensions with the NPT II gene and selected with high concentrations of G418 (50 μg/ml), we also obtained low transformation frequencies. On the other hand, starting selection at much lower concentrations of herbicide or G418 and gradually increasing the selection pressure (see Materials and methods) resulted in a four- to five-fold increase in the number of stably transformed colonies per flask. As a result, we started at low concentrations and gradually increased the selection pressure in all subsequent experiments whether herbicide or G418 was the selection agent. Under this regime, we observed that clear-cut discrimination between transformed and non-transformed cells was possible in a shorter time with G418 (four weeks) than with imazethapyr or imazaquin (six to eight weeks).

In order to monitor the frequency of stable transformation, the GUS marker gene was always used in

Table 1. Transformation of cotton cotyledon explants by *Agrobacterium* and cell suspension cultures by the biolistic method to achieve herbicide resistance.

Cotton variety	A19 gene mutation used in transformation	Transformation method	Selection agent and level	Average number of independent transformation events obtained[a]	Number of transformation events from which plants were made	Number of transformed plants regenarated	Maximum imazethapyr resistance *in vitro* (μM)
C4	642	*Agrobacterium*	G418 10 mg/l	324	5	371	10
	642	*Agrobacterium*	imazethapyr 3 μM	390	n.d[b].	n.d.	n.d.
B2	642	*Agrobacterium*	G418 10 mg/l	584	5	140	3
GC510	563	*Agrobacterium*	G418 10 mg/l	37	25	62	n.d.
B3991	642	*Agrobacterium*	imazaquin 1 μM	45	3	12	n.d.
Coker 315	563	biolistic	imazethapyr 300 μM	750	n.d.	n.d.	n.d.
B1654	563	biolistic	imazethapyr 300 μM	580	11	496	50
B1654	642	biolistic	imazaquin 100 μM	700	35	357	n.d.

[a] Per 100 *Agrobacterium*-treated seedling explants (as determined by NPT II ELISA) or 1×10^6 bombarded cells (as determined by continued growth on medium containing herbicide after six subcultures).
[b] Not determined.

parallel experiments. Figure 3 shows high frequency, stable transformation of B1654 cell suspension cultures after gradual selection with imazethapyr. Similar results were obtained when G418 or imazaquin was the selection agent (not shown). In all experiments, approximately one in a hundred transiently expressing cells proved to exhibit stable GUS gene expression. By combining multiple bombardment and gradually increasing selection pressure, we were able to obtain an average of 700 stably transformed cells from every flask of 1×10^6 cells. Selection with either antibiotic or herbicide yielded no escapes, and all of the plants regenerated from transformed colonies were positive in the recallussing assay (see Materials and methods).

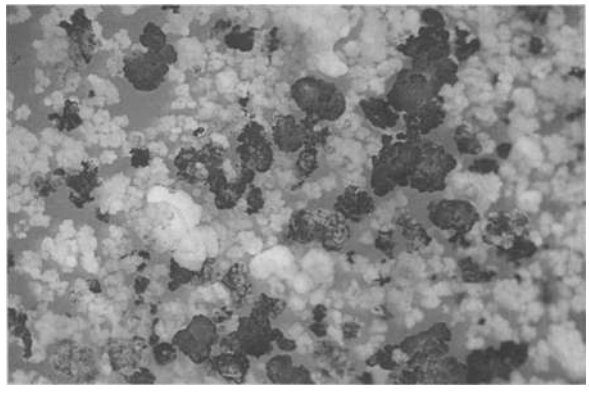

Figure 3. High-frequency GUS gene expression in Acala variety B1654 cell suspension cultures following biolistic cotransformation with a mutant form of the A19 AHAS gene (codon 563) and the GUS gene. Selection for the transformed cells employed gradually increasing concentrations of imazethapyr (1 to 300 μM). Staining for stable GUS expression was done after 8 weeks of selection.

