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Development of In Vitro Systems for Switchgrass (*Panicum virgatum*)

Final Report for 1992 to 2002

B. V. Conger

The University of Tennessee

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ORNL/SUB-02-11XSY161/01

Environmental Sciences Division

DEVELOPMENT OF IN VITRO SYSTEMS FOR SWITCHGRASS (*PANICUM VIRGATUM*)

FINAL REPORT FOR 1992 TO 2002

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TEN-YEAR PROJECT SUMMARY (1992–2002)

Our project began on July 1, 1992, with the objective of developing systems that could be used in biotechnological approaches to switchgrass improvement. Within six months after initiation of the project, we had worked out protocols in which plants could be regenerated from callus cultures through both organogenesis and somatic embryogenesis. Documentation for both modes of regeneration was provided in our progress reports and in publications. One thousand regenerated plants were established in the field during the first year. We found that Alamo (lowland type) was much more amenable to *in vitro* culture, and plants could be regenerated much more easily than from Cave-in-Rock (upland type).

During the first three years of the project, we studied the influence of genotype, culture medium components, explant type, etc., on regeneration. As mentioned, we found that the lowland cultivars Alamo and Kanlow were much easier to regenerate than upland cultivars, such as Trailblazer, Blackwell, and Cave-in-Rock. For callus induction, we initially used mature caryopses, young leaf tissue, and portions of seedlings. We were successful in inducing callus and regenerating plants from all explants.

A significant finding was the requirement of a cytokinin in the medium. Optimum production of embryogenic calli was obtained with 11.3 to 45.0 μM of culture 2,4-dichlorophenoxyacetic acid (2,4-D) and 45.0 μM of 6-benzylaminopurine (BAP). Later experiments with various explants confirmed the requirement of BAP for superior results. The cytokinin requirement appeared to be universal for all *in vitro* manipulations.

An important finding during the first 2 to 3 years was the development of inflorescences from split top nodes of plants in the 4-5 node stage. Axillary shoots proliferated from lower nodes. The protocol involved splitting the nodes longitudinally and placing the cut surface in contact with MS medium containing 30 g/L maltose and 0.0 to 25.0 μM BAP. The fully developed panicles from top nodes were subsequently used as a source of axenic explants to produce embryogenic calluses that were used for targets in gene transfer experiments.

The axillary shoots produced from lower nodes represents an effective and efficient method for micropropagation of switchgrass. It also appears to be genotype independent and should be applicable for upland as well as lowland cultivars. Theoretically, approximately 500 plantlets can be obtained from one parent plant in 12 weeks if nodal segments are cultured for 8 weeks at 29°C on MS medium containing 12.5 μM BAP and then transferred for an additional 4 weeks to induce rooting.

Two other systems developed during the 4th to 6th year period of the project included multiple shoot formation initiated from germinated seedlings and regenerable suspension cultures. The latter were initiated from embryogenic calluses produced from *in vitro* developed inflorescences. An important factor for producing multiple shoots was the presence of thidiazuron in the medium. The shoots could be easily rooted and numerous plantlets produced.

A goal of our research from the early stages was to develop a suspension culture system from which we could regenerate plants by somatic embryogenesis. Approximately 1 g fresh weight of embryogenic callus was transferred to liquid MS medium containing 30 gL^{-1} maltose and various concentrations of 2,4-D and BAP. Somatic embryos were collected on a 210 μm mesh screen. Larger cell clumps were collected on a 500 μm mesh. The embryos could be germinated directly into plants. The cell clumps produced embryogenic calluses from which plants were regenerated. Single cells and small cell clumps passing through the 210 μm mesh could be returned to suspension or plated on solid medium. Embryogenic calluses were produced from which plants could be regenerated. Development of this system allows us to produce somatic embryos in very large numbers.

As mentioned, 1000 plants regenerated from cell and tissue cultures were established in the field within 12 months after initiation of the project. This planting has been maintained as an observation and source nursery since 1993. There is great variability among individual plants for vigor, growth habit, color, culm size, etc. Yield data on an individual plant basis were collected from 300 plants in 1993 (those transplanted first) and from the entire plot in 1994 and 1995. The 20 highest yielding plants were selected, and crossing blocks with 2, 4, and 20 clones were established in 1997. Seed was harvested from these plantings in 1998 and used to establish yield trials. Dry matter yield data indicate that our synthetics are equal to or superior to Alamo and the synthetics developed by Charles Taliaferro at Oklahoma State University.

The last 3 to 4 years of the project focused on anther and microspore culture experiments to produce haploid plants and on genetic transformation. Although thousands of putative haploid plants were produced from a few anthers, they were very weak and difficult to keep alive. Chromosome counts revealed the gametic number in cells where it was possible to count chromosomes. The isolated microspore culture experiments were not successful.

Genetic transformation experiments utilizing both microprojectile bombardment and *Agrobacterium* were conducted during the final 3 to 4 years of the project. Tungsten particles coated with the plasmids pAHC25 (GUS-BAR) and psGFP-BAR were bombarded into embryogenic callus tissues with a particle inflow gun. Transformation was successful, and more than 100 transgenic plants were obtained with both constructs. Presence of both reporter genes (*gus* and *gfp*) and the selectable marker (*bar*) were confirmed by Southern blot analyses, and the transgenes were transmitted through male and female gametes.

Transformation experiments utilizing *Agrobacterium tumefaaciens* AGL1 (pDM805) were also successful. Immature somatic embryos and embryogenic calluses were incubated with *A. tumefaciens* in multiwell plates containing liquid MS medium. Several hundred transgenic plants were obtained. The presence of both *gus* and *bar* was confirmed by Southern blot hybridization, and both transgenes were transmitted through both male and female gametes and expressed in progeny.

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FIRST FIVE-YEAR PROJECT SUMMARY (JULY 1, 1992–JUNE 30, 1997)

The objective of our project during the first five years was to develop cell and tissue culture systems that might be used in biotechnological approaches to switchgrass improvement. This primarily involved the development of efficient and repeatable systems for whole plant regeneration from tissues and cells cultured *in vitro*. This is a basic requirement for almost all aspects of the technology ranging from micropropagation to gene transfer.

Prior to our work, beginning in 1992, very little *in vitro* culture work had been conducted with this species. The only published report was an abstract by C. H. Chen and coworkers in the proceedings of the VI International Congress of Plant Cell and Tissue Culture held in 1986. Our initial investigations focused on different media, medium components, explants, and upland versus lowland cultivars. Our results and progress made on the project for this period are summarized below.

- Within six months after initiation of the project, protocols were developed in which hundreds of plantlets could be regenerated with ease from various explants. We established 100 plants each of Alamo and Cave-in-Rock in a greenhouse. These were used for vegetative explants. Mature caryopses were also used as explants. Regeneration by both organogenesis and somatic embryogenesis was obtained from both young leaf tissue and mature caryopses. Best results were obtained with Murashige and Skoog (MS) medium containing 11.3 to 45.0 μM 2.4-D and 15.0 to 45.0 μM 6-benzylaminopurine (BAP). Plants could be regenerated with much greater ease from both explants of Alamo than from those of Cave-in-Rock.

- As stated above, our initial results indicated that the response of Alamo to *in vitro* culture was much superior to that of Cave-in-Rock. Additional experiments were conducted with the lowland cultivar Kanlow and the upland cultivars Blackwell, Trailblazer, and Cave-in-Rock. Explants used included mature caryopses and tissue from young seedlings and regenerated plantlets. Both the percentage of explants producing regenerants and the mean number of regenerants per explant was much greater for Kanlow than from any of the upland cultivars. For example, when tissue from young regenerated plantlets was used, nearly 60% of the explants of Kanlow produced regenerants compared to about 30% of those from Blackwell and Trailblazer. No regeneration was obtained for Cave-in-Rock. The mean number of regenerants per explant was six for Kanlow and less than two for both of the above named upland cultivars. Therefore, for unknown reasons, the regenerability of lowland cultivars was much greater than that of upland types.

- During our early experiments with node cultures, we observed the direct production of inflorescences from some of them. Subsequent studies showed that these arose from top nodes of tillers in the two to four node stage. The protocol involved splitting the nodes longitudinally and placing the cut surface in contact with MS medium containing 30 g/L maltose and 0.0 to 25.0 μM BAP. Incubation was in 16 h light (cool white fluorescent bulbs at 80 μM $m^{-2}s^{-1}$)/8 h dark at 29°C. Fully developed panicles were produced after 2- to 5-weeks culture. This was a

new and unique finding for *in vitro* culture of gramineous species. The young inflorescences were subsequently used as a source of axenic explants to initiate new cultures, which in turn produced embryogenic calluses that could be used as target tissues in gene transfer experiments. They also have potential to initiate anther cultures and for *in vitro* hybridization experiments. However, such investigations were not initiated during the entire contract period of ten years.

- The technology of most immediate and practical use and which can be used by other cooperators in the Bioenergy Feedstock Development Program, especially the breeders, is axillary shoot production from node cultures. This is an effective and efficient method for clonal propagation of switchgrass. It can be used to multiply selected and elite genotypes for breeding programs. Of major importance is that the technique appears to be genotype independent and should be applicable for upland as well as lowland cultivars. Nodes below the top nodes of tillers in the four to six node stage were split longitudinally along the sheath edge. They were plated with the cut edge in contact with MS agar medium containing 30 g/L maltose and incubated in the light/dark regime described above for inflorescence production. Shoots emerged from the nodal segments after 1-week culture. After 8 weeks, shoots were 3 to 5 cm long with 3 to 4 leaves. Roots were produced after transfer of the shoots to medium without BAP. Root development was sufficient after 4 weeks so that plantlets could be established in soil. An average greenhouse plant at the late elongation stage has approximately 15 tillers, each with 4 to 6 visible nodes. Theoretically, approximately 500 plantlets can be obtained from one parent plant in 12 weeks if nodal segments are cultured for 8 weeks at 29°C on MS medium containing 12.5 μM BAP and then transferred to MS-0 medium for an additional 4 weeks to induce rooting.

- Within one year after initiation of the project, 1000 plants (973 of Alamo and 27 of Cave-in-Rock) had been established in the field as a demonstration and source nursery. Considerable variation in color, size, vigor, maturity, etc., exists among the plants. This variability is probably due to both that which is inherent and that induced by the tissue culture process. One harvest of individual plants was made in 1994 and two were made in 1995. The summer drought of the second year adversely affected yield, especially of the second harvest. The average yield per plant was approximately 23 Mg ha^{-1}. The most productive individual plants yielded nearly twice that amount. The 20 highest yielding plants were identified multiplied in the greenhouse for establishment of three experimental synthetics consisting of 2, 4, and 20 clones.

- Genetic transformation experiments were initiated during the latter part of the first five-year project period. Early results indicated that transfer and expression of foreign genes should be possible in switchgrass. The objective was to investigate the possibility of transfer and expression of foreign DNA in switchgrass tissues. Two explant sources were used, mature embryos and *in vitro* produced florets. Plasmid pAHC25 was precipitated onto 1.1 μM tungsten particles (M17, Bio-Rad). This plasmid contains the μ*idA* (*gus*) reporter gene; coding for β-glucuronidase (GUS); and a selectable marker gene, *bar,* which provides tolerance to phosphinothricin based herbicides bialaphos and Basta™. A Particle Inflow Gun

(PIG) was used with a helium pressure of 80 PSI. A vacuum of 711-mm Hg was pulled in the chamber. Transient expression for GUS was obtained from both explants. Fifteen plants tolerant to Basta were obtained from bombardment of the *in vitro* produced florets. No molecular analyses were performed at this time. Both explant sources appeared to be suitable for genetic transformation. *In vitro* produced florets appeared an especially attractive target at that time. They had high regeneration capability through somatic embryogenesis, and since they were axenic, no additional sterilization (which may damage tissue) was needed; and unlike mature embryos, clonal fidelity could be maintained.

SECOND FIVE-YEAR PROJECT SUMMARY (JULY 1, 1997–JUNE 30, 2002)

INTRODUCTION

Research efforts to improve biomass production of switchgrass should also include the potential applications of biotechnology. Within six months after initiation of the project in 1992, we had developed protocols for plant regeneration through both organogenesis and somatic embryogenesis from tissues and cells of various explants cultured *in vitro*. Although whole plants were regenerated from several genotypes, we found that, in general, lowland cultivars, such as Alamo and Kanlow, were much more amenable to tissue culture manipulations than upland cultivars such as Cave-in-Rock and Blackwell. As pointed out in the First Five-Year Project Summary, which precedes this section, we developed several systems for regenerating plants. These included the induction of callus from several explants and regeneration by both organogenesis and somatic embryogenesis. We also developed systems for producing inflorescences directly from cultured split top nodes of tillers in the two to four node stage and axillary shoot production from split half nodes below the top node.

OBJECTIVES

Develop efficient and repeatable regeneration systems from cell and tissue cultures of switchgrass and use these systems to demonstrate stable and heritable gene transfer in this species.

SUMMARY

A ten-year summary and a summary for the first five years of the project (1992–1997) precede this section. A summary of the second five years (1997–2002) will be presented here, and this will be followed by a more detailed report of this period that includes data and other documentation of our results.

The early part of this period was spent on further development of regeneration systems. Effort was also expended toward characterization of our somatic embryogenesis systems. This involved experiments to relate specific proteins to regeneration capacity of tissue sources and genotypes. Experiments on genetic transformation became a major focus, especially during the final 2 to 3 years.

The formation of multiple shoots from young seedlings obtained from germinating caryopses was obtained in the latter part of 1997. The response was obtained from both lowland and upland cultivars and was induced by addition of thidiazuron (TDZ) to the medium. The technique was later modified so that multiple shoots were produced, without callus, directly from mature caryopses. This was accomplished by adding 1 mg/L p-chloro-phenoxyacetic acid and 3, 6, or

10 mg/L BAP to the medium. The protocol was used effectively for the cultivars Alamo, Kanlow, Blackwell, Cave-in-Rock, Trailblazer, and Shawnee. Although the method is not as effective as that from utilizing young seedlings and TDZ, it results in a time-saving step (i.e., shoots form directly from the caryopsis rather than from a shoot derived from the caryopsis).

The development of a highly regenerable (by somatic embryogeneses) suspension culture system was a goal from the beginning of our work in 1992. Although our early attempts were not successful, this was accomplished in 1997. This utilized Alamo genotype 2702 (our designation). Later experiments were directed toward obtaining regeneration from additional genotypes and optimizing the system with different pretreatments. High regeneration efficiency was obtained from six additional genotypes of Alamo. Embryogenic calluses of different ages (10, 20, or 30 days) were subjected to various osmotic pretreatments prior to culture initiation. The highest number of embryogenic calluses and highest regeneration efficiency were obtained when 10-day-old calluses were pretreated for 30 h on 0.3 M each of sorbitol and mannitol prior to initiation of the culture. More than 1100 plantlets were regenerated from 20 mL of liquid medium. As with other regeneration systems, more success was obtained with lowland than with upland types.

Experiments were conducted during the early months of 1998 to relate specific proteins to regeneration capacity of tissue sources and genotypes. Its variation is often associated with genetic differences and morphogenic changes. The esterase isoenzyme patterns of intracellular soluble proteins were examined in embryogenic and nonembryogenic calluses of ten Alamo genotypes and in plants of Trailblazer, Blackwell, and a Nebraska experimental. Esterase bands were detected in embryogenic genotypes and tissues that were not present in their nonembryogenic counterparts. However, the lack of specificity was such that it was difficult to relate genotype to regeneration capacity. The detection of differences between embryogenic and nonembryogenic calluses within a genotype appeared to have little utility for our purposes, and the work was discontinued.

Although gene transfer experiments were initiated earlier, they become a major part of our focus during the last 3 years of this period. The first experiments were conducted with microprojectile bombardment. Genes utilized were the reporter *uidA* (*gus*) in which tissues stain blue when incubated with a substrate, X-gluc, the reporter gene *gfp* (green fluorescent protein), and the selectable marker *bar* which confers tolerance to phophinothricin based selective agents. Transformation was obtained with the constructs, pAHC25 (*gus* and *bar* genes), and a new plasmid, GFP-BAR (*gfp* and *bar* genes) constructed in our laboratory. GUS was expressed in callus tissue and floral parts including pollen grains, ovaries, and lodicules. GFP was expressed in callus and in leaf tissue and pollen of T_1 plants. Plants tolerant to the herbicide Basta (*bar* gene) were obtained from both constructs.

Sexual transmission of both the *gfp* and *bar* genes and their expression in T_1 progeny was demonstrated, and their presence was confirmed by Southern blot hybridization. A Southern analysis, utilizing the restriction enzyme *Kpn*I which cuts the GFP-BAR plasmid only once, indicated copy numbers of *gfp* ranging from as few as 3–4 to numerous. Plants with the lowest copy numbers of transgenes had an approximate 1:1 ratio of GFP fluorescing: nonfluorescing

pollen grains and a higher rate of sexual transmission of transgenes than plants with high copy numbers.

Genetic transformation experiments beginning in early 2000 were also focused on use of *Agrobacterium tumefaciens*. The strain AGL1 containing the 18.15 kb transformation vector pDM805 was used to infect various explants. Success was obtained in most experiments for both *gus* and *bar* genes. Transient GUS expression was observed in tissues (calluses, caryopses, leaf, and seedling segments, etc.) incubated with the bacterium. GUS expression was also observed in pollen and ovaries of T_0 plants. Calluses selected on medium containing 10 mg^{-1} bialaphos regenerated plants that were tolerant to Basta. Transformation frequencies ranged from 14.5 to 25% indicating high efficiency. Presence of both the *bar* and *gus* genes in T_0 plants was confirmed by Southern blot hybridization. Crosses between the transgenic and control plants showed that the genes were sexually transmitted through both male and female gametes and expressed in T_1 plants. The segregation ratios for all crosses fit the expected 1:1 as determined by a χ^2 test.

The production of haploids (plants with the gametic chromosome number) was also a goal from the beginning of the project. Specifically, the creation of 2X plants from the lowland tetraploids and then redoubling of the chromosome number would have increased homozygosity. Crossing of these homozygous lines would then allow the possibility of maximizing heterozygosity. Reduction of the 8X chromosome number to 4X in upland types may facilitate crosses between the upland and 4X lowland types. Anther, and later isolated microspore, culture experiments also became a major focus of our research during the latter 2–3 years of the project. Six Alamo clones were used for anther culture experiments. From 15,720 anthers plated, hundreds of green plants were regenerated that could be established in soil. However, these were produced from only a very few anthers. Also, the period between the initial plating of anthers and receiving the first response was very long, 4–6 months. Furthermore, growth of the plantlets was slow, and the mortality rate was high. Extremely limited success was also obtained with upland cultivars. During 2001, isolated microspore liquid culture was investigated as an alternative to anther culture. Procedures were based on successful results recently obtained with rye, timothy, triticale, and wheat. The procedure involved tiller collection, microspore isolation, microspore culture, and regeneration. Although we were able to isolate and culture individual microspores, we only obtained a few divisions during culture. Further details on experiments can be found in the 2001 Annual Report that follows.

Yield trials that included Alamo; our 2-, 4-, and 20-clone synthetics, which originated from the original establishment of regenerated plants in the field; plus three experimental breeding lines from Charles Taliaferro's program at Oklahoma State University were successfully established at Knoxville and at the Highland Rim Experiment Stations at Springfield, Tennessee. Dry matter yields obtained in 2000 and 2001 indicated that the best of our synthetics are superior to Alamo and at least equal to or superior to those from Oklahoma State.

SECOND FIVE-YEAR PROJECT DETAILED REPORT (JULY 1, 1997–JUNE 30, 2002)

INTRODUCTION

An introduction to the project, including relevant background is presented above. Specific objectives for the period 1997–2002 were:

- To develop additional and improve existing regeneration systems, especially that would be genotype independent and also useful for gene transfer experiments.
  - Multiple shoot formation
  - Cell suspension cultures
- Attempt to relate specific proteins to regeneration capacity of specific tissues and genotypes.
- Produce transgenic plants, confirm integration of the transgene(s) by molecular techniques, and demonstrate sexual transmission of the transgenes through both male and female gametes.
  - Determination of bialaphos concentration in culture medium for selection of *bar* gene
  - Microprojectile bombardment
  - Inheritance of *bar* transgene from psGFP-BAR
  - *Agrobacterium*
- Produce haploid plants from anther and/or microspore cultures.
- Establish crossing blocks and yield trials from high yielding individual plants of the 1000 regenerated plant nursery set out in 1993.

The following will emphasize significant results obtained from 1997–2000. A separate detailed annual report for 2001 is in Appendix A.

RESEARCH RESULTS

Multiple Shoot Formation

Materials and Methods. Mature caryopses of Alamo, Trailblazer, and Blackwell were mechanically dehusked and presterilized in 70% ethanol for 2 min. They were then sterilized with 75% clorox and 1% Triton-X for 20 min. The surface-sterilized caryopses were washed three times with sterile water, cultured on MS medium containing 3% maltose, and supplemented with various combinations of thidiazuron, TDZ (0.0, 1.0, 2.0, and 4 mg/L), and 2,4-D (0.0, 1.0, 2.0, and 5.0 mg/L). The pH of the media was adjusted to 5.6 with 0.1 N NaOH, and the media were solidified with 8 g agar. Twenty five seeds were cultured in each petri dish (100 X 15 mm) with

ten dishes of each combination for each cultivar. The cultures were incubated at 29°C in darkness for 3 weeks and then transferred to light (80 μm mol m^{-2} s^{-1} ; 16 h photoperiod) provided by cool white fluorescent bulbs. The number of seedlings showing multiple regeneration and the number of regenerated shoots per responding seedling were counted after 4-weeks culture.

For complete plant development, multiple shoot clumps were excised into pieces (5–6 mm in diameter) and cultured on growth regulator free MS medium containing 3% maltose for an additional 4 weeks. Rooted plants were established in pots and grown in a greenhouse.

To illustrate development by scanning electron microscopy (SEM), shoot apices of Alamo at various stages of growth were fixed in 2.5% glutaraldehyde buffered with 0.067 M phosphate buffer, pH 7.0 at 4 C for 24 h, dehydrated in a graded acetone series, critical point dried, and coated with gold.

For histological analysis, 10- to 21-day old apical portions were excised from seedlings. They were then fixed in formalin:glacial acetic acid:ethanol (5:5:90 v/v) for 24 h, dehydrated through a graded series of ethanol and t-butyl alcohol and embedded in paraffin. Microtome sections (10 μm thickness) were stained with hematoxylin-alcian blue.

Results. Within 7 d culture, mature caryopses developed into typical seedlings with elongated mesocotyls, coleoptiles, and roots. Shoot apices were confined to the coleoptile-mesocotyl juncture and could easily be recognized by a localized enlargement. After 2 to 3 weeks, the mesocotyl and root portions produced callus, the growth of primary leaves was retarded, and shoot apices started to multiply. Initially, shoot apical meristems were enlarged and resulted in the formation of an early multiple shoot cluster and also callus on the mesocotyl (Fig. 1A). Shoot differentiation was observed after another week. Regenerated shoots multiplied as tight clusters (Fig. 1B).

Addition of different combinations of 2,4-D and TDZ induced germination of caryopses, followed by multiple shoot formation from the apical region and the development of a variable amount of callus at the mesocotyl portion. The ratio of 2,4-D:TDZ clearly affected shoot regeneration frequency and the number of shoots per responding explant (Table 1). Increasing 2,4-D concentration decreased multiple shoot formation. However, increasing TDZ concentration increased shoot formation at almost all 2,4-D levels. The highest frequency of regeneration and mean number of shoots per responding explant was obtained with 4.5 μM of 2,4-D in combination with 18.2 μM TDZ. Higher 2,4-D concentrations increased callus formation and suppressed mesocotyl elongation. Neither 2,4-D and TDZ alone induced multiple shoots; both were required to elicit the response. Caryopses of all cultivars responded in a similar manner but at a variable frequency (Table 1). The best response for number of shoot producing seedlings as well as the number of regenerated shoots per responding seedling was obtained with Alamo. Trailblazer responded the best of the two upland cultivars. The regeneration frequency of Blackwell was about one-half that of Alamo and about two-thirds that of Trailblazer. The pattern of development in the upland cultivars was also similar to that observed in Alamo.

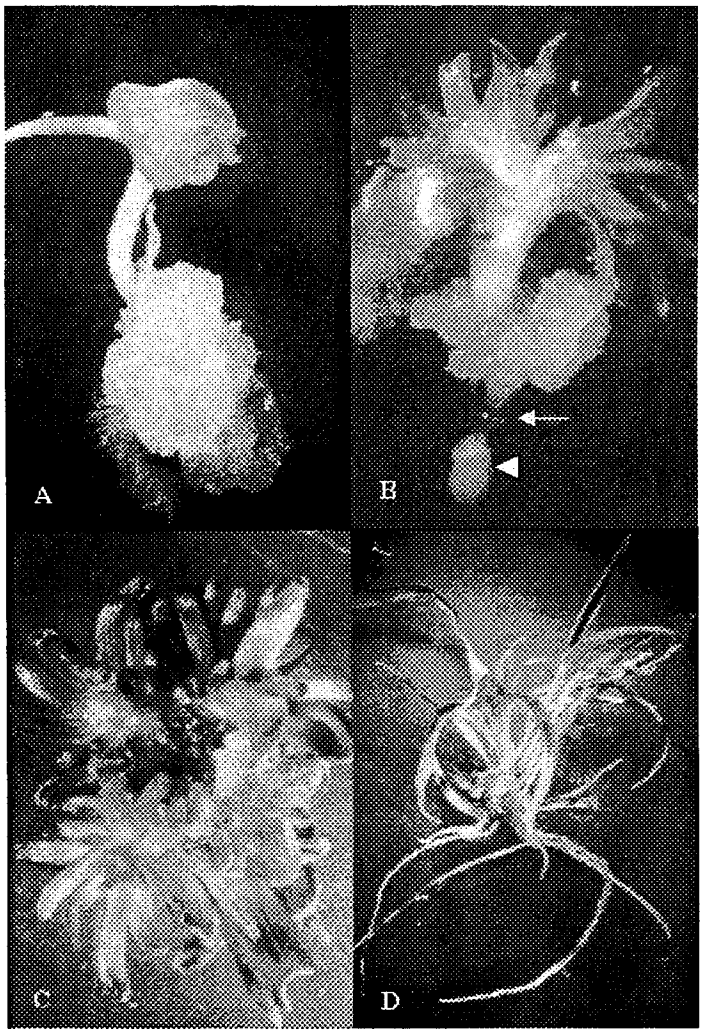

Fig. 1. Direct differentation of multiple shoots from mature caryopses of Alamo. Caryopses were cultured on MS medium supplemented with 18.2-mM TDZ and 4.5-mM 2,4-D. (A) Structure containing multiple early shoot clumps after 2-week culture. Note callusing of the mesocotyl portion X 8. (B) Proliferation of multiple shoots as tight clusters from an intact seedling after 4-week culture. Note the presence of the caryopsis (àrrow) and callusing of the root (arrowhead) X 8. (C) Multiplication of shoot clusters on growth regulator free MS medium X 8. (D) A clump of rooted plantlets prior to transfer to greenhouse X 1.5.

Table 1. The effects of different combinations of 2,4-D and TDZ on multiple shoot differentiation in three different cultivars of switchgrass (values are means of ten replicates), data for 0.0 μM, 2,4-D, and/or TDZ are not included because none were obtained.

Growth regulator		Alamo		Trailblazer		Blackwell	
2,4-D μM	TDZ μM	% shoot regeneration	Mean no. of shoots per responding explant (± SE)	% shoot regeneration	Mean no. of shoots per responding explant (± SE)	% shoot regeneration	Mean no. of shoots per responding explant (± SE)
4.5	4.5	0.0^{e}	0.0^{e}	0.0^{d}	0.0^{d}	0.0^{d}	0.0^{d}
4.5	9.1	36.8^{b}	33.1 ± 2.2^{b}	30.4^{a,b}	26.1 ± 2.0^{b}	20.8^{a}	20.3 ± 1.4^{b}
4.5	18.2	52.0^{a}	39.6 ± 2.8^{a}	32.8^{a}	29.3 ± 2.5^{a}	23.2^{a}	24.4 ± 1.9^{a}
9.0	4.5	13.2^{c}	10.1 ± 0.8^{d}	8.8^{c}	8.5 ± 0.8^{c}	5.2^{c}	6.0 ± 0.4^{c}
9.0	9.1	14.8^{c}	9.9 ± 0.8^{d}	8.0^{c}	7.8 ± 0.6^{c}	5.6^{c}	7.5 ± 0.6^{c}
9.0	18.2	34.4^{b}	29.2 ± 1.9^{c}	25.2^{b}	25.9 ± 1.8^{b}	14.8^{b}	21.8 ± 1.4^{b}
22.6	4.5	4.4^{d,e}	7.9 ± 0.6^{d}	5.6^{c}	7.0 ± 0.4^{c}	0.0^{d}	0.0^{d}
22.6	9.1	6.0^{d}	7.5 ± 0.5^{d}	7.2^{c}	8.6 ± 0.5^{c}	0.0^{d}	0.0^{d}
22.6	18.2	0.0^{e}	0.0^{e}	0.0^{d}	0.0^{d}	0.0^{d}	0.0^{d}

Values followed by the same letter are not significantly different at P = 0.05 using Fisher's LSD.

For rooting of shoots, multiple shoot clumps were divided into 3 to 5 units and subcultured on growth regulator free MS medium with 3% maltose. Further multiplication of shoot clumps also occurred on this medium (Fig. 1C), and after 3 to 4 weeks, 15 to 30 plants with roots were obtained from each clump. Clumps of rooted plantlets were divided into smaller units of 10 to 15 shoots before transfer to the greenhouse (Fig. 1D). The rooted plants were successfully grown in pots.

Early ontogenetic events during the formation of multiple shoot clumps were studied by both light and scanning electron microscopy. After 10 d culture, suppression of primary meristem growth and inhibition of internode elongation followed by proliferation of axillary buds were apparent. These events induced shoot meristems that developed in a nested array at the axil of primary leaves (Fig. 2A). Further differentiation of shoot meristems was observed after another 2 to 3 days and leafy structures with trichomes emerged (Fig. 2B). Each of the differentiated shoot meristems subsequently gave rise to multiple shoot clumps. Enlargement of shoot apices and development of leaf buttresses were evident at 15-d culture. Shoot clumps in different developmental stages are shown in Fig. 2C indicating that the development of these clumps is asynchronous. After 21 days, clusters of multiple shoot clumps could be seen at the axil of primary leaves (Fig. 2D).

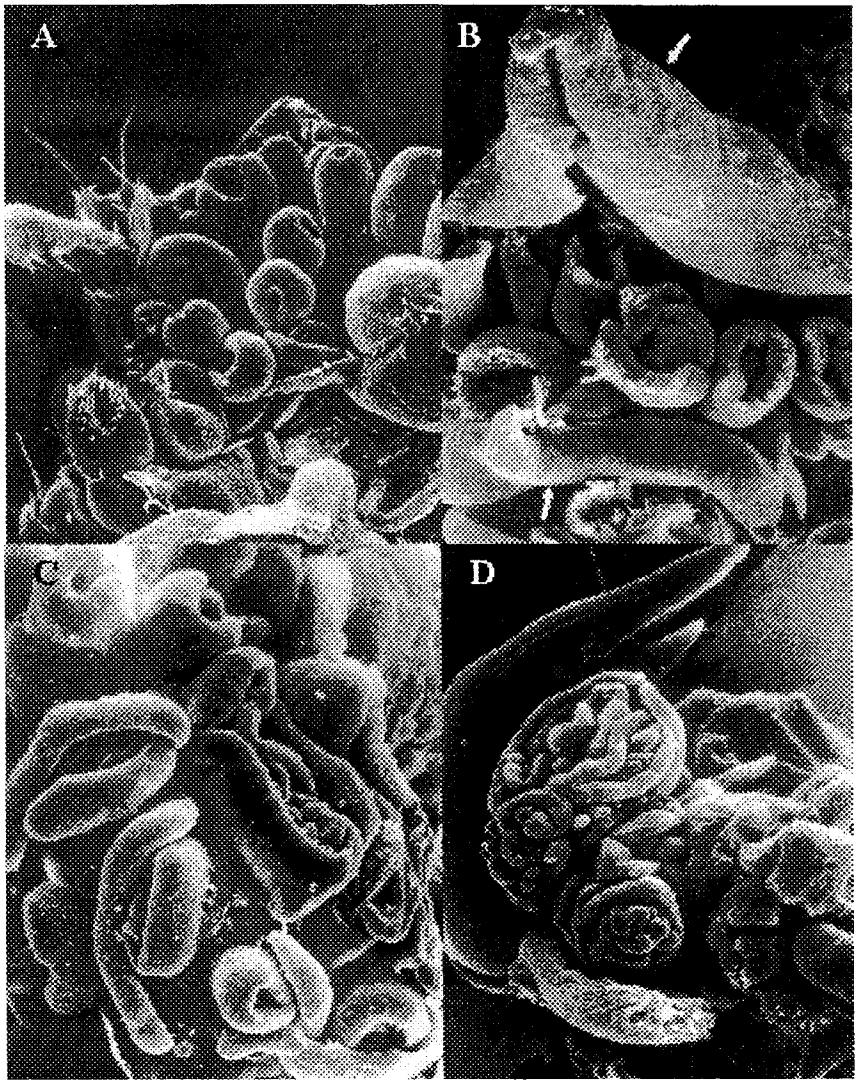

Fig. 2. SEM ontogeny of multiple shoot formation. (A) Emergence of multiple shoot meristems from a shoot apex after 10-d culture X 52. (B) Differentiation of shoot meristem into leafy structures with trichomes. Note their position at the axil of primary leaves (primary leaves are indicated by arrows) X 69. (C) A section of the early multiple shoots shown in Fig. 1A. Note the asynchronous development X 60. (D) Clusters of multiple shoot clumps at the axil of the primary leaves after 3-weeks culture X 37.

Light microscopy also supported the observed mode of multiple shoot development. A coleoptile enclosing a shoot apical meristem, a distinct internode portion, and several leaf primordia were evident in a 5-d old germinated seedling (Fig. 3a). The next developmental process included rapid activation of axillary buds with little or no internode elongation and widening of the apex (Fig. 3b). These were the first morphological changes in the shoot apex during multiple shoot formation and occurred within 10-d culture. Differentiation of leaf primordia as well as reprogramming of shoot meristems into several meristematic foci occurred at about 15 d (Fig. 3c). The development of supernumerary buds at the apical dome is shown at a higher magnification in Fig. 3d. Further differentiation and proliferation of these buds produced the multiple shoot clumps (Fig. 3e).

The multiple shoot formation technique is a new approach for inducing efficient shoot formation. It is less genotype dependent than some of our previously described techniques. Therefore, it appears applicable to both lowland and upland cultivars. The protocol utilizes direct differentiation of multiple shoots from intact seedlings. Therefore, it eliminates the labor involved in meristem isolation and culture reported in similar studies with other species (e.g., maize and oat). It was anticipated that the system might be useful in our gene transfer experiments; however, we initiated our transformation experiments with other target tissues and did not yet utilize the multiple shoot system for that purpose.

Suspension Cultures

A primary objective since initiation of our project in 1992 was to develop a regenerable (through somatic embryogenesis) cell suspension culture. The identification of a highly embryogenic genotype of Alamo (designated 2702) allowed us to accomplish this goal in 1997.

Materials and Methods. Cell suspension cultures were initiated from 3-month-old embryogenic calluses formed from *in vitro* developed inflorescences of Alamo genotype 2702. Approximately 1 g fresh weight of callus was transferred to 125-mL Erlenmeyer flasks containing 30 mL MS medium. The medium was supplemented with various concentrations of 2,4-D (2.2, 4.5, 9.0, or 18.0 μM), BAP (2.2, 4.4, or 8.8 μM), and 30 g L^{-1} maltose. The pH was adjusted to 5.6 before autoclaving at 120°C (106 kPa) for 20 min. Flasks were incubated on a gyratory shaker at 120 rpm and maintained at a temperature of 29°C in the dark. After 10 days, the suspensions were allowed to settle and 10 mL of the supernatant were removed and replaced by an equal volume of fresh medium. This procedure was repeated for 6 to 8 weeks during which time the calluses began to dissociate into single cells and small cell clumps. The suspension cells were then filtered through polypropylene meshes of pore sizes 710 and 210 μm to separate the cell clumps. Once established, the suspensions were maintained routinely by transferring 5 mL of the culture to a 125-mL flask containing 15 mL of fresh medium at 2-week intervals.

Fifty-six-day old suspensions were used for fresh weight (FW), dry weight (DW), and packed cell volume (PCV) analyses. Growth was monitored by transferring 5 mL of the culture to a 125 mL flask containing 15 mL of fresh medium at 3-d intervals from the day of subculture (day 0) up to 15 days. Measurements were taken from five flasks. For FW, the contents of a flask were filtered through a preweighed Whatman No.1 filter using a Buchner funnel. DW was determined by

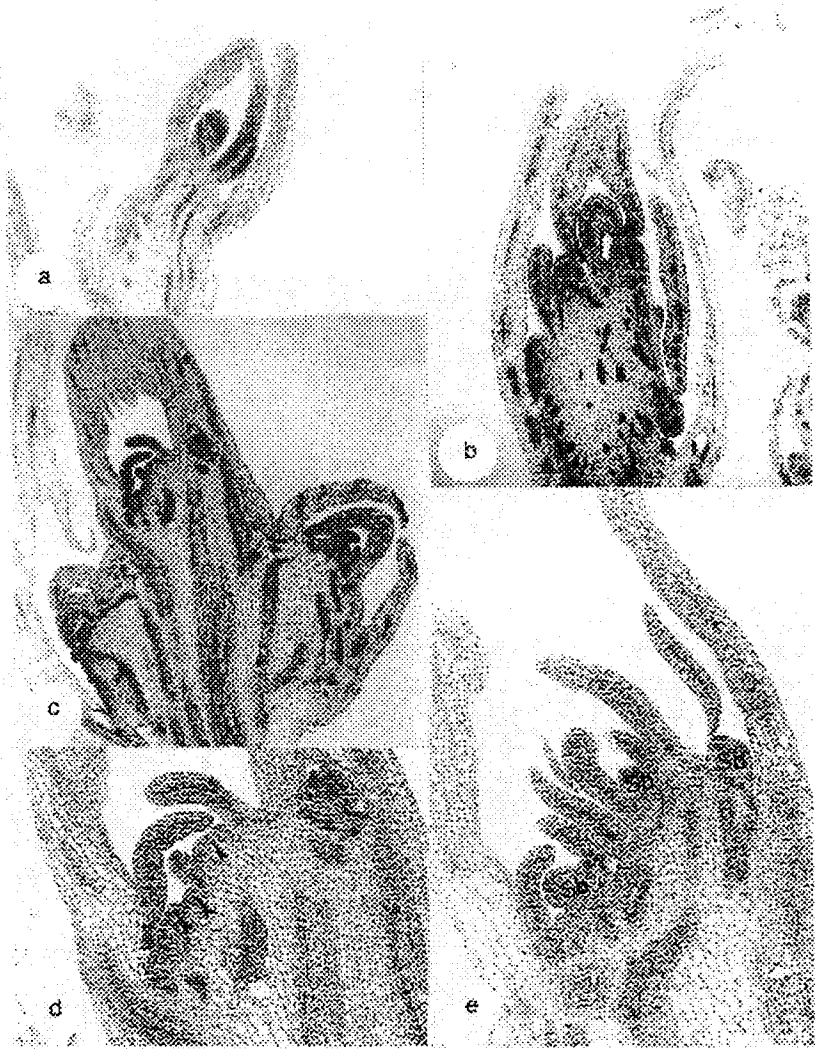

Fig. 3. Histological longitudinal sections of the axil showing differentiation of multiple shoot clumps. (a) Histology of the primary apical meristem after 5 d culture initiation X 12. (b) Shoot meristems after 10-d culture. Note the inhibition of internode elongation X 15. (c) Differentiation of shoot apices and leaf primordia corresponding to Fig. 2b X 15. (d) A higher magnification view of Fig. 3c showing the differentiation of meristematic foci (arrowheads) X 30. (e) A multiple shoot clump with differentiated supernumerary buds (Sb) X 30.

drying the cells at 70°C until a constant reading was obtained. For determination of PCV, 10 mL of the suspensions were centrifuged at 1000 *g* for 5 min and PCV was calculated as a percentage of the pellet volume compared to the total volume.

Different approaches were utilized for plant regeneration. Somatic embryos and cell clumps were collected on 210 μm and 500 μm meshes after sieving the suspension and plated on MS medium without growth regulators. One mL of the remaining suspension containing single cells and small cell clumps (2–8 cells) was pipeted onto MS solid medium supplemented with 3% maltose and various combinations of 2.4-D (2.2–18.0 μM) and BAP (4.4 or 8.8 μM). Cultures on petri dishes containing somatic embryos and cell clumps were incubated for 4 weeks at 29°C in the light (80 μmol $^{-2}s^{-1}$; 16-h photoperiod) provided by cool white fluorescent bulbs. The plated cells were incubated in the dark for induction of embryogenic callus. After 4 weeks, they were transferred to light conditions as above and incubated an additional 4 weeks for plant regeneration.

For light and scanning electron microscopy, collected embryos were fixed in 2.5% glutaraldehyde buffered to pH 6.8 with 0.1M phosphate buffer, at 4°C for 24 h under vacuum. The samples were dehydrated in a graded acetone series. Specimens for light microscopy were embedded in paraffin sectioned at 8-μm thickness and stained with hematoxylin and alcian blue. For SEM, the specimens were dehydrated with a graded acetone series, critical point dried, and sputtered with gold palladium. The samples were examined with a ETEC Autoscan SEM operated at 20 kv.

Results. Within 6 to 8 weeks in S_2 medium (MS+ 9.0 μM 2,4-D and 4.4 μM BAP), the embryogenic calli dissociated into smaller fragments resulting in the establishment of fine suspensions. The appearance of suspension cultures after filtration through a 210-μm mesh is shown in Fig. 4a. The suspensions were comprised mainly of small, round, densely cytoplasmic starch containing cells with distinct nuclei. Some large, elongated, and highly vacuolated cells with sparse cytoplasm could also be seen. Regular filtration was required to maintain these as fine suspensions as the cultures tended to form cell clusters of a few cells to aggregates larger than 1 mm. With an elevated level of 2,4-D (18.0 μM), the suspensions became thick and mucilaginous. Higher concentrations of BAP (8.8 μM) did not promote dissociation of the embryogenic callus. Growth characteristics of the suspensions indicated that there was a lag phase during the first 3 days after subculturing after which the growth was more exponential (Table 2).

Various stages of embryogenesis in suspension cultures were observed. Fig. 4b shows a densely cytoplasmic dividing cell (two-cell stage). Further divisions resulted in the formation of proembryos with a suspensor-like structure (Fig. 4c), and somatic embryos with unicellular (Fig. 4d), multicellular (Fig. 4e), and without any suspensor (Fig. 4f) could be seen. These embryos were free-floating in the medium and not attached to cell masses. A somatic embryo at a later stage of development exhibiting formation of the coleoptile notch is shown in (Fig. 4g). The notch represents the site of the shoot apex and coleoptile. Secondary somatic embryogenesis or polyembryony was also common (Fig. 4h). Secondary embryos may have their origin from the proliferation of cells of developed somatic embryos. In these experiments, we did not observe simultaneous development of several embryos from embryogenic cell masses.

Table 2. Grown characteristics of Alamo 2702 suspension cultures as measured by packed cell volume (PCV), fresh weight (FW), and dry weight (DW).

Standard errors are shown after ±.