Herbicide selection using the mutated A19 gene as the selectable marker vs. antibiotic selection with the NPT II gene selectable marker

To compare the efficiencies of herbicide and antibiotic selection using different selectable marker genes, an experiment was conducted in which explants transformed with *Agrobacterium* (containing Bin 19 derivatives with the two mutated forms of the A19 gene) were selected with either imazethapyr (3 μM) or G418 (10 μg/ml). At these concentrations, growth of callus colonies from untreated seedling explants is completely inhibited. Colonies from treated explants that continued to grow after eight weeks on imazethapyr (3 μM) or G418 (10 μg/ml) were assayed for the presence of NPT II by ELISA.

The results show that the A19 AHAS gene with the 642 mutation works as a selectable marker as well as the NPT II gene in Bin 19 (Table 2). In addition, the A19 gene with the 563 mutation worked as well as the 642 mutant under the same conditions of imazethapyr selection (data not shown).

Imidazolinone and sulfonylurea resistance conferred by the 563 and 642 mutations

The herbicide-specific resistance of callus transformed with the A19 gene containing either the 563 or the 642

Table 2. Antibiotic vs. herbicide selection in variety C4 cotyledon explants transformed with *Agrobacterium* containing the A19 AHAS gene with the 642 mutation.

Selection agent	Number of cotyledon explants	Number of colonies selected after four weeks[a]	Number of colonies showing active growth after ten weeks	Number of NPT II positive colonies	% of NPT II positive colonies
G418 10 μg/ml	230	182	43	37	86
Imazethapyr 3 μM	390	214	34	25	74

[a] Colonies were selected at random.

mutation was measured by growing a known amount of transformed cells in a liquid medium containing highly toxic levels of imazethapyr (300 μM) or chlorsulfuron (2.8 μM). The increase in fresh weight was compared to that of sensitive control cells after 14 days of growth. The data in Table 3 show that cells transformed with the A19 gene with the 563 mutation grew well in a medium containing either imazethapyr or chlorsulfuron, and exhibited an average fresh weight increase of 240%. On the other hand, cells transformed with A19 gene containing the 642 mutation grew well in medium with imazethapyr, but failed to exhibit an increase in fresh weight in medium with chlorsulfuron. These data clearly illustrate that the 642 mutation confers resistance only to imidazolinones, whereas the 563 mutation confers resistance to both imidazolinones and sulfonylureas.

Transformed cell AHAS activity in the presence of imazethapyr and chlorsulfuron

The AHAS activity in extracts from embryogenic callus colonies transformed with the mutated A19 gene (563 or 642) was examined in the presence of toxic levels of imazethapyr (100 μM), and chlorsulfuron (2.8 μM). The AHAS activity from colonies transformed with the 563 mutation was strongly resistant to both imazethapyr and chlorsulfuron. In contrast, the AHAS activity from cells transformed with the 642 mutation was resistant to imazethapyr but sensitive to chlorsulfuron (Table 4). These results again demonstrate the imidazolinone-specific resistance conferred by the 642 mutation, and also corroborates the cell growth measurements reported in Table 3.

Southern blot analyses of cotton transformants

A Southern blot analysis of one example of an Acala B1654 plant biolistically transformed with the 8.2 kb *Bam*HI/*Pst*I fragment containing the A19 gene with the 563 mutation (Figures 1 and 2) is shown in Figure 4A. The transformed plant DNA in lane 2 has two extra *Sph*I fragments (10.5 and 9.0 kb) compared to the control DNA in lane 1. Thus, we conclude this biolistically-transformed plant has two copies of the mutated A19 transgene. The transgene copy number in ten other transformants derived by this method ranged from one to four (data not shown).