Criterion Measured	Days in Culture					
	0	3	6	9	12	15
PCV, %	1.9 ± 0.04	2.5 ± 0.07	4.9 ± 0.05	8.3 ± 0.16	12.6 ± 0.36	14.8 ± 0.42
FW, mg	158.4 ± 3.6	178.5 ± 4.4	250.0 ± 8.4	495.8 ± 8.4	1066.0 ± 32.0	1364.0 ± 53.0
DW, mg	11.0 ± 0.62	13.3 ± 0.40	21.7 ± 0.80	47.9 ± 1.4	80.6 ± 1.4	107.0 ± 4.6

Well-developed embryos were collected on a 210-μm mesh screen from cultures which had previously been passed through a 710-μm mesh screen (Fig. 5A). Scanning electron microscopy showed that the suspension-derived embryos have the morphology of a typical grass embryo including a developed scutellar region, protruding coleoptile, and root apex (Fig. 5B). A longitudinal section through an embryo also showed a well-developed scutellum composed of large, starch containing cells (Fig 5C). A distinct coleoptile and first leaf were also present. The embryonal axis possessed both shoot and root apices and consisted of small densely cytoplasmic cells. Thus, switchgrass cell suspension cultures are capable of producing fully developed somatic embryos without plating onto solid medium. Dedifferentiation of cells in the scutellar region (Fig. 5D) resulted in the formation of clumps and may also account for the increase in culture mass by sloughing cells. This suggests that a cyclic pattern of somatic embryogenesis may operate in suspension cultures of switchgrass.

Somatic embryos and cell clumps (dedifferentiated embryos) germinated to form plantlets with shoots and roots when transferred to MS agar solid medium without growth regulators. Suspension cultures which were passed through a 210-μm mesh screen upon plating onto various combinations of 2,4-D and BAP formed a lawn of embryogenic callus which included various developmental stages of somatic embryos. Somatic embryos formed in these calli, on growth regulator free MS medium, germinated to form complete plantlets that were successfully established in soil in the greenhouse.

Genotype, Osmotic Pretreatment, and Inoculum Age

Alamo genotypes 3125, 3921, and 2118 (our designations) were used in these experiments. Embryogenic suspension cultures of genotypes 3125 and 2118 were established according to former protocols. Both callus initiation and the suspension initiation media were the same: S1 medium (MS + 9 μM 2,4-D + 5 μM BAP + 3% maltose, pH = 5.6 to 5.8).

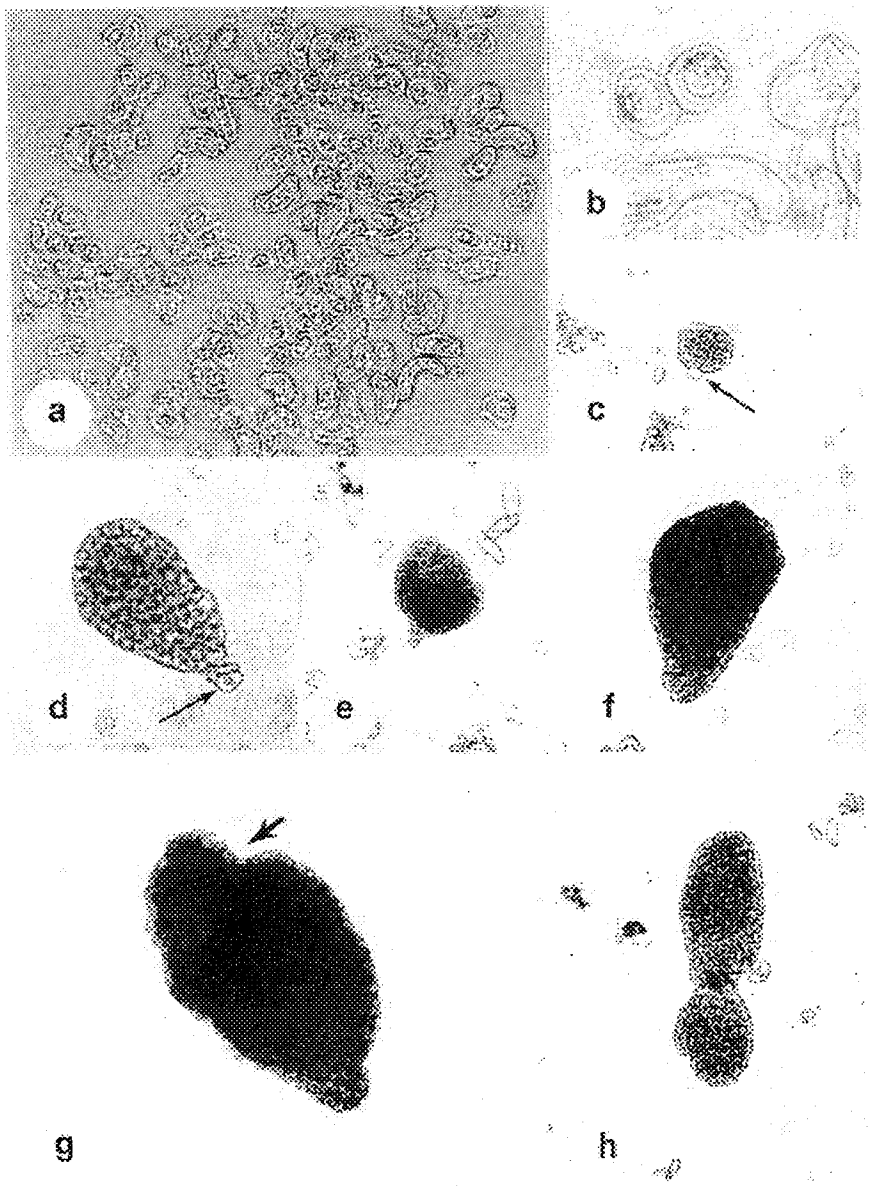

Fig. 4. A series of events of somatic embryogenesis observed in cell suspension cultures of switchgrass. (a) Single cells from suspensions. (b) Dividing cell (two-cell stage). (c) Proembryo with suspensor like structure (arrow). (d) Globular embryo with unicellular suspensor (arrow). (e) Embryo with multicellular suspensor. (f) Mature embryo without any suspensor. (g) Embryo in a later stage of development with scutellar notch (arrow). (h) Polyembryony.

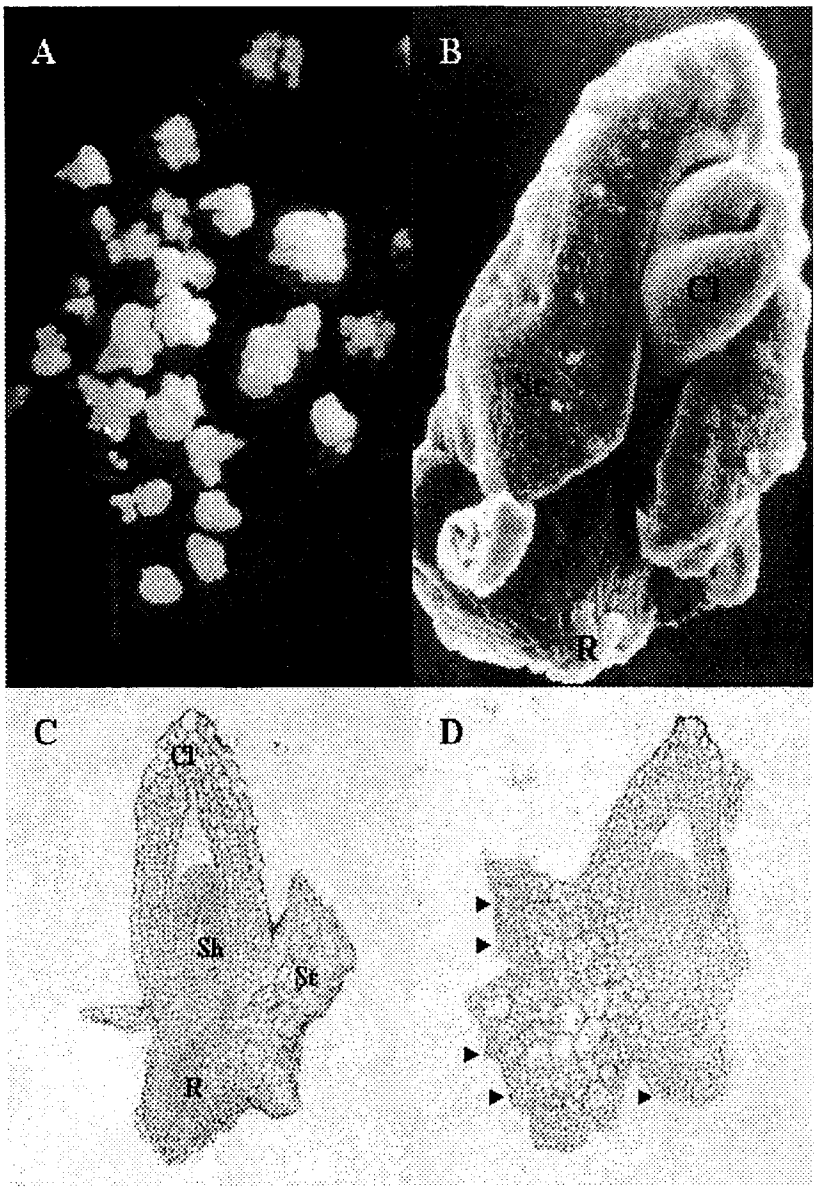

Fig. 5. Scanning and light micrographs of somatic embryos recovered from a suspension culture on a 210-mm mesh screen. (a) Embryos at various stages of development. (b) Scanning electron micrograph of a fully developed embryo; Cl, coleoptile; Sc, scutellum; R, root apex. (c) Median longitudinal section through a fully developed embryo; Cl, coleoptile; Sc, scutellum; Sh, shoot apx; R, root apex. (d) Longitudinal section through an embryo beginning de-differentiation from the scutellar region (arrowhead).

New protocols were established for suspension cultures of genotype 3921. Embryogenic calluses were produced from inflorescence cultures. The calluses were then transferred to a sterile petri dish and broken up gently with forceps. The small pieces were transferred to a sterile flask that contained 20 mL of MS liquid medium supplemented with 22 μM 2,4-D, 5 μM BAP, and 3% maltose (C1 medium). The pH was adjusted to 5.6 to 5.8. Culture was in the dark at 29°C, and the flasks were rotated at 120 rpm on a gyratory shaker. After 2 days, the suspensions were allowed to settle and approximately 15 mL of the liquid was removed by pipette and discarded. Fifteen mL of fresh medium were then added to the cultures at 1-week intervals for 4 to 6 weeks until the embryogenic cultures began to dissociate into single cells and small cell clumps. The suspensions were then filtered through a nylon mesh of 710 μm to separate the cell clumps. Once established, the suspensions were maintained routinely by transferring 5 mL of the culture to a 125-mL flask containing 15 mL of fresh medium at 2-week intervals.

Compared to embryogenic suspension cultures of 2702, 3125, and 3921, the suspension of 2118 was a mixture of embryogenic and nonembryogenic cell masses. This genotype did not perform as well *in vitro* culture, either in liquid or on solid medium, as the other two genotypes.

In vitro produced inflorescences from Alamo genotype 2702 were used to initiate *in vitro* cultures. Individual spikelets were plated on MS solid medium (0.8% agar) supplemented with 3% maltose, 5 μM BAP, and 9 μM 2,4-D. This medium is hereafter referred to as MS1. Incubation was at 29°C in the dark for 30 days. Resultant calluses were subcultured onto fresh medium for an additional 10, 20, or 30 days. These will be referred to as 10-, 20-, and 30-d inocula.

Compact white calluses (10-, 20-, and 30-d inocula) were selected, transferred to MS1 solid medium containing 0, 0.1, 0.2, or 0.3 M each of sorbitol and mannitol and cultured for 30 h. For initiation of cell suspensions, small clumps (~ 1 to 2 mm dia) were transferred (one per well) to sterile 24-well Multiwell™ Tissue Culture Plates. Each well contained 2 mL of MS1 liquid medium. The pH was adjusted to 5.7 with 0.1 N NaOH. Plates were incubated on a gyratory shaker at 120 rpm at 29°C in the dark. After 2 weeks, the suspensions were allowed to settle, and 1 mL of the supernatant was removed and replaced by an equal volume of fresh medium. After an additional 2 weeks, data were recorded for number of callus clusters per explant and percentage of embryogenic calluses. The individual 2 mL suspensions were then transferred to 125-mL Erlenmeyer flasks containing 20 mL of MS1 medium. The flasks were rotated (120 rpm) at 29°C in the dark. After 2 weeks, the suspensions were filtered through polypropylene meshes of pore sizes 710 and 510 μm. Cell clumps collected on 710 μm were plated on MS solid medium without growth regulators (MS0) and incubated for 4 weeks at 29°C in the light (80 μmol $m^{-2}s^{-1}$, 16-h photoperiod) provided by cool white fluorescent bulbs for plant regeneration.

Tissues collected on the 510-μm meshes were returned to 20-mL fresh liquid medium in 125-mL flasks. This procedure was repeated at 2-week intervals, and the cultures remained embryogenic from 5 months.

Within 4 weeks in MS1 liquid medium, single callus clumps used as explants dissociated into fragments. With 0.1 to 0.3 M each of sorbitol and mannitol, dissociation of calluses decreased, but the frequency of formation of embryogenic white opaque calluses increased. Inoculum age was of

prime importance for producing an embryogenic response. Suspensions initiated with 30-d-old inocula produced mainly nonembryogenic clusters. In contrast, 10-d-old inocula produced cultures with high embryogenic potential especially when 0.3 M each of sorbitol and mannitol was included in the medium. Figs. 6 and 7 show the effect of osmotic pretreatment on callus production and on the embryogenic capacity of suspension cultures initiated from 10, 20, and 30-d-old inocula.

Various stages of embryogenesis in 2-week old suspension cultures were observed. Histological observation also indicated somatic embryogenesis (not shown). Polyembryony and simultaneous development of several embryos from embryogenic cell masses were common. Pretreatment with sorbitol and mannitol resulted in an increase in the recovery of regenerable embryogenic suspensions. Plants were regenerated by somatic embryogenesis from callus clusters collected on a 710-μm mesh screen. The duration of callus growth on MS1 medium (inoculum age) used to

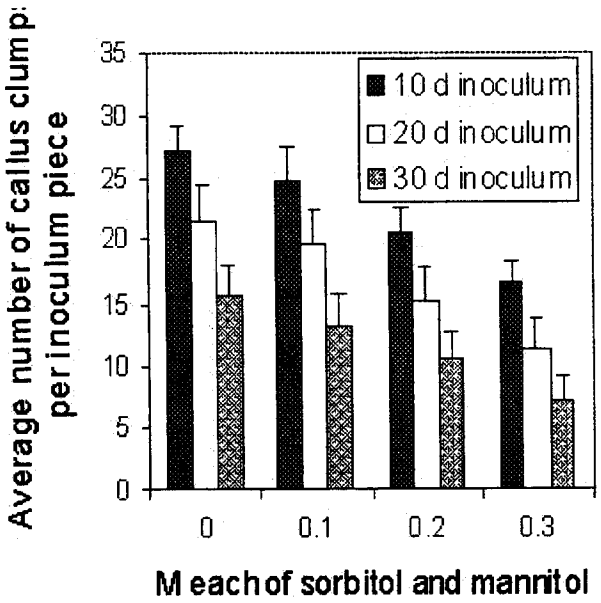

Fig. 6. Effect of osmotic pretreatment (equal concentrations each of sorbital and mannitol) on callus production in suspension cultures initiated from 10-, 20-, and 30-d inocula. For the osmotic treatments, calluses were transferred after the 10-, 20-, and 30-d inoculum period to solid medium containing the osmoticum and cultured for 30 h prior to initiation of the suspensions. The number of callus clusters per 2-mL culture well was counted after 4 weeks. Each treatment consisted of 48 replications.

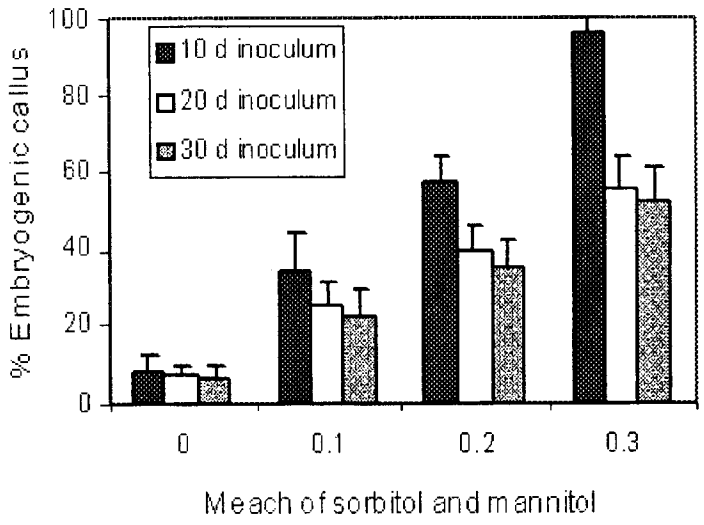

Fig. 7. Effect of osmotic pretreatment (equal concentrations of sorbitol and mannitol) on the embryogenic capacity of suspension cultures initiated from calluses grown for 10, 20, and 30 days on solid medium. For the osmotic treatments, calluses were transferred after the 10-, 20-, and 30-d inoculum period to solid medium containing the osmoticum and cultured for 30 h prior to initiation of the suspensions. The percentage of embryogenic calluses was calculated after 4 weeks as the number of embryogenic callus clusters/total number of callus clumps X 100. Each treatment consisted of 48 replications.

initiate the suspensions affected regeneration efficiency. Best results were obtained when 10-d-old inocula were used to initiate the suspensions. Less satisfactory results were obtained with 20- and 30-d-old inocula.

The results demonstrate the importance of induction conditions for successful initiation of switchgrass suspension cultures. The same protocol was also used successfully for the induction of suspensions from Alamo 2118 and 3125 genotypes. The regeneration efficiency of suspensions from different Alamo genotypes was established as a total number of plants growing on MS0 solid medium without growth regulators after 60-d culture (Fig. 8).

The results show that modification of protocols, viz, osmotic pretreatment, and inoculum age can dramatically improve the initiation, growth, and regeneration capacity of Alamo suspension cultures. Similar modifications would also be expected to improve regenerability from suspensions of other lowland cultivars. However, preliminary results with the Nebraska experimental cultivar indicate that other modifications may be needed to improve culture and regeneration from suspensions of upland types.

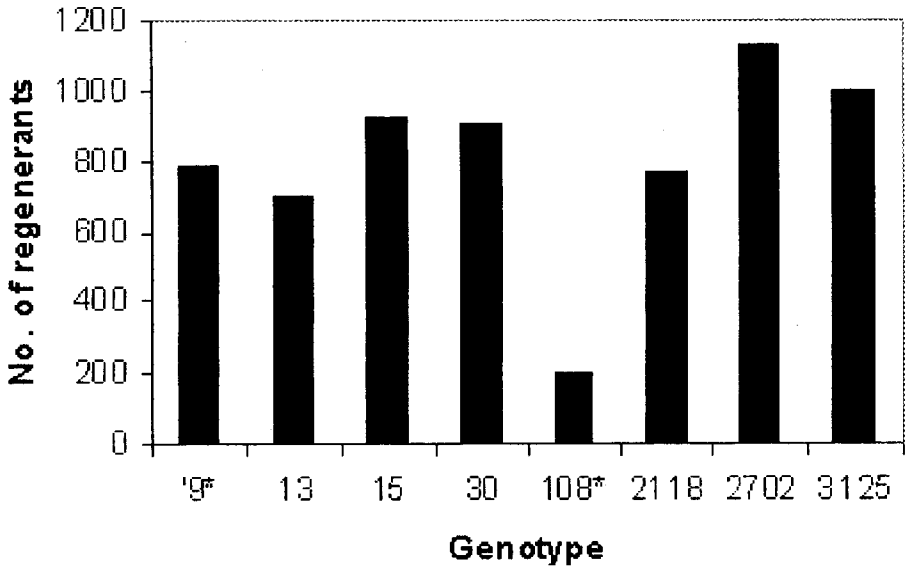

Fig. 8. Regeneration efficiency of suspensions obtained from different Alamo genotypes. Suspensions were initiated from 10-d inocula. For the osmotic pretreatment, calluses were transferred to MS1 medium containing 0.3 M each sorbitol and mannitol and were cultured 30 h prior to initiation of the suspensions. Genotypes 9* and 108* represent results obtained from pretreatment with 0.1 M each sorbitol and mannitol. This osmotic treatment gave a better regeneration response for these genotypes than 0.2 or 0.3 M each sorbitol and mannitol. For plant regeneration, 100 callus clumps collected on a 710-mm mesh screen were randomly selected and plated on MS0 medium. The data show the total number of regenerants after 60 d incubation in the light.

Relation of Specific Proteins to Regeneration Capacity and Regenerable Genotypes

We were interested in characterizing biochemical and molecular events linked to somatic embryogenesis with the objective of identifying markers related to embryogenic potential. These markers could aid in identifying genotypes, explants, culture conditions, etc., related to embryogenesis and may also provide information on the mechanisms underlying the process. Our focus was on studying the expression of peroxidase and esterase isoenzymes from embryogenic and nonembryogenic suspension cultures.

Preliminary results indicated that embryogenic calli were associated with the presence of specific intracellular isoesterases and the lack of extracellular esterase activity. They also suggested the possibility that esterase isozymes may be useful biochemical markers for somatic embryogenesis.

Experiments were continued during the early part of 1998. The esterase isoenzyme patterns of intracellular soluble proteins were examined in embryogenic and nonembryogenic calluses of ten

Alamo genotypes and in plants of Trailblazer, Blackwell, and a Nebraska experimental line. Some esterase isoenzymes were present in all embryogenic cultures. On the other hand, embryogenic calluses from different genotypes showed a specific distribution and appearance of various isoenzymes bands depending on genotype. Although each genotype showed a specific isoenzyme pattern, it was difficult to relate genotype to regeneration capacity. The detection of differences between embryogenic and nonembryogenic calluses within a genotype appeared to have little utility for our purposes. Therefore, this work was discontinued.