Figure 4B shows Southern blot analyses of a transformation event obtained with *Agrobacterium*. In this case the T-DNA included the 10.0 kb *Bam*HI/*Sal*I fragment containing the A19 gene with the 642 mutation (Figures 1 and 2) positioned downstream from the NPT II marker gene of binary vector Bin 19 [10]. Lanes 2 and 3 of Figure 4B show DNAs from a sample of transformed callus and an R_0 plant (variety C4) regenerated from the same callus. Lanes 4 and 5 contain DNA from two different progeny plants (both of which scored positively in an NPT II ELISA) derived from the R_0 plant represented in lane 3. Lane 1 is the negative control. These data show the transformed callus, the R_0 and the progeny plants each contain one copy of the mutated A19 transgene, which is revealed as an extra 4.3 kb *Sph*I fragment.

The size of this *Sph*I fragment indicates incomplete transfer of the T-DNA probably occurred during the original transformation event. According to the map of the 10 kb fragment with the A19 gene, an *Sph*I fragment of at least 6.8 kb would be expected in any transformant which received a complete T-DNA copy (Figure 1). To confirm the 5′ end of the A19 transgene and at least 0.6 kb of upstream DNA were transferred intact, we probed a Southern blot of the DNAs represented in Figure 4B with the 0.75 kb *Hin*dIII fragment that contains this region (Figure 1). The results verified the complete integration of the entire A19 transgene and at least 0.6 kb of its upstream promoter region (data not shown). Further confirmation of this is provided

Table 3. Growth of cells transformed with mutant forms of the A19 AHAS gene (at codon 563 or 642) in the presence of toxic levels of an imidazolinone herbicide, imazethapyr, and a sulfonylurea herbicide, chlorsulfuron. Fresh weight increase was determined after 14 days of culture.

Herbicide concentration	% increase in FW[a]		
	Control (untransformed) cells	Cells transformed with A19 gene mutation	
		codon 563	codon 642
No herbicide	240	238	260
Imazethapyr (300 μM)	6	230	195
Chlorsulfuron (2.8 μM)	15	251	32

[a] Each value is an average of five replicates. Increase was calculated from the ratio of the increase in fresh weight to the weight of the inoculum (see Materials and methods).

Table 4. AHAS enzyme activity (average units per mg protein per hour) from colonies transformed with mutant forms of the A19 AHAS gene (codon 563 or 642) in the presence of imazethapyr and chlorsulfuron. The herbicides were used at a level 100-fold greater than the IC_{50} concentrations. Transformants with the 563 mutation were obtained by the biolistic method and transformants with the 642 mutation were obtained by the *Agrobacterium* method.

A19 gene mutation	AHAS enzyme activity (AU mg $^{-1}$ h^{-1})					
	imazethapyr (μM)			chlorsulfuron (nM)		
	0	100	% activity	0	2800	% activity
None/control	0.69	0.24	35	0.56	0.19	34
563	0.70	0.58	83	0.60	0.49	82
642	0.55	0.39	71	0.58	0.23	40

by the herbicide-resistant phenotype of transformed plants (below). Finally, we also hybridized a Southern blot of the DNAs represented in Figure 4B with an NPT II gene probe. The results (not shown) confirmed the presence of one T-DNA, including an intact copy of the NPT II marker gene.

Evaluation of herbicide resistance in regenerated R_0 plants and their progeny

The ability of leaf petiole explants from transformed R_0 plants to form callus *in vitro* in the presence of imazethapyr (3, 10, 25 and 50 μM) was evaluated to obtain an approximation of the herbicide resistance that might be expected on the whole plant level. Plants derived from both the *Agrobacterium* and biolistic transformation methods were routinely screened in this manner before transferring them to soil. The results are presented in Figure 5 and Table 1.

Leaf and petiole explants from control plants bleach on a medium containing 3 μM imazethapyr within three weeks (Figure 5). However, all of the C4 plants derived from *Agrobacterium* transformation with the A19 gene containing the 642 mutation produced large amounts of green callus on this same level of imazethapyr (3 μM) within three weeks (Figure 5). Explants from randomly selected C4 plants representing five independent transformation events recallussed on an even higher level of imazethapyr (10 μM). Furthermore, all the Acala B1654 plants transformed biolistically with the A19 gene containing the 563 mutation consistently produced callus on a medium containing 50 μM imazethapyr, the highest level tested (Table 1).