Genetic Transformation

Gene transfer experiments were first initiated in 1995 but were temporarily discontinued because of a change of personnel working on the project. The experiments were reinitiated in 1997 with a Ph.D. student. The student later withdrew in 1999. Significant progress was made in this area from 1999 through 2001 with two Visiting International Research Scholars. The initial experiments were with microprojectile bombardment utilizing a homemade particle inflow gun (PIG). The constructs used were pAHC25 and GFP-BAR. The former possesses the reporter gene β-glucuronidase (*uid*A or *gus*) and the selectable marker *bar* that confers tolerance to phosophinothricin based selective agents, such as bialaphos and the herbicide Basta. Phosphinothricin inhibits glutamine synthase causing a rapid accumulation of ammonia that leads to plant cell death. The *bar* gene codes for phosphinothricin acetyl-transferase (PAT). The resulting acetylated phosphinothricin is no longer inhibitory to glutamine synthase. GFP-BAR was constructed in our laboratory and possesses the reporter gene green fluorescent protein (*gfp)* and *bar*. GFP has advantage over GUS as a reporter because the assay to detect its presence or absence is nondestructive. Transformation experiments with *Agrobacterium tumefaciens* were conducted during the last 2 years of the project. The construct used was pDM805 with the *gus* and *bar* genes. This construct had been previously used successfully with barley by the Australian workers at CSIRO, and we obtained it from that institute.

Determination of Bialaphos Concentration in Selection Medium. Both of the plasmids (pAHC25 and psGFP-BAR) in our microprojectile experiments possess the *bar* gene as mentioned above. Experiments were conducted to determine concentrations of bialaphos in the medium that would provide adequate selection of tolerant calluses and other tissues in petri dishes and other culture vessels. Two-month old calluses from *in vitro* produced inflorescences of Alamo genotype 2702 were cultured on MS basal medium supplemented with 2 mg/L 2,4-D, 1 mg/L BAP, and various concentrations of bialaphos ranging from 2 to 12 mg/L. The medium was solidified with 8% agar and maltose at 30 g/L served as the carbohydrate source. Incubation was at 29°C in the dark. The tissues were subcultured after 14 d. After 30 d culture, calluses on medium containing 2 to 6 mg/L bialaphos became light brown in color but continued to grow slowly indicating that they were still alive. Those on 10 or 12 mg/L became necrotic and turned dark brown. Based on these results, 10 mg/L was chosen for the transformation experiments. In a second experiment with embryogenic calluses, the tissues were exposed to 5 or 10 mg/L of the selective agent. Tissue browning was observed within 10 d culture on medium with 5 mg/L. The effect was more pronounced with 10 mg/L, and the tissues turned brown within 7 d of culture.

Microprojectile Bombardment. As mentioned, plasmid pAHC25 possesses the reporter gene *uid*A encoding for β-glucuronidase (GUS), and psGFP-BAR contains the reporter gene, *gfp*, which codes for the green fluorescent protein (GFP). Transformed cells and tissues expressing GUS stain blue when incubated with the substrate 5-bromo-4-chloro-3-indoyl-D-glucuronic acid (X-Gluc). Cells and tissues expressing GFP fluoresce bright green under blue light.

Plasmids mentioned above were precipitated onto 1.1 μm tungsten particles. Target tissues were precultured either on 0.3 M each of sorbitol and mannitol or 150 g/L sucrose for 4 h before bombardment. A particle inflow gun (PIG) was used for delivery. Tissues, either calluses or multiple shoot clumps, were placed in the center of petri dishes. These were placed 16.5 cm from the screen in the syringe filter. A baffle (200 μm nylon screen) was positioned at a distance of 10 cm above the tissue for proper deflection of the particles. A vacuum pressure of 711 mm Hg was drawn in the chamber, and a helium pressure of 80 PSI was used to propel the particles.

Bombarded shoot clumps of Alamo and Kanlow were transferred to MS0 medium supplemented with 5 mg/L or 10 mg/L of bialaphos and subcultured every 2 weeks. Three selection procedures were employed: continuous selection of 5 mg/L bialaphos for 8 weeks; continuous selection on 10 mg/L bialaphos for 8 weeks; and selection on 5 mg/L bialaphos for 4 weeks followed by 10 mg/L for 4 weeks.

After 56 days, 58 plants derived from shoot clumps continued to grow and produce roots on the selection medium (Table 3). All of these plants were transferred to soil and established in the greenhouse. Leaves of the plants were brushed with 0.1% Basta, and all were found to be sensitive to the herbicide. Therefore, shoot clumps, as subjected to the protocols of these experiments, were not satisfactory target tissues for transformation.

Embryogenic callus from immature inflorescences was induced as previously described. Calluses with transient expression of GUS are shown in Fig. 9A. In most experiments the two-step selection procedure was used. The first was on C1 medium supplemented with 5 mg/L bialaphos for 3 to 4 weeks and then on MS0 or MSG medium with 10 mg/L bialaphos until plants were regenerated (Tables 4 and 5). In some experiments continuous selection on 5 mg/L or 10 mg/L bialaphos was performed to test the possibility of obtaining transgenic plants with this type of selection (Table 4). A plantlet selected and growing on MS0 medium with 5 mg/L bialaphos is shown in Fig. 9B. A transgenic callus producing green shoots on MSG medium with 10 mg/L bialaphos is shown in Fig. 10A. We found that escapes could occur on the bialaphos selection medium especially with 5 mg/L of the agent. Therefore, we also initiated testing with direct application of Basta on young plantlets in petri dishes (Fig. 10B).

The use of MSG instead of MS0 medium shortened the time required to obtain the regeneration of putative transgenic plantlets. In some cases, plants were obtained in only 3 months. There were differences in transformation efficiency between Alamo genotypes used. The largest number of putative transgenic plants were obtained with Alamo genotypes 2702, 9, 22, and BAP 26. Experiments were also performed with the upland cultivars, but no transgenic plants were obtained.

Numbers of possible transgenic plants obtained to date by bombardment with both plasmid constructs are summarized in Tables 4 and 5. A total of 27 putative transgenic plants were obtained with pAHC25 and 30 were obtained with ps-GFP-BAR. Two mature transgenic plants are shown in Fig. 9C. These were obtained from experiments conducted in late 1998 and early 1999 with bombardment of immature inflorescence derived calluses with psGFP-BAR. Pollen grains obtained from these plants fluoresced bright green with GFP under blue light (Fig. 9D), and the plants exhibited tolerance to 0.1% Basta when brushed on their leaves (Fig. 9E). Tissues obtained from the plants also produced callus and regenerated plants on medium containing 10 mg/L bialaphos (Fig. 9F). A Southern blot hybridization for the *bar* gene confirming genetic transformation of these two plants is shown in Fig. 11.

Table 3. Results of selection of shoot clumps bombarded with pAHC25 and cultured for different periods (d) on medium with different bialaphos concentrations.

Continuous selection 5 mg/L bialaphos						
Cultivar	Experiment Number	No. of shoot clumps bombarded	Number of surviving clumps			
			14 d	28 d	42 d	56 d
Alamo	1	68	47	20	14	13
	2	60		16	13	12
	3	62			2	2
Kanlow	4	66		18	10	7
Continuous selection 10 mg/L bialaphos						
Cultivar	Experiment Number	No. of shoot clumps bombarded	No. of surviving clumps			
			14 d	28 d	42 d	56 d
Alamo	5	62	34	15	9	6
	6	72		31	12	3
	7	65			6	4
	8	70			7	1
Two-step selection (5 and 10 mg/L bialaphos)						
Cultivar	Experiment Number	No. of shoot clumps bombarded	No. of surviving clumps			
			14 d	28 d	42 d	56 d
Alamo	9	75		22	10	6
Kanlow	10	72		34	11	4

Efforts during the early part of 2000 were directed toward further quantification and analyses of transgenic plants obtained by microprojectile bombardment with the two constructs described above. We obtained approximately 270 plants of Alamo, 9 of Shawnee, and 5 of Kanlow (Table 6).

A Southern blot confirming presence of the *bar* gene was presented above. Two Southern blots were performed to confirm presence of the *gfp* gene. In the first, a double digest with *Nco* I and *Not* I of 10 mg of genomic DNA from transformed and control plants released a 0.7-kb fragment containing the *sgfp* gene and a 1.4-kb fragment containing *sgfp*, the *nos* 3' terminator, the *Ubi*1 promoter, and part of the intron for the *bar* gene (Fig. 12). The gene was present in all five plants tested. The signal for the 1.4-kb fragment was much weaker than that of the 0.7-kb fragment because only approximately 100 bp of *sgfp* were in the larger fragment. Trl and Tr4, which had a 1:1 ratio for GPF fluorescing versus nonfluorescing pollen grains, produced the weakest signals. The 1:1 ratio would be expected for a single copy with full expression. The other plants, with less than 50% glowing pollen, showed a stronger signal for *sgfp* indicating more copies and probably transgene silencing.

This method of specifically isolating the gene with restriction enzymes cutting the plasmid in two places shows presence of the gene upon Southern hybridization. However, it does not provide definitive proof of integration in the plane genome. Also, it does not provide an indication of copy number. Therefore, a second Southern was performed with a restriction enzyme that cut the plasmid only once. These results with *Kpn* I are shown in Fig. 13. The results again show that *gfp* is present in the five transgenic plants (lanes 3 to 7) and not in the control (lane 2). Plants represented in lanes 3 and 7 may have as few as 4 and 3 copies, respectively. These two plants also had 1:1 ratios of fluorescing to nonfluorescing pollen grains. Plants represented by lanes 4, 5, and 6 had lower than 50% fluorescing grains, and the plant with the heaviest bands (lane 6) had only 10 to 20% fluorescing grains. The grains were also late in development and showed abnormalities. Pollen grains from a T_o plant (primary transformant) with approximately 50% of them fluorescing is shown in Fig. 14A. Leaf tissue from a T_0 plant expressing GFP is shown in Fig. 14B.

Inheritance of the *bar* Transgene. Transgenic plants exhibiting a high tolerance to the herbicide Basta when 0.1% of the herbicide was brushed on their leaves were described above. During 2000, sexual crosses were made with one of the transgenic plants (Trl) and a nontransformed Alamo plant. The *bar* gene was transmitted through both male and female gametes and expressed in T_1 progeny (Table 7). Trl exhibited 50% fluorescing pollen, and assuming that 50% also possessed the *bar* gene, a 1:1 ratio of Basta tolerant:nontolerant offspring would be expected. A ratio not significantly different from 1:1 was observed for only one of the crosses. In this case, Trl was used as the female parent.

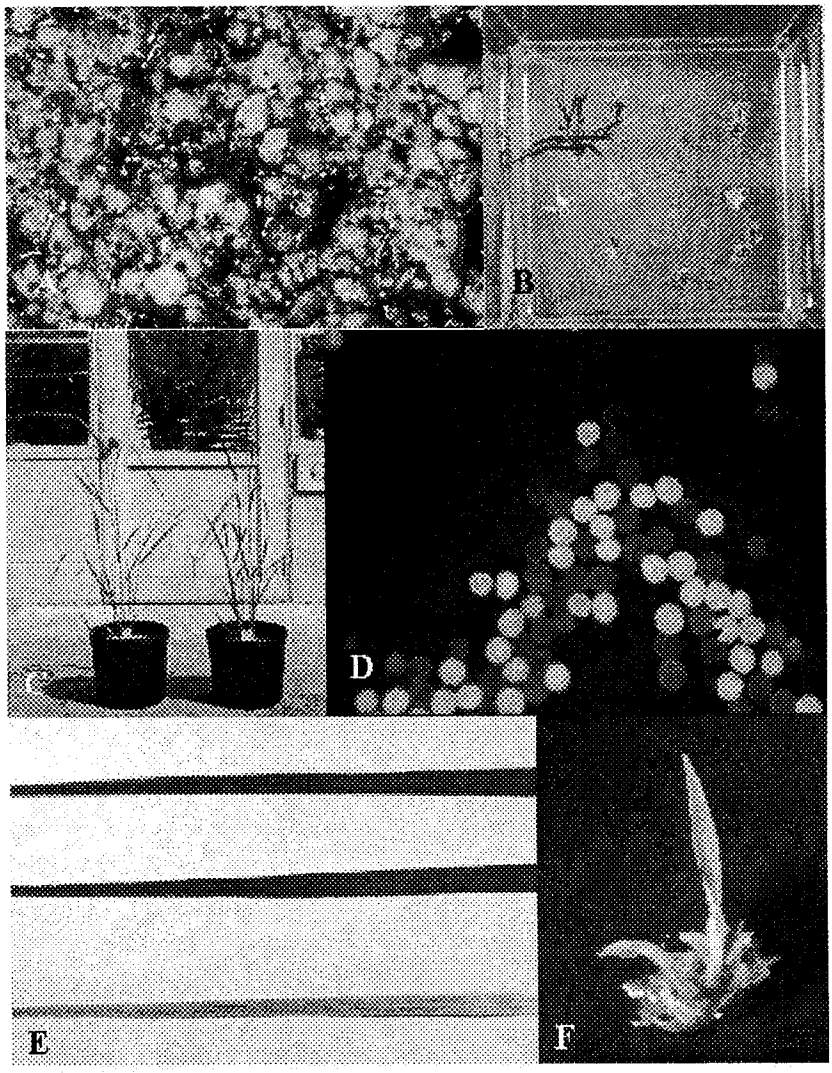

Fig. 9. Switchgrass transformation. (A) Transient expression of GUS in calluses bombarded with pAHC25. (B) Regenerated plantlet in Magenta box growing on medium supplemented with 5 mg/L bialophos. (C) Transgenic plants of Alamo obtained by microprojectile bombardment of immature inflorescence derived calluses with psGFP-BAR. (D) Pollen grains from one of the plants shown in (C) fluorescing with GFP. (E) Top and center – leaves from the plants shown in (C) exhibiting tolerance to Basta after applying 0.5% of the herbicide. Bottom – leaf of control plant treated similarly. (F) Plantlet regenerated from callus derived from one of the plants in (C) growing on medium supplemented with 10 mg/L bialophos.

Table 4. Results of selection of calluses bombarded with a pAHC25 plasmid on medium with different bialaphos concentration.

Genotype	Selection	Number of plates bombarded[a]	Number of putative transgenic plants[b]
Alamo 2702	Continuous selection on 5 mg/L bialaphos	20	1
Alamo 22	Continuous selection on 5 mg/L bialaphos	12	2
Kanlow S0	Continuous selection on 5 mg/L bialaphos	4	1
Alamo 2702	Continuous selection on 10 mg/L bialaphos	8	2
Alamo 2702	Two-step selection (5 and 10 mg/L bialaphos)	8	0
Kanlow S0	Two-step selection (5 and 10 mg/L bialaphos)	8	1
Total		60	7

[a]There were approximately 30 callus pieces bombarded per petri dish.

[b]Number of regenerated plantlets indicating tolerance to Basta after brushing portions of leaves one time with 0.1% of the herbicide.

Table 5. Results of two-step selection of calluses bombarded with plasmid pAHC25 or psGFP-BAR.

Genotype	Number of plants bombarded with plasmid pAHC25[a]	Number of transgenic plants[b]	Number of plants bombarded with plasmid psGFP-BAR[a]	Number of transgenic plants[b]
Alamo 2	8	0	7	4
Alamo 4	2	0	4	2
Alamo 6	4	0	7	1
Alamo 9	8	5	4	4
Alamo 11	10	0	0	0
Alamo 13	4	0	4	2
Alamo 19	4	0	4	0
Alamo 22	20	4	10	1
Alamo 23	12	1	8	1
Alamo 30	4	1	12	0
Alamo 2702	12	2	11	8
Alamo C 5	4	0	4	0
Alamo C 10	0	0	4	0
Alamo C 21	4	2	4	3
Alamo C 44	4	1	4	2
Alamo C 50	4	3	7	6
Alamo S 0	4	0	3	0
Alamo S 01	0	0	4	0
Alamo S 07	0	0	8	0
Alamo S 011	0	0	4	0
Alamo BAP 26	8	7	4	1
Total	120	27	117	35

[a]There were approximately 30 callus pieces bombarded per petri dish.

[b]Number of regenerated plantlets indicating tolerance to Basta after brushing portions of leaves one time with 0.1% of the herbicide.

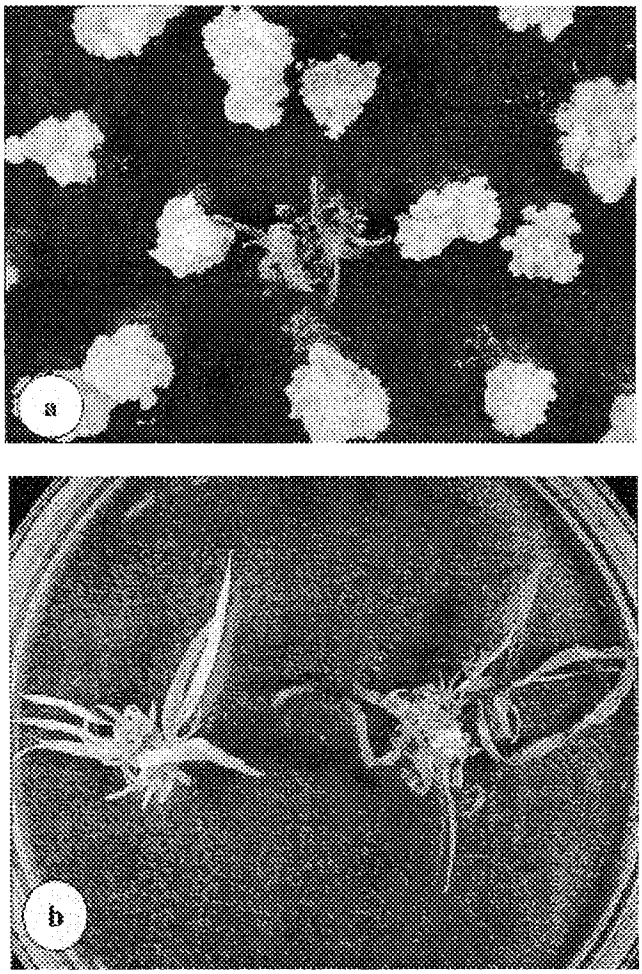

Fig. 10. (a) Selection of transgenic calluses of Alamo 2702 producing green shoots on MSG medium supplemented with 10 mg/L bialophos. (b) Leaves of two putative transgenic plants selected on 5 mg/L bialophos were rubbed with 0.1% Basta. Left, nontolerant plant; right, tolerant plant.

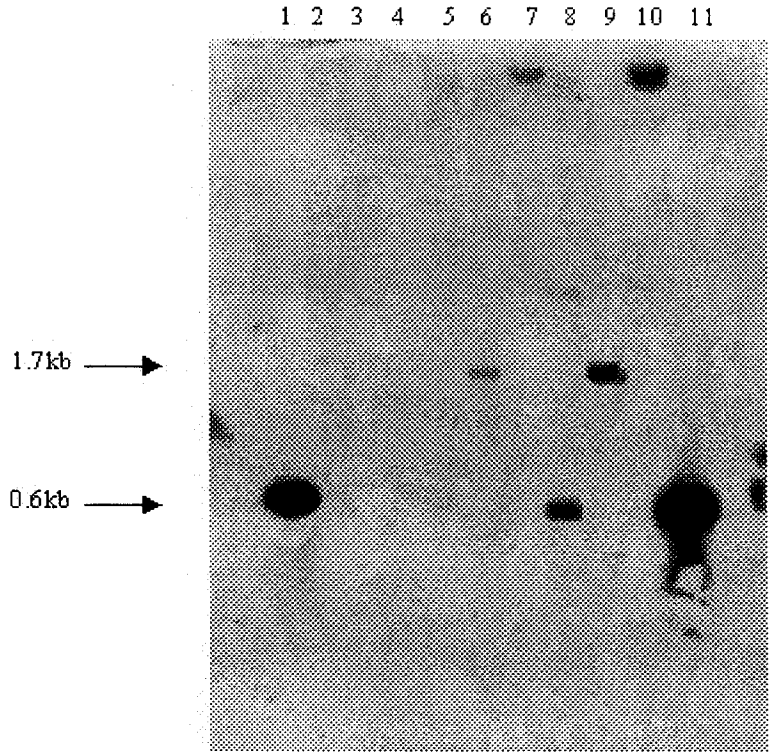

Fig. 11. Southern blot hybridization of two transgenic plants for the *bar* gene. *Lane 1*, psGFP-BAR (10 ng); *lane 2*, *pst* I digest of control DNA; *lane 3*, *Bgl* II digest of control DNA; *lane 4*, uncut control DNA; *lanes 5* and *8*, *Pst* I digests of Tr1 and Tr2; *lanes 6* and *9*, *Bgl* II digests of Tr1 and Tr2; *lanes 7* and *10*, uncut Tr1 and Tr2; *lane 11*, psGFP-BAR (20 ng).

Table 6. Number of transgenic plants tolerant to Basta obtained with psGFP-BAR (GFP reporter) and pAHC25 (GUS reporter) by microprojectile bombardment.