To evaluate herbicide resistance on the whole plant level, seeds derived from outcrosses involving the transformed R_0 plant previously demonstrated to have one copy of the mutated A19 transgene (lane 3 of Figure 4B) were germinated in the presence of imazaquin at a concentration of 175 μg/l (560 nM). As expected, the R_0 plant progeny included both transformed (herbicide-resistant) and non-transformed (herbicide-sensitive) siblings in a 1:1 ratio, due to segregation of the single T-DNA (transformation was confirmed by NPT II ELISA). Severe herbicide toxicity symptoms

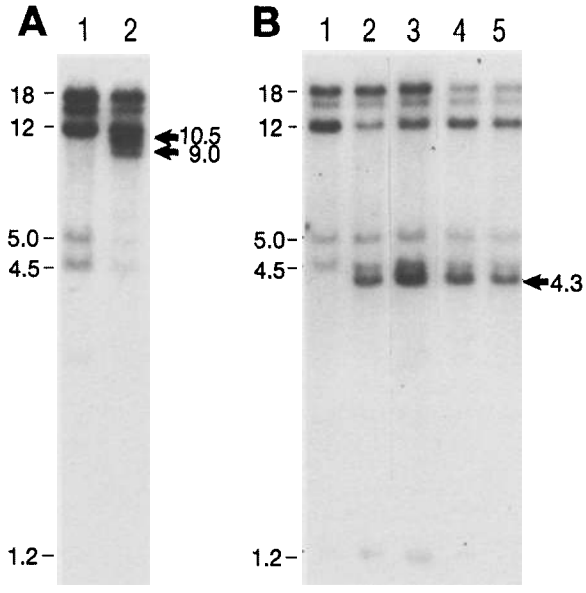

Figure 4. Southern blot analyses of cotton DNAs transformed with mutant forms of the A19 gene using either a biolistic device (A) or *Agrobacterium* (B). Each lane contains 5–10 μg of DNA digested with *Sph*I. The probe for each blot was the insert of cDNA clone pAHAS2 [12]. The sizes (in kb) of the *Sph*I fragments containing the native AHAS genes of cotton are indicated on the left side of each blot. The sizes of *Sph*I fragments containing the mutant A19 transgenes (marked by arrows) are indicated on the right side of each blot. Lane 1 in each blot contains control callus DNA (A) or control leaf DNA (B). Lane 2 of (A) contains biolistically transformed Acala B1654 plant DNA. Lane 2 of (B) contains variety C4 embryogenic callus DNA transformed with *Agrobacterium*. Lane 3 of (B) contains leaf DNA from an R_0 plant regenerated from the callus represented in lane 2 of (B), while lanes 4 and 5 contain leaf DNA from two progeny plants derived from outcrosses involving the R_0 plant.