Number with psGFP-BAR	Number with pAHC25
141 Alamo	128 Alamo
9 Shawnee	5 Kanlow

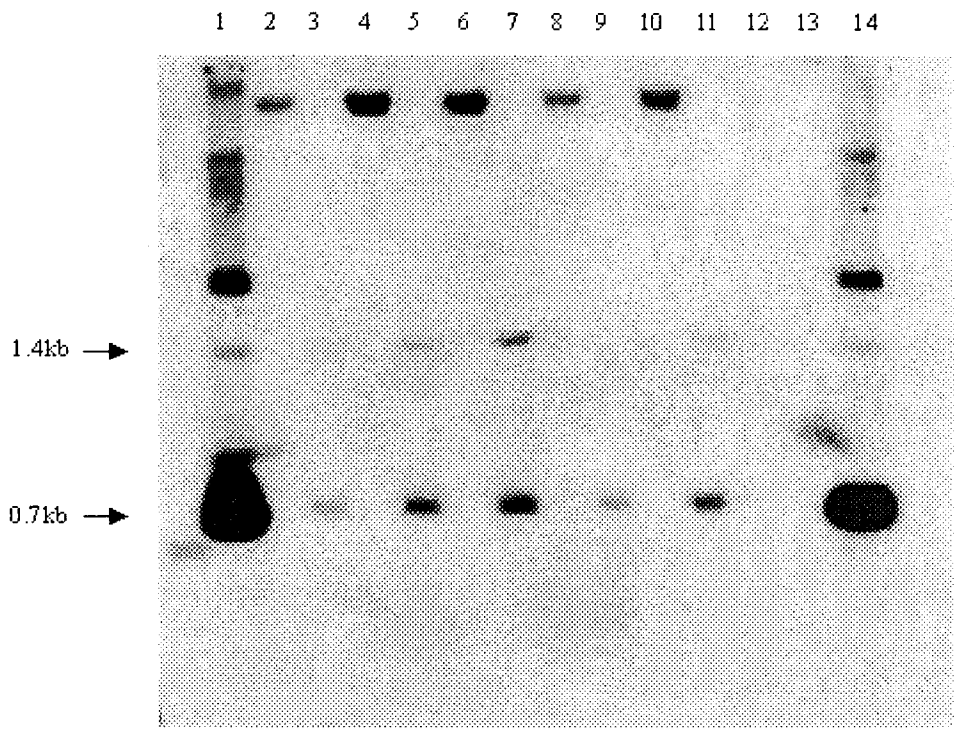

Fig. 12. Southern blot hybridization of five transgenic plants for the *gfp* gene. *Lane 1*, psGFP-BAR (1.0 ng); *lanes 2, 4, 6, 8,* and *10*, uncut DNA of Tr1 through Tr5; *lanes 3, 5, 7, 9,* and *11*, *Nco* I-*Not* I digests of Tr1 through Tr5; *lane 12*, uncut control DNA; *lane 13*, *Nco* I-*Not* I digest of control DNA; *lane 14*, psGFP-BAR (0.5 ng).

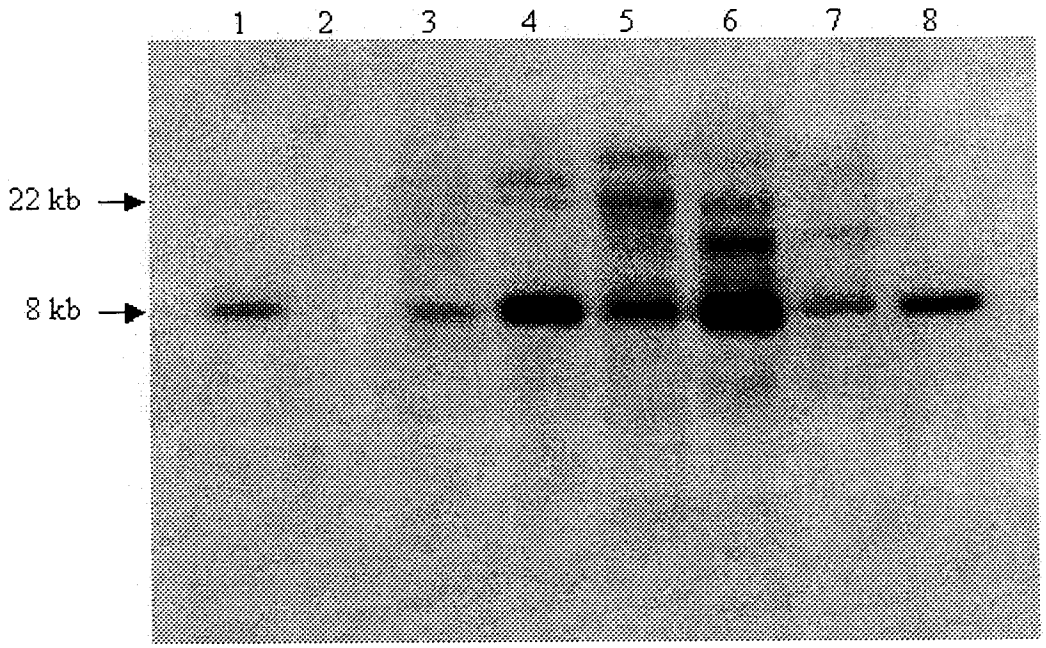

Fig. 13. Southern blot hybridization of five transgenic plants estimating copy number of the *gfp* gene. *Lane 1* psGFP-BAR (1 pg), *lane 2* control plant DNA, *lanes 3, 4, 5, 6, and 7* transgenic plants DNA, *lane 8* psGFP-BAR (2 pg). All DNA was digested with *Kpn* 1 which linearized the plasmid.

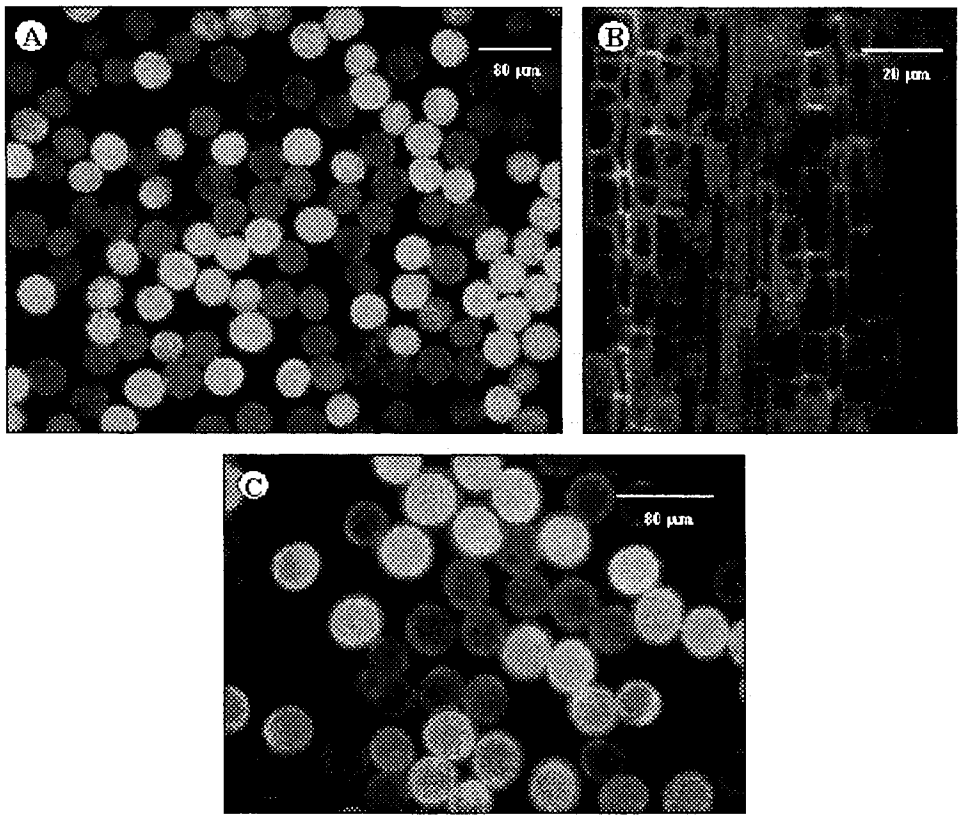

Fig. 14. GFP expression in pollen of a T_0 transgenic plant (A), leaf tissue of a T_0 plant (B), and pollen of a T_1 progeny plant (C).

Table 7. Number of Basta tolerant and nontolerant offspring obtained from four separate crosses using Trl as male parent and two crosses using Trl as female parent with an Alamo nontransgenic control plant.

Chi-square values for fit to 1:1 expected ratio are also given.

Cross	Number tolerant	Number nontolerant	Ratio	χ^2
Alamo X Trl	20	56	1:2.8	8.53**
Alamo X Trl	14	79	1:5.6	22.72**
Alamo X Trl	16	49	1:3.1	8.35**
Alamo X Trl	46	92	1:2.0	7.67**
Trl X Alamo	24	49	1:2.0	4.28**
Trl X Alamo	48	57	1:1.2	0.39ns[a]

**Significant at $P < 0.01$

[a]$P = 0.38$

Possible reasons for a higher frequency of nontolerant to tolerant T_1 plants include: (1) gene silencing (mentioned above) and (2) less than 50% of the pollen participating in pollination possessed the *bar* gene. The latter relates to the phenomena of "haplontic" and/or "diplontic" selection. These terms, coined many years ago for induced mutations, are based on the hypothesis that cells carrying a mutation (perhaps also a transgene) often have reduced vitality. Competition among cells results in the loss of some of those genetically altered. Male gametes with alterations are likely to be at an even greater competitive disadvantage than female gametes because of the requirement to germinate and produce a pollen tube. Another possibility is that some self pollinations occurred. However, switchgrass is cross-pollinated and predominately self-incompatible. We have not obtained any seed set on selfed greenhouse grown plants. Further verification of incorporation of the transgene is fluorescing pollen (approximately 50%) in T_1 plants (Fig. 14C).

Plants obtained with the pAHC25 construct and showing tolerance to Basta were also examined for expression of GUS in pollen and other floral parts. Pollen expressing GUS is shown in Figs. 15A and 15B indicating, as with GFP, presence of the transgenes in the male germ cells. Expression was also observed in ovaries (female germ cells) as shown in Fig. 15C. (Expression for GFP has not yet been detected in female tissues.) Fig. 15D shows expression in lodicules. This is somatic tissue at the floret base.

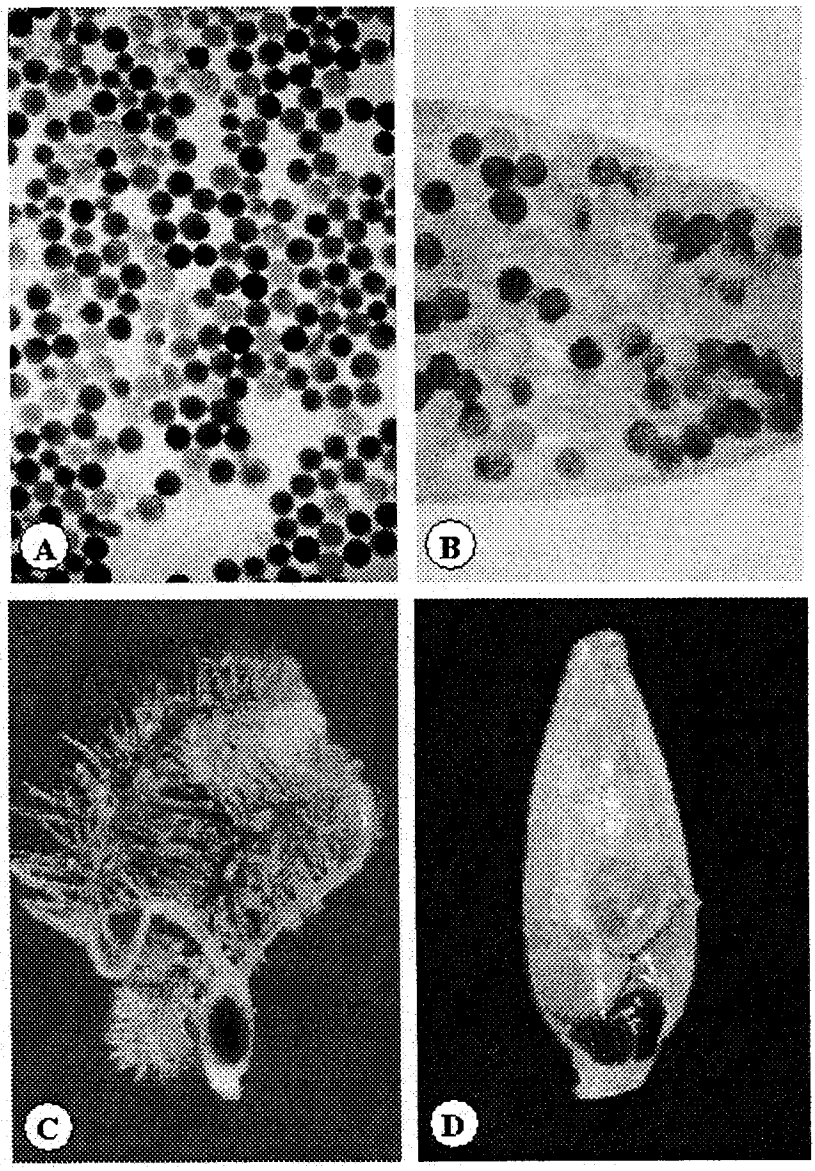

Fig. 15. GUS expression in floral parts from transgenic plants obtained by microprojectile bombardment with the plasmid pAHC25. (A) Pollen grains spread on a microscope slide. (B) Pollen grains *in situ* in an anther. (C) Expression in an ovary. Note the attached stigmas and styles. (D) Expression in lodicules (scale-like structures at the base of the ovary).

As with *gfp,* expression of the *gus* transgene in germ cells indicated the probability that they can be transmitted sexually. Results of experiments utilizing transgenic plants as both male and female parents in crosses with nontransgenic plants are reported in the 2001 Annual Report that follows. Southern hybridization experiments to confirm incorporation of both the *gus* and *bar* genes into the plant genome were also conducted in 2001 and are presented in that Annual Report.

Agrobacterium-Mediated Transformation. Genetic transformation experiments using the above mentioned *Agrobacterium tumefaciens* strain AGL1 containing the 18.15 kb transformation vector pDM805 were initiated in 2000. The *Agrobacterium* was grown from a single colony in mg/L medium supplemented with 20 mg/L rifapmicin and 5 mg/L tetracycline for 40 h at 27°C. Standard and full strength inocula (OD_{600} 0.560) were prepared; and 50, 100, or 200 μM acetosyringone were added to mg/L medium for pre-induction of the *vir* genes.

Embryogenic calluses and somatic embryos were initiated from various explants of different Alamo genotypes (Table 8). They were transferred into an *A. tumefaciens* suspension in multiwell plates for inoculation. This was accomplished by incubation of the tissues with the bacterium at 27°C for 3 min or 3 h in the dark. After inoculation, the explants were transferred with a wide-mouth pipette onto MS medium for callus maintenance. The medium was supplemented with 0, 50, or 100 μM acetosyringone to improve bacterial virulence. Cocultivation (continued incubation with the bacterium) was performed at 27°C in the dark for 3 d.

Table 8. Explant source of various Alamo genotypes (our designations) used to initiate callus cultures for *Agrobacterium*-mediated transformation.

Alamo genotype	Explant
C10	Mature caryopses
C21	Mature caryopses
C50	Mature caryopses
S01	Seedlings
S07	Seedlings
2	Immature inflorescence
23	Immature inflorescence
0108	Immature inflorescence
2702	Immature inflorescence

Basal parts (5 to 6 mm) of plantlets obtained from somatic embryos were cut transversely into smaller pieces (1 or 2 mm) in the presence of *A. tumefaciens* and inoculated in the bacterial suspension at 27°C in the dark for 1 h. In some experiments, the tissues were wounded with carborundum. Cocultivation was performed on MS medium supplemented with 5 mg/L 2,4-D and

1 mg/L BAP at 27°C in the dark for 5 d. Acetosyringone at 100 μM was also added to the medium.

Cuttings of plantlet segments (produced from somatic embryos) in the presence of *A. tumefaciens*, followed by inoculation and cocultivation, resulted in GUS expression only on the edges (Fig. 16A). When the segments were wounded with carborundum and incubated with the bacterium, expression was observed on the entire segment (Fig. 16B). Preculture of nonwounded segments on callus induction medium for 5 d increased GUS expression (Figs. 16C and 16D). This suggests an increase in the competence of cells for T-DNA transfer.

Bialaphos was used as the selective agent for the *bar* gene. At transfer to fresh selection medium, as well as to regeneration medium at 14-d intervals, each piece of callus derived from one somatic embryo or from one piece of inoculated embryogenic callus was divided into several small pieces. During the selection process, 150 mg/L of the bactericide Timentin was included in the medium to kill the *Agrobacterium*. In some experiments, half of the explants were precultured for 7 to 10 days with Timentin and without the selective agent before plating onto selection medium. The other half were grown on MS medium supplemented with 3 mg/L bialaphos, also for 7 to 10 days (semi-selection). Calluses cultured both with and without the selective agent were then transferred to fresh MS medium containing 10 mg/L bialaphos and cultured an additional 4 weeks. In another experiment, direct selection of somatic embryos was conducted by transferring them onto medium with 10 mg/L bialaphos immediately after cocultivation with the *Agrobacterium*.

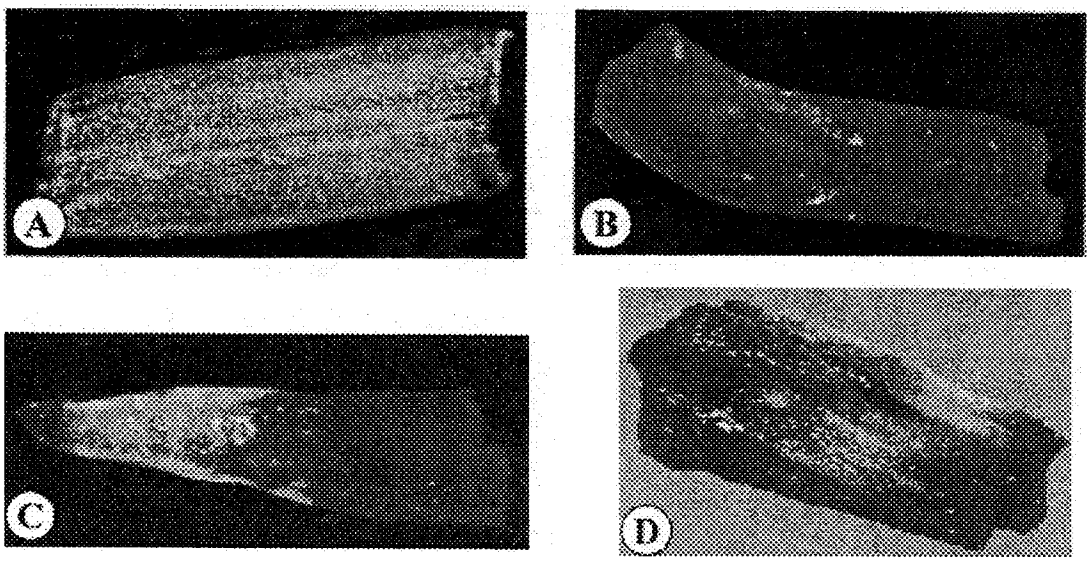

Fig. 16. GUS expression in plantlet segments inoculated with *Agrobacterium tumefaciens*. (A) Expression on cut edges of segment. (B) Expression on entire segment. The segment was wounded with carborundum before inoculation with the bacterium. (C-D) Expression on nonwounded segments precultured for 5 d before inoculation.

Data for the influence of *A. tumefaciens* induction, the subsequent selection on callus viability, and the frequency of embryogenic callus formation are shown in Tables 9–12. It is evident that the presence of acetosyringone during both inoculation and cocultivation is very important for callus formation from isolated somatic embryos (Table 9). Although somatic embryos were produced from calluses transformed with noninduced *A. tumefaciens* (culture without acetosyringone), the highest percentage of embryogenic calluses from genotype C50 was obtained after cocultivation in the presence of 200 μM acetosyringone (Table 9).

Table 9. Number of inoculated somatic embryos and number of calluses derived from them (in parentheses) from Alamo genotype C50.

Somatic embryos were either precultured on MS medium without bialaphos or first selected on MS medium with 3 mg/L bialaphos (semiselection) for 1 week. Calluses from both treatments were then transferred to MS medium with 10 mg/L bialaphos for an additional 4-week selection period. Different concentrations of acetosyringone (AS) were used during inoculation and cocultivation.

Type of selection	AS concentration during inoculation [μM]	AS concentration during cocultivation [μM]		
		0	50	200
Preculture on MS without bialaphos	0	30 (0)	36 (0)	22 (0)
	50	36 (0)	24 (0)	26 (6)
	200	24 (0)	32 (0)	34 (8)
Semiselection on MS + 3 mg/L bialaphos	0	24 (0)	22 (0)	30 (2)
	50	22 (0)	30 (2)	22 (2)
	200	26 (0)	24 (2)	36 (6)

Cultures initiated from genotypes C10, C21, and 2702 produced higher percentages of embryogenic calluses than those initiated from genotypes 2 and S07 (Table 11). Data for somatic embryos inoculated with the *Agrobacterium* and then subjected to direct selection on medium with 10 mg/L bialaphos are presented in Table 12. The highest number of calluses were obtained from genotypes C10 and 0108 in the presence of 200 μM acetosyringone.

After 4 weeks of selection on medium with 10 mg/L bialaphos, cultures with somatic embryos were transferred to regeneration medium (MS with 0.5 mg/L gibberellic acid) also containing 10 mg/L bialaphos. More than 800 plantlets were tested for their response to Basta by rubbing 0.1% solution of the herbicide on their leaves (Fig. 17). The test was performed at different stages of plant growth under *in vitro* and greenhouse conditions. The first treatment was applied when the plantlets were growing on the selection medium and had 3 to 4 leaves. Application of Basta at this stage of plant growth allowed us to eliminate most of the untransformed escapes early, which increased the efficiency of the transformation procedure. Plants that showed no reaction or only a localized reaction were again treated with the herbicide before transferring to larger culture vessels.

Table 10. Number of inoculated calluses (IC), number of IC that survived (SC), and number of SC that produced embryos (EC) from Alamo genotype C50.

The IC were either precultured on MS medium without bialaphos or first selected on MS medium with 3 mg/L bialaphos (semiselection) for 1 week. All calluses were then transferred to MS medium with 10 mg/L bialaphos for an additional 4-week selection period. Different concentrations of acetosyringone (AS) were used during inoculation and cocultivation.

Type of selection	AS concentration during inoculation [μM]	AS concentration during cocultivation [μM]								
		0			50			200		
		IC	SC	EC	IC	SC	EC	IC	SC	EC
Preculture on MS without bialaphos	0	38	11	5	26	13	5	25	12	7
	50	52	20	13	51	16	10	22	6	4
	200	32	22	6	27	12	6	35	16	11
Semiselection on MS+3 mg/L bialaphos	0	23	11	7	34	14	4	26	14	12
	50	56	9	8	33	24	8	37	25	8
	200	34	20	6	30	15	9	35	18	6

Only a few escapes were identified after the second treatment. From this experiment, we recovered more than 500 plants that exhibited tolerance to the herbicide.