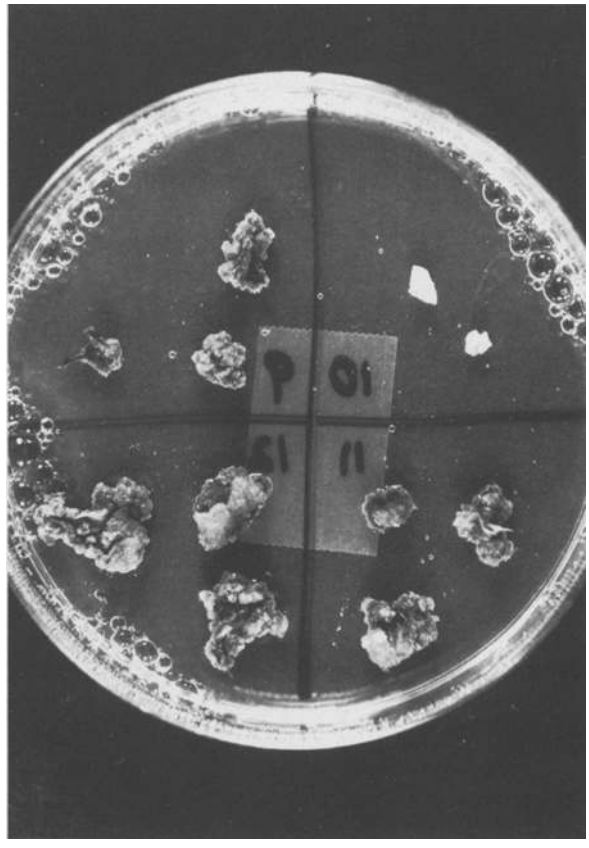

Figure 5. Recallussing assay on a medium containing 3 μM imazethapyr. Leaf and/or petiole segments were cultured for three weeks. Leaf segments from control C4 variety plants bleached on this medium (upper right). Leaf segments from three C4 plants transformed with *Agrobacterium* carrying the A19 gene with the 642 mutation produced profuse callus, indicating a significant level of imazethapyr resistance.

(i.e., reduced internode length, stunted growth, dead root and shoot tips and irreversible yellowing of leaves) were observed in the seedlings that were not transformed. In contrast, the transformed seedlings grew normally except for a slight, short-lived yellowing of their leaves (Figure 6).

Herbicide-resistant R_0 plants derived from biolistic transformation of Acala variety B1654 are weak, take a long time to produce flowers and are usually infertile (both male and female). However, some seed has been obtained using these cell suspension-derived R_0 plants as pollen parents in backcrosses to B1654. Some seedling progeny from these crosses exhibited a level of resistance to imazaquin (175 μg/l) equal to the resistant Coker seedlings (Figure 6).

Discussion

Acala and Coker variety cotton plants transformed with a native gene (A19) encoding mutant forms of AHAS have been obtained using both *Agrobacterium* and biolistic transformation methods. We have shown that transformation with the A19 gene mutated at codon 642 confers resistance to imidazolinone herbicides only, whereas a mutation at codon 563 produces resistance to both sulfonylurea and imidazolinone herbicides (Tables 3 and 4). Similar observations were made recently by Hattori *et al.* [14] in their study of a *Brassica napus* AHAS gene with a mutation at codon 557 (equivalent to codon 563 in the A19 gene). These investigators demonstrated this mutation provides resistance to four different classes of herbi-

Figure 6. Phenotype of herbicide-resistant (left) and sensitive (right) sibling progeny plants (variety C4) derived from outcrosses involving a transformed R_0 plant carrying one copy of the A19 transgene with the 642 mutation (see lane 3 of Figure 4B). Seeds were germinated in a solution containing 175 μg/l of imazaquin, which is five-fold higher than the field application (pre-emergence) rate. The photograph was taken after three weeks of growth.

cides which inhibit AHAS enzyme activity, including imidazolinones and sulfonylureas. The imidazolinone-specific resistance conferred by the codon 642 mutation has been previously demonstrated for the equivalent mutation in the *A. thaliana* AHAS gene [38]. Thus, our results concerning the specificity or generality of the herbicide resistance conferred by different AHAS gene mutations is consistent with other investigations.

In most previous efforts to engineer resistance to imidazolinones and sulfonylureas, a mutant AHAS gene from a chlorsulfuron-resistant line of *A. thaliana* was transferred into heterologous plants such as tobacco [6], flax [24], oilseed rape [25], poplar [5], rice [22] and maize [11]. In all of these cases, the native promoter of the mutant *A. thaliana* AHAS gene was replaced with the CaMV 35S promoter. In one case where a native gene was used to engineer herbicide resistance, Hattori *et al.* [14] produced sulfonylurea resistant *B. napus* plants after transformation with a mutant allele of the AHAS3 gene, which was isolated from a *B. napus* cell line. In both this case as well as the study reported here, the homologous transgenes apparently retain relatively high levels of expression with their native promoters, and in both cases confer significant levels of herbicide resistance. Thus, both our study and that of Hattori *et al.* [14] suggest there are viable alternatives to using the mutant AHAS gene from *A. thaliana* and heterologous promoters such as the 35S promoter.