For practical breeding purposes, transformation efficiency is probably less important in perennial than annual crops. Switchgrass is a perennial that can be both macro- and micropropagated with ease. We reported, in our earlier work, that with utilization of an *in vitro* node culture technique, approximately 500 plants could be obtained from one plant in 12 weeks. Therefore, a single transgenic plant with the desired traits is theoretically adequate. However, as reported below, our transformation efficiencies with *Agrobacterium* are quite high.

All plants that showed no reaction or a localized reaction to Basta were again treated with the herbicide before transferring to larger culture vessels. Only a few escapes were identified after the second treatment. After another 2 to 3 weeks of culture, the number of Basta-tolerant plantlets was estimated and the transformation efficiency was determined. Data for the effect of acetosyringone on transformation efficiency in Alamo genotype C50 from two experiments are presented in Table 13. The presence of acetosyringone, especially during cocultivation of somatic embryos improved the frequency of transgenic plant recovery. Some of the somatic embryos transformed without acetosyringone also formed vigorously growing calluses during subsequent selection, but none produced plantlets.

Table 11. Number of inoculated calluses (IC), number of IC that survived (SC), and number of SC that produced embryogenic calluses (EC).

The inoculated calluses originated from various explants of different Alamo genotypes (see Table 2). The calluses were either precultured on MS medium without bialaphos or first selected on MS medium with 3 mg/L bialaphos (semiselection). Further selection of all calluses was with 10 mg/L bialaphos for another 4 weeks. The acetosyringone (AS) concentrations during inoculation and cocultivation are also listed.

Alamo genotype	Type of selection	AS concentration during inoculation [μM]	AS concentration during cocultivation [μM]								
			0			50			200		
			IC	SC	EC	IC	SC	EC	IC	SC	EC
C10	Preculture without bialaphos	0	29	8	5	39	15	10	26	10	8
		200	37	14	10	43	20	16	23	7	6
	Semiselection 3 mg/L bialaphos	0	26	6	3	23	15	10	28	16	4
		200	35	15	4	32	14	9	35	15	9
C21	Preculture without bialaphos	0	22	6	6	19	8	5	23	6	4
		200	18	6	4	22	8	6	24	8	6
	Semiselection 3 mg/L bialaphos	0	24	4	4	30	5	3	27	7	5
		200	21	7	5	34	10	6	32	6	4
2702	Preculture without bialaphos	0	35	10	5	26	15	13	20	8	4
		200	29	9	6	32	17	11	28	9	5
	Semiselection 3 mg/L bialaphos	0	22	8	2	34	14	11	28	10	4
		200	32	12	4	50	20	16	22	6	4
2	Preculture without bialaphos	0	20	6	5	32	8	6	48	17	9
		200	24	8	6	32	4	2	26	6	4
	Semiselection 3 mg/L bialaphos	0	25	0	0	26	0	0	30	0	0
		200	31	0	0	22	0	0	27	0	0
S07	Preculture without bialaphos	0	55	14	4	23	5	5	30	8	2
		200	30	6	2	20	6	4	22	6	2
	Semiselection 3 mg/L bialaphos	0	38	2	0	20	2	0	36	8	2
		200	24	2	0	26	8	6	28	6	2

Table 12. Number of inoculated somatic embryos and number of calluses derived from them (in parentheses) from different Alamo genotypes.

The embryos were transferred to MS medium with 10 mg/L bialaphos for direct selection for 5 weeks. The acetosyringone (AS) concentrations were 0, 50, or 200 [μM] during both inoculation and cocultivation.

Alamo genotype	Acetosyringone concentration [μM]		
	0	**50**	**200**
C10	28 (3)	32 (4)	39 (13)
C21	40 (0)	38 (4)	34 (3)
2702	39 (3)	40 (9)	67 (13)
2	23 (2)	60 (7)	61 (11)
23	30 (0)	36 (2)	24 (5)
0108	32 (0)	23 (6)	34 (10)
S01	34 (0)	26 (1)	32 (4)
S07	44 (0)	51 (0)	38 (2)

The response of embryogenic calluses from the same genotype was different than that for somatic embryos. The highest number of Basta-tolerant plantlets was obtained from calluses inoculated and cocultured with the *Agrobacterium* on medium without acetosyringone. When 200 μM acetosyringone were added to the inoculation medium, overgrowth of the bacterium was observed during the selection and resulted in the inhibition of callus growth. Therefore, increase of the *Agrobacterium* virulence by acetosyringone may not be necessary for transformation of calluses from this genotype.

Data for the transformation efficiency of somatic embryos and embryogenic callus cultures from other (different) Alamo genotypes are shown in Tables 14 and 15, respectively. Experiments were performed with eight genotypes, but some failed to produce any transgenic plants; therefore they are not included in these tables. There were definite genotype differences with regard to transgenic plant recovery and efficiency. The highest numbers of transgenic plants were recovered from genotypes C10 and C21. Data for transformation efficiency from all experiments that resulted in production of at least one transgenic plant are summarized in Table 16.

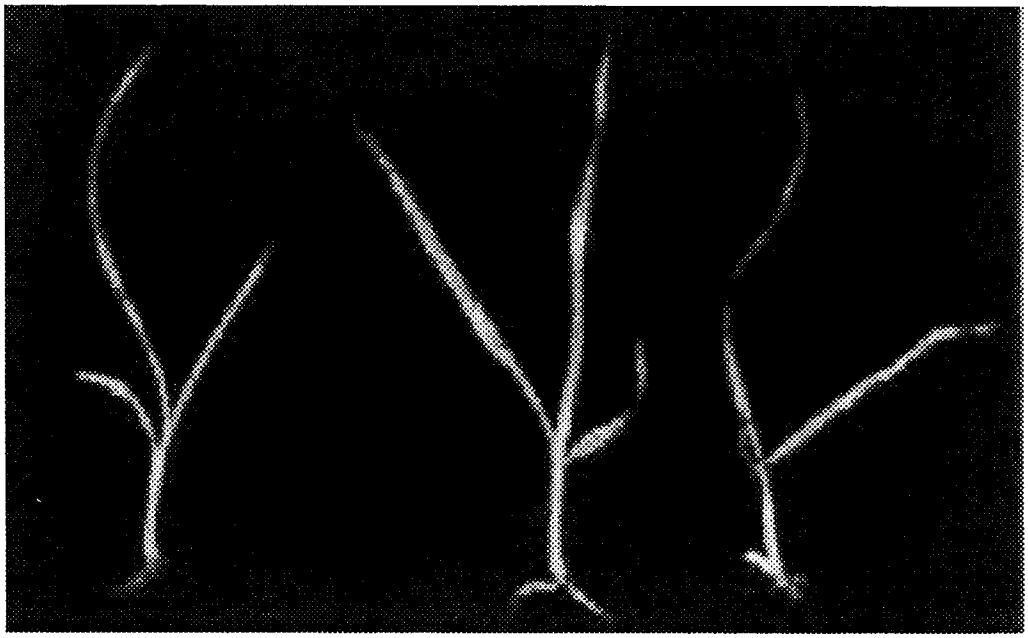

Fig. 17. Response to Basta after rubbing leaves with the herbicide (*left*, control; *center* and *right,* putative transformants).

Table 13. Effect of acetosyringone (AS) on the efficiency of *Agrobacterium*-mediated transformation of somatic embryos and embryogenic calluses from Alamo genotype C50.

Number of inoculated explants (IE), number of bialaphos resistant calluses derived from them (transformation events, TE), and number of Basta-tolerant plantlets (R) were used for estimation of the transformation efficiency (R/IE and R/TE). Different concentrations of AS were used during inoculation and cocultivation.

Type of inoculated explants	AS concentration during inoculation	AS concentration during cocultivation [μM]														
		0					50					200				
		IE	TE	R	R/IE	R/TE	IE	TE	R	R/IE	R/TE	IE	TE	R	R/IE	R/TE
		#	#	#	%	%	#	#	#	%	%	#	#	#	%	%
Somatic embryos	0	54	32	0	0	0	20	6	0	0	0	24	5	8	33.3	160.0
	50	40	5	0	0	0	57	37	49	86.0	132.4	24	8	3	12.5	37.5
	200	50	8	0	0	0	24	6	0	0	0	74	41	72	97.3	175.6
Calluses	0	108	41	69	63.9	168.3	61	27	0	0	0	51	28	8	15.7	28.6
	50	108	29	11	10.2	37.9	131	48	10	7.6	20.8	59	31	6	10.2	19.4
	200	49	42	0	0	0	19	27	0	0	0	117	67	5	4.3	7.5

Table 14. Effect of acetosyringone (AS) on the efficiency of *Agrobacterium*-mediated transformation of somatic embryos from various Alamo genotypes.

Number of inoculated explants (IE), number of bialaphos resistant calluses derived from them [transformation events (TE)], and number of Basta-tolerant plantlets (R) were used for estimation of the transformation efficiency (R/IE and R/TE). Different concentrations of AS were used during inoculation and cocultivation.

	AS concentration during inoculation and cocultivation [μM]															
	0					50					200					
	IE	TE	R	R/IE	R/TE	IE	TE	R	R/IE	R/TE	IE	TE	R	R/IE	R/TE	
Alamo genotype	No.	No.	No.	%	%	No.	No.	No.	%	%	No.	No.	No.	%	%	
C10	28	3	0	0	0	32	4	8	25.0	200.0	39	13	33	84.6	253.8	
C21	40	0	0	0	0	38	4	3	7.9	75.0	34	3	9	26.5	300.0	
2	23	2	0	0	0	60	7	0	0	0	61	11	9	14.8	81.8	
0108	32	0	0	0	0	23	6	6	26.1	100.0	34	10	0	0	0	

Table 15. Effect of acetosyringone (AS) on the efficiency of *Agrobacterium*-mediated transformation of embryogenic calluses from various Alamo genotypes.

Number of inoculated explants (IE), numbers of bialaphos resistant calluses [transformation events (TE)], and number of Basta-tolerant plantlets (R) were used for estimation of the transformation efficiency (R/IE and R/TE). Different concentrations of AS were used during inoculation and cocultivation.

Alamo genotype	AS concentration during inoculation [μM]	AS concentration during cocultivation [μM]														
		0					50					200				
		IE	TE	R	R/IE	R/TE	IE	TE	R	R/I	R/TE	IE	TE	R	R/IE	R/TE
		#	#	#	%	%	#	#	#	%	%	#	#	#	%	%
C10	0	55	14	0	0	0	62	30	55	88.7	183.3	54	26	21	38.9	80.8
	200	72	29	0	0	0	75	34	8	10.7	23.5	58	22	18	31.0	81.8
C21	0	46	10	0	0	0	49	13	19	38.8	146.2	50	13	48	96.0	369.2
	200	39	13	0	0	0	56	18	34	60.7	188.9	56	14	20	35.7	142.9
2702	0	57	18	0	0	0	60	29	10	16.7	34.5	48	18	0	0	0
	200	61	21	0	0	0	82	37	11	13.4	29.7	50	15	15	53.6	100.0
2	0	45	6	12	26.7	200.0	58	8	0	0	0	78	17	0	0	0
	200	55	8	0	0	0	54	4	0	0	0	53	6	0	0	0
S07	0	93	16	0	0	0	43	7	0	0	0	66	16	0	0	0
	200	54	8	0	0	0	46	14	1	2.2	2.2	50	12	0	0	0

Table 16. Efficiency of *Agrobacterium*-mediated transformation of embryogenic calluses and somatic embryos from various Alamo genotypes.

The data are summarized from Tables 13, 14, and 15.

Type of explant	Inoculated explants (number)	Transformed plantlets (number)	Transformation efficiency %
Calluses	2550	370	*14.5*
Somatic embryos	811	200	*24.7*

The data can be summarized as follows:

- Induction of *Agrobacterium vir* genes by acetosyringone during inoculation and cocultivation increases the production of transgenic plants from transformed somatic embryos.

- Although transformed plants were obtained from embryogenic calluses infected without acetosyringone, the presence of this compound during cocultivation is important for production of transgenic plants at higher frequency.

- Transformation efficiency of both somatic embryos and embryogenic callus cultures is genotype dependent. The highest number of transgenic plants was obtained from genotypes C10, C12, and C50. As presented in previous reports, these genotypes also have high regeneration frequencies.

- Somatic embryos are preferable to callus cultures because:

  - During subsequent selection, transformed somatic embryos form highly embryogenic callus which produced numerous transformants.

  - Most of the recovered plantlets were transgenic while about 2/3 of the plantlets obtained from calluses were untransformed escapes.

More than 100 of the transformed plantlets were again treated with Basta and transferred to nonsterile conditions in plastic bags. None of the plants showed any reaction to the herbicide. Sixty-one of these primary transformants (T_0 plants) were randomly selected and transferred to soil in pots and established in the greenhouse. Their response to Basta was tested again by rubbing at least one leaf of each tiller with the herbicide solution. Tolerance indicated that the *bar* gene was transmitted from cells of the infected somatic embryos and calluses and was present in the regenerated plants. All plants were fertile, and they produced seeds after open pollination in the greenhouse.

Most of the Basta-tolerant plantlets were also characterized by histochemical analysis of GUS expression in young leaf tissues during selection. The greenhouse plants were additionally tested for GUS expression in young leaf tissues. These usually had higher GUS activity than the older tissues (Table 17). The data showed that the coexpression of *gus* and *bar* genes in T_0 plants was 90% (55/61).

In general, stable GUS expression was detected in leaf tissues (Fig. 18A), shoots (Fig. 18B), pollen grains (Fig. 19), and young ovaries (Fig. 20). GUS activity was not observed in roots. Some plants that showed weak GUS activity in leaf tissues had high GUS activity in floral parts and vice versa.

Table 17. Stable GUS expression in leaf tissues of Basta tolerant T_0 plants grown in the greenhouse.

GUS activity	Number of plants	% of total number
+++	9	14.8
++	23	37.7
+	23	37.7
-	6	9.8
Total number of plants:	61	

+++Detection of signal within 6 h of adding X-Gluc substrate
++Strong signal after 16 h incubation
+Weak signal after 16 h (only a few leaf cells show GUS activity)
-No detectable activity

Integration of both transgenes into the plant genome was confirmed by Southern blot hybridization. Plant genomic DNA was isolated from 200 mg of young leaf tissue of individual plants exhibiting tolerance to Basta using the Puregene DNA isolation Kit (Gentra Systems). Fifteen micrograms of genomic DNA was digested with the restriction enzyme *Bam* HI, size-fractionated by agarose gel electrophoresis, and transferred to a Hybond-N nylon membrane following the manufacturer's protocol (Amersham Pharmacia Biotech). The *gus* probe (638 bp) and the *bar* probe (437 bp) were generated by PCR amplification (PCR DIG Probe Synthesis Kit, Roche Molecular Biochemicals). The membrane was hybridized with the denatured probes overnight, and detection was performed by an enzyme immunoassay and enzyme-catalyzed color reaction using the DIG Nucleic Acid Detection Kit (Roche Molecular Biochemicals).

The binary plasmid vector pDM805 was restricted with *Bam* HI. The T-DNA region of the plasmid has three *Bam* HI sites. The fragment containing the *gus* gene is 14.2 kb. One internal 3.7 kb fragment is also released that contains the *bar* gene. Additional sites restricted by the enzyme must be derived from the plant genome, and the number of hybridizing bands around and greater than 5 kb reflects the number of copies of the integrated *gus* gene (Fig. 21A).

The mobility of the bands differed from plant to plant, indicating independent transformation events and random integration. The banding pattern observed demonstrated that most of the tested plants contained one copy (lanes 1, 3, 4, 8, 9, and 10) or two copies (lanes 2, 5, and 6) of the transgene. Integration of a low copy number is one of the advantages of the *Agrobacterium*-mediated gene transfer. Only one of the plants (lane 7) contained at least eight copies of the *gus* gene. This plant also had a heavier band than the others for the *bar* gene indicating also the possibility of multiple copies of this gene (Fig. 21B). The *bar* gene was present in all ten plants tested.

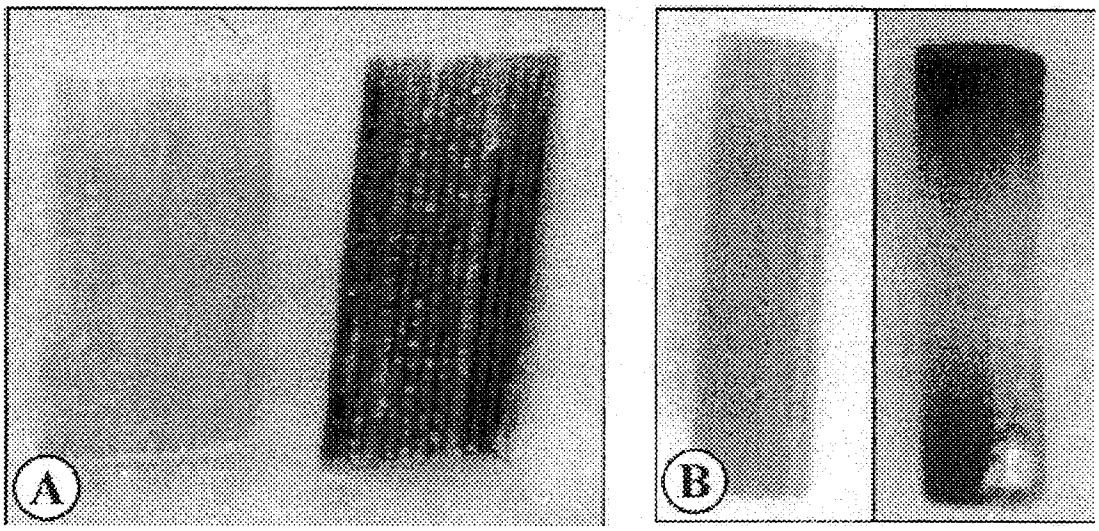

Fig. 18. Stable GUS expression in leaf tissues (A) and shoots (B) of T_0 plants (*left,* control; *right,* tissues from transgenic plants).

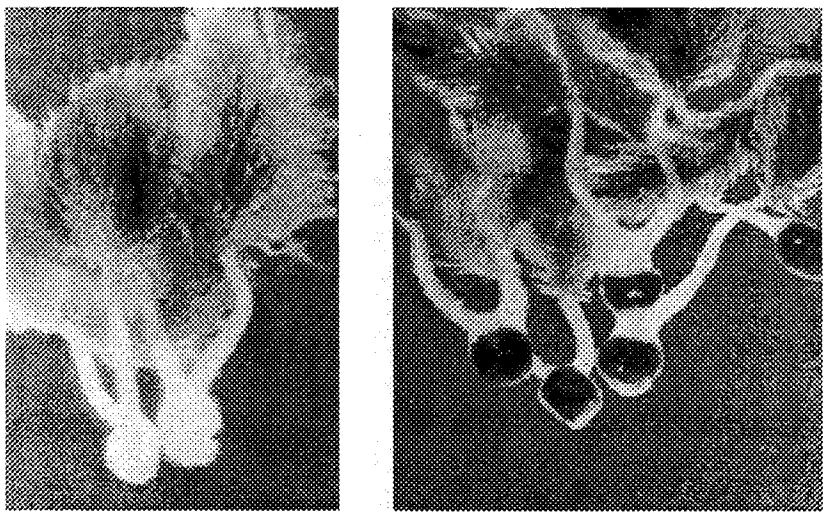

Fig. 19. GUS expression in pollen grains from T_0 plants (*left*, control; *right,* a transgenic plant).

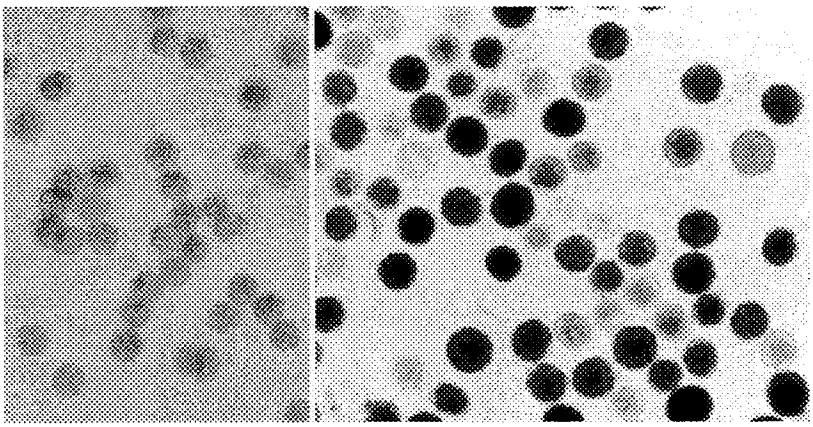

Fig. 20. GUS expression in young ovaries from T_0 plants (*left*, control; *right*, a transgenic plant).

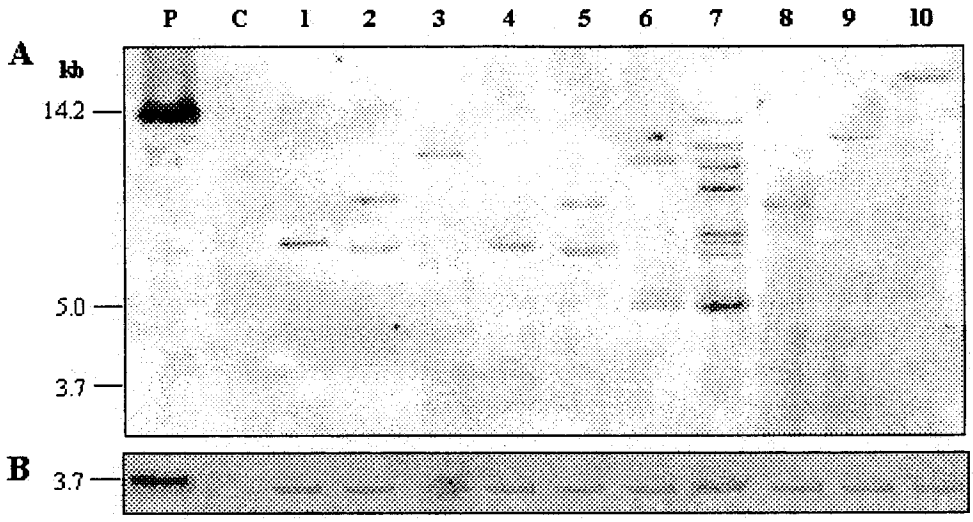

Fig. 21. DNA gel blot hybridization analysis of transformed plants (T_0). DNA from nontransgenic switchgrass (C), purified plasmid pDM805 (P), and transformants (lanes 1–10) was digested with *Bam*HI, fractionated by electrophoresis, transferred to a nylon membrane, and hybridized with the *gus* probe (A) or the *bar* probe (B).

Further detailed results on *Agrobacterium*-mediated transformation, molecular analyses, and sexual transmission of the transgenes through both male and female gametes are detailed in the 2001 annual report that follows in the appendix.

Anther Culture

Considerable effort was expended during the last 2 to 3 years of the project toward attempts to obtain haploid and doubled haploid plants. Whereas most of our experiments (*in vitro* culture and gene transfer) over the ten years were very successful, success with anther cultures was limited. Experiments to obtain haploid plants using isolated microspore techniques were conducted during 2001 and are detailed in the 2001 annual report (see Appendix).

Anther and pollen cultures have been used in various species to obtain plants with the gametic (haploid) number of chromosomes. These can be used to develop homozygous breeding lines, which in turn, can be crossed to maximize heterozygosity. In switchgrass, they also have potential for attempting crosses between tetraploid and octaploid cytotypes. Experiments were initiated in 1998 and very little success was obtained. They were continued in 1999 with both field and greenhouse plants. Six Alamo genotypes (615, 2017, 2702, and 3025) were used in experiments involving plating of nearly 16,000 anthers. Main factors studied were genotype, medium composition, and cold pretreatment. Data emphasizing these three factors are summarized in Tables 18, 19, and 20. The best responding genotypes were 615 and 3025 from field established plants and greenhouse plants of 2702. The superior response of greenhouse compared to field grown plants is opposite to results obtained in 1998. The best medium appeared to be C_{17} with 0.5 mg/L BAP and 1.5 mg/L 2,4-D. Anthers subjected to no cold pretreatment produced a higher response than those subjected to 4°C for either 7 or 14 days.

Spikelets of switchgrass possess one perfect and one staminate floret (Fig. 22a). The smaller anthers shown plated on the left of Fig. 22b are from staminate florets, and the larger ones are from perfect florets. After several months culture, a low percentage of the anthers produced callus and/or embryo-like structures (Fig. 22c) and, as shown in Fig. 22d, the response was sometimes quite dramatic. A somatic embryo and regenerated plantlet are shown in Figs. 22e and 22f, respectively. It was not determined whether the shoots were haploid, doubled haploids, or had their origin from somatic tissue.

Six Alamo (lowland type, tetraploid) clones (615,1991, 2017, 2702, and 3025) were used as doner plants of anthers in 1999. From 15,720 plated anthers, 106 green plantlets were established in soil in 2000 (Table 21, Fig. 23). The frequency of initiation of anther-derived calluses remained very low. Additionally, some of the obtained calluses were nonembryogenic. From embryogenic calluses it was possible to regenerate a high quantity of green plants.

Table 18. Effect of genotype on anther culture response.

Genotype	Days of cold pretreatment	Number of anther plated	Number of responding anthers [a]	Percent response
615-f	0	960	17	1.77
615-f	7	960	4	0.42
615-f	14	960	0	0.00
Total		2880	21	0.73
1913-f	0	360	0	0.00
1913-f	7	960	2	0.21
1913-f	14	960	0	0.00
Total		2280	2	0.09
2017-f	0	960	2	0.21
2017-f	7	960	0	0.00
2017-f	14	960	0	0.00
Total		2880	2	0.07
2702-f	0	960	7	0.73
2702-f	7	480	0	0.00
2702-f	14	960	0	0.00
Total		2400	7	0.29
2702-gh	0	960	25	2.60
2702-gh	7	960	16	1.67
Total		1920	41	2.14
3025-f	0	1440	18	1.25
3025-f	7	960	5	0.52
3025-f	14	960	0	0.00
Total		3360	23	0.68
Total		15720	96	0.61

Growing conditions of donor plants; f-field; gh-greenhouse

[a]Anthers producing callus and/or embryo-like structures

Table 19. Effect of medium composition on anther culture response.

Medium	No. of anthers plated	No. of responding anthers[a]	Percent response
C_{17}	1920	13	0.68
$C^{17}D$	1920	33	1.72
N_6	1980	11	0.56
N_6BD	1980	5	0.25
PM	1980	13	0.66
PM BD	1980	18	0.91
Yu Pei	1980	2	0.10
Yu Pei BD	1980	1	0.05
TOTAL	15720	96	0.61

BD modification of original medium to include 0.5 mg/L BAP and 1.5 mg/L 2,4-D

[a]Anthers producing callus and/or embryo-like structures

Table 20. Effect of cold pretreatment on anther culture response.

Days of cold pretreatment	No. of anthers plated	No. of responding anthers[a]	Percent response
0	5640	69	1.22
7	5280	27	0.51
14	4800	0	0.00
TOTAL	15720	96	0.61

[a]Anthers producing callus and/or embryo-like structures

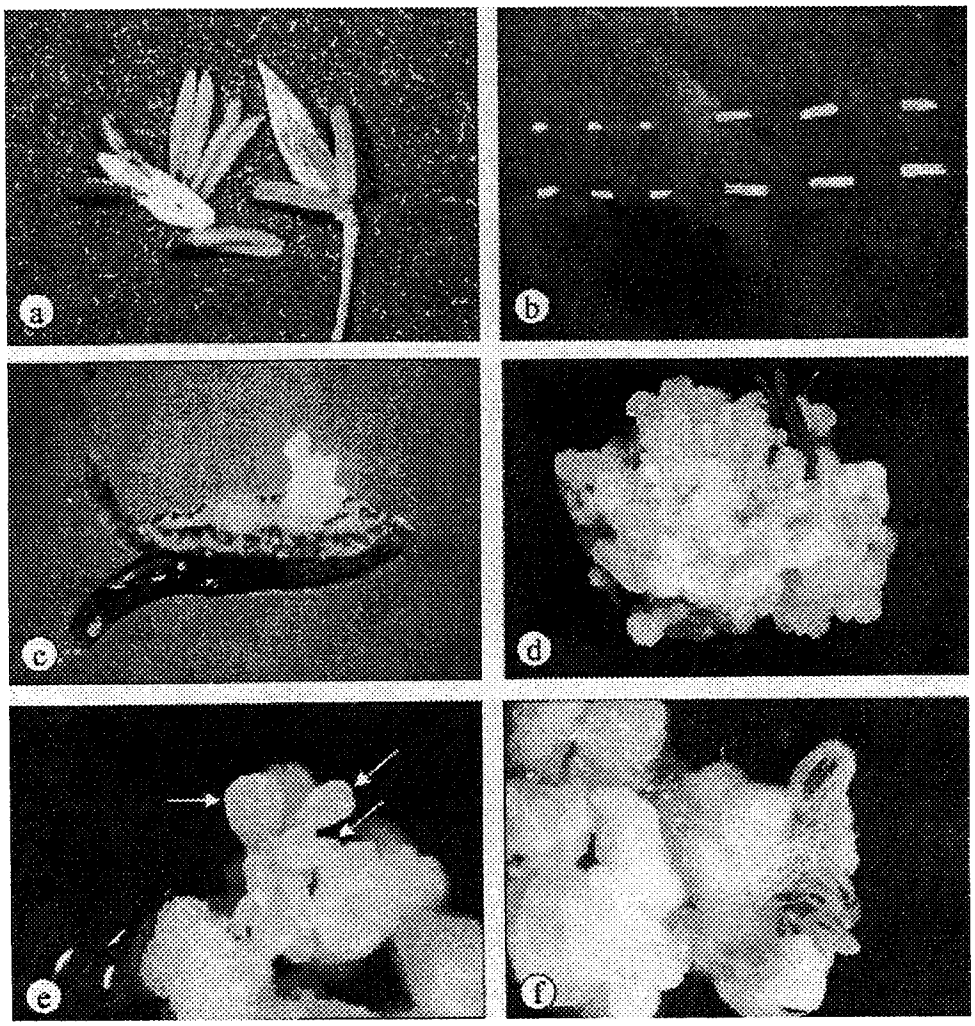

Fig. 22. Anther culture of switchgrass. (a) Spikelets have two florets with the upper floret being perfect (left) and the lower one staminate (right). (b) Anthers from both florets plated onto medium. The small anthers on the left are from the staminate floret. The large ones on the right are from the perfect floret. (c) Embryo-like structures and callus tissue emerging from inside of anther after 4–6 months of culture. (d) High embryogenic response with numerous structures developed. (e) Somatic embryos (arrows) produced from anther. (f) Shoot regeneration.

A negative aspect is the long period between the initial plating of anthers and receiving the first response (anthers producing callus and/or embryo-like structures). This can be as long as 4-6 months. This is much longer than for most other Poaceae species. Successful regeneration of green plants took as long as 16 months (Table 22) because of poor plantlet rooting. For improved rooting, additional transfer and culture in a liquid medium was required. The next obstacle was proper growth and development of regenerates in greenhouse conditions. After transferring regenerates from *in vitro* culture into soil, the growth of the plantlets was slow. Occasionally, the plants turned yellow and died in spite of proper maintenance (Fig. 24). Ten out of a total of 73 plantlets regenerated from clone 2702 did not survive. A similar situation existed for clone 3025 where 5 out of a total of 33 regenerants were lost.

Anther culture was also conducted with field-established plants of Shelter and Cave-in Rock (octaploid cultivars, 2n=8x=72). Experiments conducted in 1999 showed no advantage of cold pretreatment. Also, medium C_{17} supplemented with 0.5 mg/L[1] BAP and 1.5 mg/L 2,4-D was superior to others tried. Therefore, all anthers (total of 21,244) were plated on this medium with either 0-d or 1-d cold pretreatment. Anthers from both florets (perfect and staminate) were put serially in one row. Each petri dish contained ten rows. In addition, ovaries from perfect florets were plated onto the medium near each corresponding anther row (Fig. 25). This consisted of 10,200 anthers with 1,700 ovaries from Shelter, and 11,044 anthers with 1,840 ovaries from Cave-in Rock. After 5 months of cultivation, only 27 anthers responded. These were mainly from the no cold pretreatment of Cave-in Rock.

Crossing Blocks of Superior Clones

The top 20 yielding plants from a 1000 plant nursery of regenerants were identified and multiplied. They were used to establish three crossing blocks consisting of 2, 4, and 20 clones with 20 replications of each. Seeds were harvested in October 1998. Yield trials were seeded at two Tennessee locations, Knoxville and Springfield, in 1999. However, primarily due to weather conditions, a satisfactory establishment of these plots was not obtained at either location.

Successful establishment was obtained in 2000, and the stands were very uniform at Knoxville. They were less uniform at Springfield, but stands were obtained for all entries. The entries included our 2-, 4-, and 20-clone synthetics, TN-2, TN-4, and TN-20, respectively; three clones from Charles Taliaferro's program at Oklahoma State University, SL93-3, SL94-1, and NL94-1; and Alamo.

Table 21. Anther culture with tetraploid (2n=4x=36) Alamo genotypes: 615, 1913, 2017, 2702, 3025.

Genotype	No. of plated anthers	No. of responding anthers[b]	Percent response	No. of regenerated plantlets	Percent regeneration
615	2880	21	0.73		
1913	2280	2	0.09		
2017	2880	2	0.07		
2702	4320	48	1.11	73	1.69
3025	3360	23	0.68	33	0.98
TOTAL	15720	96	0.61	106	0.67

[b]Anthers producing callus or embryo-like structures

Fig. 23. Anther-derived green plants after transfer from *in vitro* conditions to soil.

Table 22. Time period between anther plating and first response/regeneration/transfer of plantlets to nonsterile conditions.

Genotype	Plating date	Time-period (months)			
		First response (callus initiation)	Beginning of regeneration	Transfer to peat pellets	Transfer to soil
A1 3025	07/16/99	5.9	7.5	8.1	9.1
A1 3025	07/16/99	5.9	7.5	10.4	11.6
A1 3025	07/16/99	5.9	7.5	14.9	16.4*
A1 2702	08/08/99	5.2	6.7	8.3	9.0
A1 2702	08/25/99	4.5	9.3	15.2	15.6*

*Including additional transfer to liquid medium for improved rooting

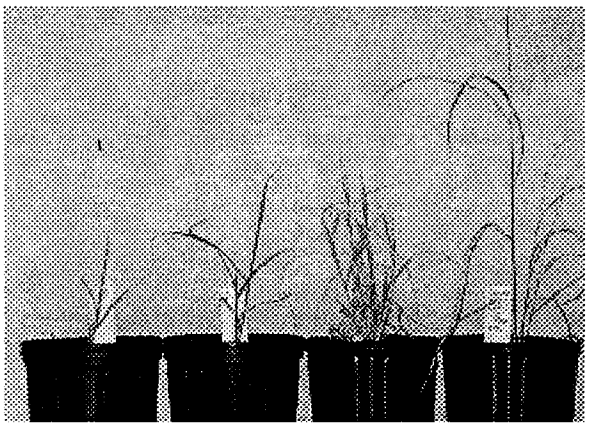

Fig. 24. After transferring of regenerants to soil, growth of the plantlets was slow. Occasionally the plants did not develop properly, turned yellow, and died.

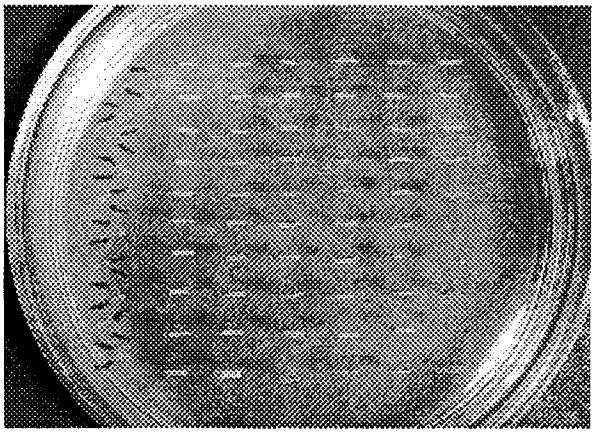

Fig. 25. Anthers from both florets (perfect, the first three anthers in each row; and staminate, the other three anthers in the same row). They were plated serially onto the medium. Each petri dish contained ten rows. Ovaries from the perfect florets were also planted near each corresponding anther row.

The plots were harvested after the first frost and dry weights in lb per acre are listed in Table 23 for Knoxville and Table 24 for Springfield. At Knoxville, for which the data are more reliable, TN-2 and TN-4, yielded more than Alamo. The yield of TN-20 was less. It appeared the more clones that make up the synthetic, the less the yield. The 2-clone synthetic is analogous to an F1 hybrid between two parents. The yield of TN-2 was about the same as that of SL93-3 and superior to the other two entries of OSU.

Table 23. Mean dry weights in lb per acre, standard deviations, and standard errors of switchgrass entries established and harvested at Knoxville, Tennessee, in 2000.

There were four replications of each entry.

Entry	Mean	SD	SE
Alamo	7095	477	238
TN-2	7864	592	296
TN-4	7348	397	198
TN-20	7083	180	90
SL93-3	7822	140	70
SL94-1	6910	644	322
NL94-1	5230	353	177

Table 24. Mean dry weights in lb per acre, standard deviations, and standard errors of switchgrass entries established and harvested at Springfield, Tennessee, in 2000.

There were four replications of each entry.

Entry	Mean	SD	SE
Alamo	5787	1259	629
TN-2	6669	1588	794
TN-4	5221	2220	1110
TN-20	6646	1206	603
SL93-3	6585	2088	1044
SL94-1	3979	1182	591
NL94-1	4142	725	363

The yields at Springfield were slightly different. Again, the yields of TN-2 and SL93-3 were about the same and superior to Alamo. However, the yield of TN-20 was also equivalent to TN-2 and SL93-3. The yield data at both locations are for the establishment year. Yields for the first full year, 2001, are presented in the 2001 annual report that follows.

DISCUSSION

Most of the objectives during the second five-year period were accomplished. The development of the multiple shoot system, and especially the suspension culture system, greatly added to our regeneration capabilities. The suspension system with the production of somatic embryos directly in liquid medium provides the potential to mass produce plants on much larger scale than with other in vitro systems. Furthermore, target tissues for genetic transformation studies can be produced in very large numbers. This provides the possibility of recovering numerous transgenic plants if transformation frequencies are high and a higher probability of recovering at least a few plants if frequencies are low.

Differences were observed in esterase isozyme banding patterns between Alamo genotypes and between embryogenic and nonembryogenic calluses. However, it was difficult to relate these differences to regeneration capacity and capability. Therefore, the work was discontinued after less than one year.

Obtainment of positive results for the transformation experiments were slow in the beginning, mainly because of personnel turnover and disruption of effort. However, a more concentrated effort resulted in a high degree of success during the last three years of the project and continues as we begin our project with Metabolix Corporation, Cambridge, Massachusetts, to genetically engineer switchgrass to produce polyhydroxybutrates. Transgenic plants were obtained in large numbers with microprojectile bombardment of two plasmids, pAHC25 and GFP-BAR, mentioned above. Support from the BFPD project was important in our construction of the new GFP-BAR plasmid. As mentioned, this construct uses the *gfp* gene as a reporter rather than *gus*. We have received several requests for this construct from various laboratories around the world. Successful gene transfer by *Agrobacterium* to switchgrass is the first demonstration of such in a forage grass. Importantly, we were able to demonstrate incorporation of the transgenes into the genome by molecular techniques and show that they are transmitted through both male and female gametes and expressed in progeny. In fact, we have demonstrated sexual transmission of the transgenes through at least two sexual generations and that they are inherited in the expected 1:1 Mendelian ratio for a dominant gene. Stability appears to be even greater in the later generations.

Experiments designed to produce haploids (plants with the gametic chromosome number) from anther and microspore cultures produced limited success. Numerous plants were produced but from only a few anthers. The time from plating anthers to the regeneration of plantlets was extremely long, up to several months. The plantlets were difficult to root. Many became necrotic and died. Chromosome counts were extremely difficult to make because there were few meristematic (actively dividing) cells. Experiments with isolated microspore culture were conducted during the last year of the project period. Although cells could be induced to divide, no plantlets were regenerated. It was decided to terminate this aspect of the project.

Yield trials involving 2-, 4-, and 20-clone synthetics developed from our original 1000 regenerated space nursery were promising and indicated that the dry matter yields were as equal to or greater than the experimental breeding lines from Oklahoma State University.

APPENDIX

ANNUAL REPORT 2001: DEVELOPMENT OF IN VITRO SYSTEMS FOR SWITCHGRASS *(PANICUM VIRGATUM)*

APPENDIX - ANNUAL REPORT 2001

DEVELOPMENT OF IN VITRO SYSTEMS FOR SWITCHGRASS (*PANICUM VIRGATUM*)

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for
U.S. DEPARTMENT OF ENERGY

Objectives:

To develop efficient and repeatable regeneration systems from cell and tissue cultures of switchgrass (*Panicum virgatum* L.) including production of haploid plants from anther and microspore cultures.

To develop efficient and reproducible gene transfer systems for this species.

Focus:

Experiments the calender year 2001 were focused on:

(1) Molecular and genetic analyses of transgenic plants obtained by microprojectile bombardment with pAHC25 (GUS-BAR).

(2) Sexual transmission of GFP-BAR from the T_1 to T_2 generation.

(3) *Agrobacterium*-mediated transformation of upland cultivars utilizing mature caryopses.

(4) Molecular analyses of transgenic plants (Alamo) obtained by the *Agrobacterium* method.

(5) Sexual transmission of transgenes in transgenic plants obtained by the *Agrobacterium* method.

(6) Anther and microspore culture.

(7) Yield trials of experimental synthetics.

Analyses of pAHC25 Transgenic Plants

Description of methods to obtain these transgenic plants were described in previous reports. We also reported GUS expression in various parts of the plants, including pollen and ovaries, and tolerance of T_0 plants to Basta™. During the past few months, we performed Southern blot hybridization for both the *uid*A (*gus*) and *bar* genes. Genomic DNA was isolated from tissue of both transformed and control plants using the PURE-GENE DNA Isolation kit (Gentra Systems). Ten µg of DNA from each plant was digested with *Kpn* I, which linearized the plasmid. The fragments were separated by electrophorsis on 0.8% agarose (25 V, overnight). DNA was then transferred to a Hybond N nylon membrane following the manufacturer's protocol (Amersham Pharmacia Biotech). Probes were made using a 0.6 kb *Pst* I bar fragment and 1.9 kb *Xba* I-*Sac* I *uid*A fragment. They were [^{32}P]-labeled using the RadPrime DNA hybridization solution (Gentra).

A Southern blot for the *bar* gene is shown in Fig. 1. Bands are present in varying numbers in all six transgenic plants tested. No bands were observed in the control plant. The number of bands indicate different copy numbers and the position of bands indicate different sizes of DNA fragments contianing the *bar* gene. Copy number ranged from one in plant No. 7 to five in plant No. 5. A Southern blot for the *uid*A (*gus*) gene is presented in Fig. 2. Again, presence of the transgene was confirmed in the seven transgenic plants tested. Only one band of light intensity was present in plants Nos. 7 and 8 indicating a probable single copy. Plants with intense bands, e.g., Nos. 2 and 3, probably have multiple copies of the gene.