We have also demonstrated the utility of mutated forms of the A19 gene as markers for the imidazolinone selection of transformed cotton cells (Table 2). This means that antibiotics and antibiotic resistance genes are not necessary to transform cotton, which may offer advantages in some cases. Other examples of herbicide selection for plant transformation include flax [24], rice [22], maize [11] and poplar [5], in which a mutant allele of the *A. thaliana* AHAS gene was the selectable marker. However, in all of these cases the sulfonylurea herbicide chlorsulfuron was the selective agent. Here we show successful selection with imidazolinone herbicides.

One of the most significant results reported here is the transformation of elite Acala varieties, which are valued for their fiber characteristics, by both *Agrobacterium* and biolistic methods. All previous reports of cotton transformation by these two methods [2, 8, 9, 30, 45] utilized highly regenerative Coker varieties [43]. One possible factor for our success in transforming Acala varieties is the regeneration protocol we employed [31]. For example, we have determined that the regeneration protocols for Coker varieties, which use other hormone combinations for callus induction [9, 43, 45], are not generally applicable to Acala varieties [31; unpublished]. Despite this improvement, the efficiency of Acala transformation with *Agrobacterium* is still low compared to Coker varieties. Contributing to this problem is the low frequency with which Acala varieties produce embryogenic callus. In addition, the few embryogenic colonies which are obtained take a long period to develop (see Results).

On the other hand, our procedure for biolistic transformation of Acala and Coker embryogenic cell suspension cultures is very efficient. Hundreds of stable transformants were obtained from each experiment. The efficiency of stable biolistic transformation (three to four percent of transiently expressing cells) was

higher than the 0.7% reported by Finer and McMullen [8]. We attribute our higher transformation efficiency to multiple shootings of highly embryogenic cell suspension cultures during the logarithmic phase of growth (two days to six days after subculture), and a gradual increase in selection pressure. Regardless of the transformation method employed, it has been shown that rapidly dividing cells in the logarithmic phase of growth are more easily transformed [1, 7, 29].

Another innovation we report here is the use of cloned cotton DNA fragments with the mutated forms of the A19 gene in our biolistic transformation protocol. We show that a DNA fragment is transferred into cotton cells as effectively as plasmid DNA, integrates into the chromosomes and is sufficiently expressed to produce the desired herbicide-resistant phenotype. This achievement and the utility of the mutated A19 gene as a selectable marker have broader implications for the genetic manipulation of cotton and other crops. In principle, it is now possible to transform any crop without the necessity of introducing non-plant DNA. Even further, we provide here the methodology by which cotton may be genetically engineered without the introduction of any non-cotton DNA. This may have functional advantages as well as render new cotton varieties derived by this approach more acceptable to regulatory agencies and the general public.

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References

1. An G, Ebert PR, Mitra A, Ha SB: Binary vectors. In: Gelvin SB, Schilperoort RA, Verma DPS (eds) Plant Molecular Biology Manual. Kluwer Academic Publishers, Dordrecht. pp. A3/1–19 (1988).
2. Bayley C, Trolinder N, Ray C, Morgan M, Quisenberry JE, Ow DW: Engineering 2,4-D resistance into cotton. Theor Appl Genet 83: 645–649 (1992).
3. Bevan M: Binary *Agrobacterium* vectors for plant transformation. Nucl Acids Res 12: 8711–8721 (1984).
4. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72: 248–254 (1976).
5. Brasileiro ACM, Tourneur C, Leplé J-C, Combes V, Jouanin L: Expression of the mutant *Arabidopsis thaliana* acetolactate synthase gene confers chlorsulfuron resistance to transgenic poplar plants. Transgenic Res 1: 133–141 (1992).
6. Charest PJ, Hattori J, DeMoor J, Iyer VN, Miki BL: *In vitro* study of transgenic tobacco expressing *Arabidopsis* wild type, mutant acetohydroxyacid synthase genes. Plant Cell Rep 8: 643–646 (1990).
7. de Kathen A, Jacobsen H-G: Cell competence for *Agrobacterium*-mediated DNA transfer in *Pisum sativum* L. Transgenic Res 4: 184–191 (1995).
8. Finer JJ, McMullen MD: Transformation of cotton (*Gossypium hirsutum* L.) via particle bombardment. Plant Cell Rep 8: 586–589 (1990).
9. Firoozabady E, DeBoer D, Merlo D, Halk E, Amerson L, Rashka K, Murray E: Transformation of cotton: (*Gossypium hirsutum* L.) by *Agrobacterium tumefaciens* and regeneration of transgenic plants. Plant Mol Biol 10: 105–116 (1987).
10. Frisch DA, Harris-Haller LW, Yokubaitis NT, Thomas TL, Hardin SH, Hall TC: Complete sequence of the binary vector Bin 19. Plant Mol Biol 27: 405–409 (1995).
11. Fromm ME, Morrish F, Armstrong C, Williams R, Thomas J, Klein TM: Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. Bio/technology 8: 833–839 (1990).
12. Grula JW, Hudspeth RL, Hobbs SL, Anderson DM: Organization, inheritance and expression of acetohydroxyacid synthase genes in the cotton allotetraploid *Gossypium hirsutum*. Plant Mol Biol 28: 837–846 (1995).
13. Hartnett M, Chui C-F, Mauvais J, McDevitt RE, Knowlton S, Smith JK, Falco SC, Mazur BJ: Herbicide-resistant plants carrying mutated acetolactate synthase genes. In: Green MB, Moberg WK, LeBaron H (eds) Managing Resistance to Agrochemicals: From Fundamental Research to Practical Strategies, pp. 459–473. American Chemical Society (1990).
14. Hattori J, Brown D, Mourad G, Labbé H, Ouellet T, Sunohara G, Rutledge R, King J, Miki B: An acetohydroxyacid synthase mutant reveals a single site involved in multiple herbicide resistance. Mol Gen Genet 246: 419–425 (1995).
15. Haughn GW, Smith J, Mazur B, Somerville C: Transformation with a mutant *Aradidopsis* acetolactate synthase gene renders tobacco resistant to sulfonylurea herbicides. Mol Gen Genet 211: 266–271 (1988).
16. Hood EE, Jen G, Kayes L, Kramer J, Fraley RT, Chilton M-D: Restriction endonuclease map of pTiBo542, a potential Ti plasmid vector for genetic engineering of plants. Bio/technology 2: 702–709 (1984).
17. Jefferson RA: Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5: 387–405 (1987).
18. Klessig, DF, Berry JO: Improved filter hybridization method for detection of single copy sequences in large eukaryotic genomes. Plant Mol Biol Rep 1: 12–18 (1983).
19. Kunkel TA: Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc Natl Acad Sci USA 82: 488–492 (1985).
20. Lee KY, Townsend J, Tepperman J, Black M, Chui C-F, Mazur B, Dunsmuir P, Bedbrock J: The molecular basis of sulfonylurea herbicide resistance in tobacco. EMBO J 7: 1241–1248 (1988).
21. Levitt G: Sulfonylureas, new herbicides. In: Miyamoto J, Kearney PC (eds) Pesticide Chemistry, Human Welfare and the Environment, pp. 243–250. Oxford University Press, New York (1983).

22. Li Z, Hayashimoto A, Murai N: A sulfonylurea herbicide resistance gene from *Arabidopsis thaliana* as a new selectable marker for production of fertile transgenic rice plants. Plant Physiol 100: 662–668 (1992).
23. Mazur BJ, Falco SC: The development of herbicide-resistant crops. Annu Rev Plant Physiol Plant Mol Biol 40: 441–470 (1989).
24. McHughen A: *Agrobacterium*-mediated transfer of chlorsulfuron resistance to commercial flax cultivars. Plant Cell Rep 8: 445–449 (1989).
25. Miki BL, Labbé H, Hattori J, Oullet T, Gabard J, Sunohara G, Charest PJ, Iyer VN: Transformation of *Brassica napus* canola cultivars with *Arabidopsis thaliana* acetohydroxyacid synthase genes and analysis of herbicide resistance. Theor Appl Genet 80: 449–458 (1990).
26. Murashige T, Skoog F: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497 (1962).
27. Norrander J, Kempe T, Messing J: Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene 26: 101–106 (1983).
28. Odell JT, Caimi PG, Yadav NS, Mauvais CJ: Comparison of increased expression of wild-type and herbicide-resistant acetolactate synthase genes in transgenic plants, and indication of posttranscriptional limitation on enzyme activity. Plant Physiol 94: 1647–1654 (1990).
29. Paszty C, Lurquin PF: Improved plant protoplast plating/selection technique for quantification of transformation frequencies. BioTechniques 5: 716–718 (1987).
30. Perlak FJ, Deaton RW, Armstrong TA, Fuchs RL, Sims SR, Greenplate JT, Fischoff DA: Insect resistant cotton plants. Bio/technology 8: 939–943 (1990).
31. Rangan TS: Regeneration of cotton. US Patent 5,244,802 (1993).
32. Rajasekaran K: Regeneration of plants from cryopreserved embryogenic cell suspension and callus cultures of cotton (*Gossypium hirsutum* L.). Plant Cell Rep 15: 859–864 (1996).
33. Ray TB: Site of action of chlorsulfuron. Inhibition of valine and isoleucine biosynthesis in plants. Plant Physiol 75: 827–831 (1984).
34. Saari LL, Cotterman JC, Thill DC: Resistance to acetolactate synthase inhibiting herbicides. In: Powles SB, Holtum JAM (eds) Herbicide Resistance in Plants, Biology and Biochemistry, pp. 83–139. Lewis Publishers, Boca Raton, FL (1994).
35. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
36. Sanford JC, Smith FD, Russell JA: Optimizing the biolistic process for different biological applications. Meth Enzymol 217: 483–509 (1993).
37. Sanger F, Micklen S, Coulson AR: DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467 (1977).
38. Sathasivan K, Haughn GW, Murai N: Molecular basis of imidazolinone herbicide resistance in *Arabidopsis thaliana* var. Columbia. Plant Physiol 97: 1044–1050 (1991).
39. Shaner DL, O'Conner SL (eds): The Imidazolinone Herbicides. CRC Press, Boca Raton, FL (1991).
40. Singh BK, Stidham MA, Shaner DL: Assay of acetohydroxyacid synthase from plants. Anal Biochem 171: 173–179 (1988).
41. Singh M, Krikorian AD: White's standard nutrient solution. Ann Bot 47: 133–139 (1981).
42. Skuzeski JM, Nichols LM, Gesteland RF: Analysis of leaky viral translation termination codons *in vivo* by transient expression of improved β-glucuronidase vectors. Plant Mol Biol 15: 65–79 (1990).
43. Trolinder NT, Goodin JR: Somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.). Plant Cell Rep 6: 231–234 (1987).
44. Umbarger HE: Amino acid biosynthesis and its regulation. Annu Rev Biochem 47: 533–606 (1978).
45. Umbeck P, Johnson G, Barton K, Swain W: Genetically transformed cotton (*Gossypium hirsutum* L.) plants. Bio/technology 5: 263–266 (1987).
46. Wiersma PA, Schmiemann MG, Condie JA, Crosby WL, Moloney MM: Isolation, expression and phylogenetic inheritance of an actolactate synthase gene from *Brassica napus*. Mol Gen Genet 219: 413–420 (1989).
47. Yenofsky RL, Fine M, Pellow JW: A mutant neomycin phosphotransferase II gene reduces the resistance of transformants to antibiotic selection pressure. Proc Natl Acad Sci USA 87: 3435–3439 (1990).