Inheritance of the *bar* Gene

We reported on sexual transmission of the *bar* gene from the GFP-BAR construct previously and the data were also included in publication (Plant Cell Rep. 20:48-54, 2001). In that case, we did not obtain the expected 1:1 ratio of Basta tolerent to nontolerant plants. In early 2001, we made controlled crosses between transgenic plants obtained with pAHC25 and nontransgenic 'Alamo' plants. Eight crosses were made using transgenic plants as the male parent and four crosses were made using the transgenic plants as female. After 3 months, the seeds were harvested and germinated on filter paper in petri dishes. Resultant T_1 seedlings were transferred to soil and placed in the greenhouse. Their leaves were brushed with 0.1% Basta. The data (Table 1) show ratios not significantly different from 1:1 for all crosses regardless of whether the transgenic plant was used as male or female. Also, copy number did not influence the ratio as was the case with the GFP-BAR construct. At this time, we do not have an explanation for the difference in inheritance pattern of transgenes from the two different constructs. The expected 1:1 ratio represents a desired situation.

Sexual Transmission of Transgenes to the T_2 Generation

Sexual transmission of the *bar* gene from the psGFP-BAR construct from primary transformants (T_0 plants) to progeny T_1 plants) was presented in previous reports and in publication. During 2001, we made crosses between T_1 offspring and nontransgenic Alamo plants. Of the 44 T_2 plants obtained when a transgenic T_1 plant was used as the male parent, 24 were tolerant to Basta and 20 were nontolerant (Table 2). Furthermore, all Basta tolerant T_2 plants produced pollen that expressed GFP. The 24:20 ratio is not significantly different from 1:1 (P=0.55) using a chi-square test. The data show that transgenes in switchgrass are sexually transmitted for at least two generations. Also, the transmission appears more predictable and, perhaps more stable, in subsequent, i.e., T_2 and later generations. The ratio of Basta tolerant to nontolerant T_1 plants obtained from crossing primary transformants as male parent to control Alamo female parents ranged from 1:2 to1:5.6 and all were significantly different from the expected 1:1 (Richards et al., Plant Cell Rep. 20:48-54, 2001).

Agrobacterium-mediated Transformation of Upland Cultivars

Infection with *Agrobacterium*

To produce transgenic plants from upland cultivars we performed transformation experiments exploring the regeneration system for multiple shoot formation. The germination rate and callus formation ability of mature caryopses from three cultivars were compared by culture of the explants on MS medium supplemented with 4.5 mM 2,4-D and 18.2 mM thidiazuron (Table 3).

Mature caryopses from cv. Blackwell were infected with *Agrobacterium tumefaciens* by incubation in the bacterial suspension at 27°C for 1h in the dark followed by transfer to MS medium containing 4.5 mM 2,4-D and 18.2 mM 1-phenyl-3(1,2,3-thiadiazol-5yl)-urea (thidiazuron) for initiation of multiple shoots. After 3 d co-culture with *Agrobacterium* under the conditions described above, the explants were transferred to the same medium supplemented with 150 mg L^{-1} Timentin for elimination of the bacterium and 10 mg L^{-1} bialaphos for selection of the transformed explants. In a second experiment, mature caryopses from the same cultivar were precultured on callus induction medium for 2 wk. Explants with formed callus were infected with *Agrobacterium* following the procedure described above.

In both experiments, the virulent system of *Agrobacterium* was stimulated by addition of 100 mM acetosyringone to the media for inoculation and/or cocultivation. Data for the effect of this compound on the viability of transformed explants are presented in Table 4. All mature caryopses inoculated with *Agrobacterium* in the presence of acetosyringone and co-cultured on an acetosyringone-free medium formed calluses after 10 wk selection with10 mg L^{-1} bialaphos. Both types of explants showed high GUS activity immediately after cocultivation (Figs. 3 A, B) as well as during the first 4 wk of subsequent selection (Fig. 3 C). However, none of these cultures formed multiple shoots.

In an attempt to improve the initial steps of the transformation procedure, mature caryopses of Blackwell and Trailblazer were sonicated before inoculation with the *Agrobacterium*. This was followed by transfer to callus induction medium (MS with 4.5 mM 2,4-D and 22.5 mM BAP). Data for the effect of this treatment as well as the presence of acetosyringone on the explant response are shown in Table 5. Most of the resultant calluses were nonembryogenic and they grew very slowly.

Molecular analysis of T_0 Alamo plants

Presence of GUS activity in T_0 Alamo plants grown in a greenhouse (listed in Table 6) was confirmed by polymerase chain reaction (PCR) analysis. An example is shown in Fig. 4. All plants possessed both the *bar* and *gus* genes.

In a previous report, we showed data for individual copy numbers of the *gus* gene in some of the primary transformants. However, determination of gene copy number does not necessarily indicate the number of insertion loci. To analyze integration patterns, Southern blot analysis was performed with genomic DNA digested

with *Spe* I, which cleaves the T-DNA at a unique restriction site. After electrophoresis, DNA fragments were transferred to a nylon membrane and hybridized with the [^{32}P]-labeled *gus* probe overnight. The probe hybridized to bands greater than 9 kb (the size of the T-DNA), which indicated that the whole T-DNA was inserted in the genome of the analyzed plants (Fig. 5). The number of the bands in each lane corresponds to the number of the T-DNA inserts. It is evident that the majority of plants have 1-2 copies of complex T-DNA inserts. To further analyze integration patterns, Southern blot analysis was performed with genomic DNA digested with the restriction enzyme *Hind* III. This enzyme does not cut the plasmid pDM805 (Fig. 6). All six plants had at least one band with a molecular size larger than 9 kb (the size of the T-DNA), which confirmed integration of the whole T-DNA into the plant genome. The number of insertion copies is in accordance with the data obtained with *Spe* I and with *Bam* HI (reported in 2000). We previously showed that one of the analyzed primary transformants (T_0 14) contained more than five inserts. The presence of multiple copies in this plant was confirmed with the *Hind* III analysis (lane 3). Some truncated DNA was detected in two of the analyzed plants (lanes 1 and 2), which suggested rearrangements of the expression cassette. Rearrangements of T-DNA are characteristic of direct gene delivery methods, but are also found in dicots and monocots transformed by *Agrobacterium*.

Data for DNA blot analysis of T_0 plants are summarized in Table 7. Based on the banding pattern and number of insertions, the analyzed plants can be divided into two groups. Group 1 (all except plants 14 and 39) showed one or two insertions with no apparent rearrangement. Plants 14 and 39 showed multiple insertions. The difference in the number of bands detected in DNA from T_0 plant 14 (Fig. 5, lane 3), after digestion with *Bam* HI and *Spe* I, suggests a rearrangement of some of the inserts. This often occurs in primary transformants containing multiple insert copies.

The data demonstrate one of the purported major advantages of *Agrobacterium*-mediated gene transfer, i.e., integration of single insert copies with no rearrangement. Single copies of transgenes are preferable to achieve predictable patterns of inheritance and to reduce gene silencing.

Transgene inheritance in the offspring (T_1 plants) of primary transformants

In our semiannual report submitted in early July 2001, we reported on inheritance of transgenes from both uncontrolled (open pollination) and controlled crosses in the greenhouse. We now have more extensive data from controlled crosses, which we consider to be more meaningful, and therefore will not repeat the data from open pollination. GUS activity was detected histochemically by incubation of young leaf tissues with X-Gluc Fig. 7 A). Testing for presence of the *bar* gene in T_1 plants was conducted when the seedlings had 3-4 leaves. The young leaves were rubbed with 0.1% Basta solution. Tissue of transgenic plants showed no reaction while that of nontransformed plants became necrotic and died (Fig. 7 B).

More than 600 T_1 seedlings were obtained from controlled crosses of four primary transformants using the transgenic plants as both male and female parents

Table 8). A segregation ratio of 1:1 was obtained from all crosses. The transgenic plants used in the crosses had one or two insert copies as shown by DNA blot hybridization. The presence of the transgenes in the offspring was confirmed by PCR analyses (Figs. 8 A,B) and Southern hybridization of the PCR gels (Figs. 8 C,D). Some T_1 plants expressed GUS only, Basta tolerance only, both traits or neither trait (Table 9). The fact that some plants were Basta tolerant but did not show GUS activity, and vice versa, suggests a rearrangement in the expression cassette during meiosis. Further and more extensive analyses, may reveal other mechanisms that would result in alternate hypotheses.

Haploids – Anther and Microspore Culture

A major effort was expended during 2001 on anther and microspore cultures to induce and recover haploid plants. The work was conducted by Dr. Zygmunt Tomaszewski who was not supported directly on the contract.

Establishment and maintenance of anther derived cultures and plants

Anthers from both staminate and perfect florets, along with ovaries from the latter, of 'Alamo' genotypes 2702 and 3025 (our designation) were plated onto various media as described in previous reports (Figs. 9 A-C). Anthers produced somatic embryos and embryogenic calluses (Figs. 10 A-F). However, the response was very slow and the time from plating anthers to obtaining embryos or calluses was very long (4-6 months). These had the potential to regenerate hundreds of plantlets which could be established in the greenhouse (Figs. 11 A-G). Because of the slow responses, successful regeneration of green plants took up to as long as 16 months. The next obstacle was obtaining proper growth and development of regenerants in the greenhouse. Occasionally, after transfer from *in vitro* culture to soil, the plants turned yellow and died even though we practiced proper and careful maintenance.

In spite of these difficulties, we successfully established about 200 green plants in the greenhouse from lines 2702. Even though growth was slow and the plants had low vigor, we were able to maintain them in order to determine their chromosome numbers in root tips. As shown in Fig. 11C, the plants did possess the gametic chromosome number.

Similar problems existed with the upland (octaploid) cultivars Shelter and Cave-in-Rock. Despite the high number of anthers (11,044) and ovaries (1840) plated during 2000, only limited success was achieved with Cave-in-Rock. The most common problems were: low regeneration frequency, long time period between anther plating and plant regeneration, difficulty with plantlet rooting and establishment of regenerants in nonsterile conditions. Of 36 green plantlets transferred to soil, only one survived, and it exhibited abnormal growth. Fifteen plantlets are still being cultured under aseptic conditions. Several ovaries also responded but the calluses obtained were nonembryogenic.

Shelter did not respond to anther culture (10,200 plated anthers). Because of the extremely low regeneration frequency obtained with anther culture, we decided to focus on isolated microspore culture in 2001.

Microspore culture.

As mentioned, isolated microspore liquid culture for obtaining androgenic embryogenesis was investigated as an alternative to anther culture. Procedures were based on successful results recently obtained with rye (Plant Cell Rep. 19:875-880, 2000), timothy (Plant Cell Rep. 19:761-767, 2000), triticale (Plant Cell Tissue Organ Cult. 61:221-229, 2000) and wheat (Plant Cell Rep. 20:685-690, 2001 and proprietary information of Northwest Plant Breeding Company, NPB). The procedure involved tiller collection, microspore isolation, microspore culture and regeneration.

(a) *Tiller collection*

Fresh tillers were cut at two nodes below the top and placed in clean erlenmeyer flasks containing distilled water with an "inducing agent" (proprietary information of NPB). Plastic bags (thin walled) were wrapped and sealed, around the necks of the flasks to minimize evaporation. Flasks containing the inflorescences were stored in the dark at 33°C for 1-4 days.

(b) *Microspore isolation*

Tillers were removed from the flasks under sterile conditions and panicles were cut from the remaining tiller portion at the top node. We utilized a two-step sterilization process. In the first-step, whole panicles were wrapped in a paper towel, which was sprayed to saturation with 70% ethanol. After 15 min (or until the towel began to dry), the spikelets were aseptically removed from the panicles with forceps and placed into a 200 ml beaker. A solution containing 50% commercial bleach and two drops of Triton X-100 were added to the beaker and the spikelets were further sterilized by stirring in the solution for 10 min. They were then rinsed three times with sterile water.

The sterilized spikelets were placed into a 200 ml blender container (Sorvall Omni-mixer type: OM) containing 90-100 ml of autoclaved cold mannitol (0.2 M or 0.3 M). Microspores were isolated by pulsing the blender three times (7-8 sec each) at 16,000 rpm. The slurry was filtered through a sterile 100 mm metal sieve and then through an 80 mm sieve to remove the raw debris. The filtrate was put into centrifuge tubes (Fig. 12 A) and centrifuged at 90 *g* for 5 min. The pellet (Fig 12 B) was resuspended in 3 ml of 0.2 M or 0.3 M mannitol. These 3 ml of the microspore suspension was then carefully layered over 10 ml of 21% autoclaved maltose solution (Fig. 12 C) and centrifuged at 70 *g* for 10 min. The microspores were collected in a band at the maltose/mannitol gradient interphase (Figs. 12 D and E , white arrows). The pellet containing the debris (Figs. 12 D and E, black arrows) was discarded. The microspores were resuspended and washed in 10 ml of 0.3 M mannitol (Fig. 12 F) and centrifuged at 60 *g* for 10 min. The pellet (Fig. 12 G, black arrow) was resuspended in 2-3 ml of induction medium (NPB) and the quantity of microspores was determined with a hemocytometer.

(c) *Microspore culture*

Microspore density was adjusted to 20,000 per ml and dispensed to the culture containers (erlenmeyer flasks, petri dishes or multiwell plates). The microspores were

cultured in the dark at 27°C (Fig. 13 A). We observed the first microspore divisions after 3-5 wk of culture (Figs. 13 B and C). Multicellular structures developed (Figs. 13 C-F) but no plants were regenerated. Various treatments and pretreatments such as, the chemical inducers used by NPB, heat stress, medium composition, different osmolalities and pHs, sterilization modifications, culture densities, inflorescence age, etc., were tried without success.

Yield Trials of 2, 4 and 20 Clone Synthetics

As mentioned in previous reports, the top 20 yielding plants from the 1000 plant nursery established in 1993, were identified and multiplied. Crossing blocks of 2, 4, and 20 clones, with 20 replications each were established and seeds were harvested in 1998. Yield trials were established at Knoxville and Springfield in 2000. Entries in the trial included the three Tennessee synthetics designated as TN-2, TN-4 and TN-20, three entries from Charles Taliferro's program at Oklahoma State University and Alamo. The plots were harvested in the establishment year (2000) and in 2001. Data for 2001 are presented in Table 10. The data for the trial at Knoxville are less variable and therefore more reliable. The highest yielding entry at Knoxville was TN-4 and the highest yielding entry at Springfield was TN-20. The Tennessee synthetics yielded more than Alamo at both locations and were overall equal to or superior to those from Oklahoma State.

Appendix Table 1. Number of Basta tolerant and nontolerant plants from crosses between six of the pAHC25 transformed plants and Alamo control plants

Cross	No. tolerant	No. nontolerant	Ratio	χ^2
Alamo x TrA4	19	21	1 : 1.11	0.10
Alamo x TrA6	9	3	1 : 0.33	3.00
Alamo x TrA2	8	11	1 : 1.38	0.47
Alamo x TrA3	16	24	1 : 1.50	1.60
Alamo x TrA2	6	9	1 : 1.50	0.60
Alamo x TrA4	30	35	1 : 1.17	0.38
Alamo x TrA4	31	28	1 : 0.90	0.15
Alamo x TrA7	9	10	1 : 1.11	0.05
TrA1 x Alamo	34	39	1 : 1.15	0.34
TrA1 x Alamo	12	10	1 : 0.83	0.18
TrA4 x Alamo	11	10	1 : 0.91	0.05
TrA2 x Alamo	15	16	1 : 1.07	0.03

Appendix Table 2. No. of T_2 Basta tolerant and nontolerant plants from a cross T_1 tolerant plant and a control Alamo plant

Cross	No. tolerant	No. nontolerant	Ratio	χ^2
Alamo x T_1	24	20	1:0.83	0.18

Appendix Table 3. Germination rate of mature caryopses from various switchgrass cultivars. Seeds were dehusked, sterilized and plated on MS medium supplemented with 4.5 mM 2,4-D and 18.2 mM thidiazuron for initiation of multiple shoot formation (70 caryopses per petri dish in two replications for each cultivar). Germination rate and callus formation frequency were determined after 10 d. Standard errors are indicated as ±

Cultivar	Germinated mature caryopses No.	Germination percent	Mature caryopses with callus No.	Percent with callus	Caryopses producing callus %
Alamo	12.5 ± 1.5	9.0	5.5 ± 0.5	44.0	3.9
Blackwell	46.5 ± 6.5	33.2	12.5 ± 4.5	27.7	8.9
Trailblazer	4.5 ± 1.5	3.2	2.0 ± 1.0	44.4	1.4

Appendix Table 4. Number of inoculated explants (IE) from cv. Blackwell and number of calluses derived from them (C). After infection with *Agrobacterium*, the explants were selected on medium with 10 mg L^{-1} bialaphos for 10 wk. Different concentrations of acetosyringone (AS) were applied during inoculation and cocultivation

Type of explant	AS concentration during inoculation [mM]	AS concentration during cocultivation [μM]					
		0			100		
		IE	C	%	IE	C	%
Mature caryopses	0	10	5	50	10	3	33
	100	10	10	100	10	3	33
Mature caryopses derived callus	0	12	5	42	12	7	58
	100	12	9	75	12	11	92

Appendix Table 5. Number of inoculated mature caryposes (E) from Blackwell and Trailblazer and number of calluses derived from them (C) after selection on medium with 10 mg L^{-1} bialaphos for 3 wk. Sonication was applied to some of the explants before inoculation with *Agrobacterium* (S treated explants, NS nontreated explants). Different concentrations of acetosyringone (AS) were used during inoculation and cocultivation

Cultivar	AS concentration during inoculation [mM]	AS concentration during cocultivation [mM]											
		0						100					
		S			NS			0			100		
		E	C	%	E	C	%	IE	C	%	IE	C	%
Blackwell	0	24	21	86	29	22	76	30	28	93	30	24	80
	100	31	28	90	30	28	93	24	17	71	30	27	90
Trailblazer	0	8	5	62	9	7	78	6	5	83	8	7	88
	100	8	6	75	8	5	63	8	7	88	8	8	100

Appendix Table 6. List of primary transformants grown in a greenhouse. Target explants were somatic embryos (SE) and calluses (C). Acetosyringone (AS) concentrations during inoculation and cocultivation are also indicated. All of the plants are Basta tolerant. GUS activity in young leaf tissues was determined histochemically and indicated as follows: +++ detection of signal within 6 h of adding X-Gluc substrate; ++ strong signal after 16 h incubation; + weak signal after 16 h (only a few leaf cells show GUS activity); -- no detectable activity

Plant No.	Alamo genotype	Target explant	AS [mM]	GUS activity
1	C50	SE	200	++
2	C50	SE	200	+
3	C50	SE	200	-
4	C50	SE	200	++
5	C50	SE	200	+
6	C50	SE	200	++
7	C50	SE	200	+
8	C50	SE	200	++
9	C50	SE	50	+
10	C50	SE	50	+
11	C50	SE	50	+
12	C50	SE	50	+
13	C50	SE	200	+++
14	C50	SE	200	+++
15	C50	SE	200	+
16	C50	SE	200	+
17	C50	SE	200	+
18	C50	SE	200	+
19	C50	SE	200	+
20	C50	C	0	+
21	C50	C	0	+
22	C50	C	0	++
23	C50	SE	200	+
24	C50	SE	200	+
25	C50	SE	200	+
26	C50	SE	200	-
27	C50	SE	200	++
28	C50	SE	200	-
29	C50	SE	200	++
30	C50	SE	200	++
31	C50	SE	200	+
32	C50	SE	50	++
32	C50	SE	200	+
34	C50	SE	50	+++
35	C50	SE	200	++

Appendix Table 6. Continued

Plant No.	Alamo genotype	Target explant	AS [mM]	GUS activity
36	C21	C	0->50	+++
37	C21	C	0->50	+
38	C21	C	0->200	++
39	C21	SE	200	++
40	C21	SE	200	++
41	C21	SE	200	++
42	C10	SE	200	++
43	C10	SE	200	++
44	C10	SE	200	++
45	C10	SE	200	++
46	0108	C	0->50	+
47	0108	C	0->50	+++
48	0108	C	0->50	+
49	2	SE	200	++
50	2	SE	200	++
51	2	SE	200	++
52	C10	SE	200	++
53	C10	SE	200	+++
54	C10	SE	200	-
55	C10	SE	200	+
56	C21	C	0->50	+++
57	C50	SE	200	-
58	C50	SE	200	-
59	C50	SE	50	+++
60	C50	SE	50	+++
61	S07	C	200->50	++

Appendix Table 7. Number of gene copies and insertion (T-DNA) copies in T_0 plants. Plant genomic DNA (15 mg) was digested with the restriction enzymes *Bam*HI or *Spe*I and the resultant fragments were separated by electrophoresis, transferred to a nylon membrane and hybridized with the *gus* gene probe. The probe was digoxigenin-labeled by PCR for gene copy detection or radiolabled in a random primer reaction for the insertion copy number determination

T_0 plant*	Gene copy** No.	Insertion copy*** No.
2	1	n.a.
4	2	n.a.
5	1	1
6	1	1
7	2	n.a
8	2	n.a.
9	n.a.	1
13	1	1
14	>8	>5
16	1	1
17	1	n.a.
18	1	n.a.
19	1	1
20	n.a.	2
22	2	2
33	n.a.	2
35	1	1
38	1	1
39	3	3
40	n.a.	1
49	2	2

* our indication;
** detected in *Bam*HI-digested DNA (3 restriction sites in the T-DNA);
*** detected in *Spe*I-digested DNA (one restriction site in the T-DNA);
n.a. not analyzed by the indicated method.

Appendix Table 8. Segregation analysis of T_1 plants derived from reciprocal crosses between T_0 and control (nontransgenic) Alamo plants. The c^2 test is based on expected segregation ratio of 1:1

Cross	T_1 plants No.	T_1 plants assayed for Basta tolerance				T_1 plants assayed for GUS activity			
		Tolerant	Sensitive	Ratio T:S	X^2	Positive	Negative	P:N	X^2
3025x$T_0$7	51	30	21	1:0.7	1.59	31	20	1:0.6	0.27
$T_0$7x3025	63	31	32	1:1	0.02	35	28	1:0.8	0.78
3125x$T_0$35	110	60	50	1:0.8	0.91	59	51	1:0.9	0.58
$T_0$35x3125	60	25	35	1:1.4	1.67	28	32	1:1.1	0.27
2017x$T_0$17	18	8	10	1:1.2	0.22	10	8	1:0.8	0.22
$T_0$17x2017	71	33	38	1:1.2	0.35	29	42	1:1.4	2.38
3125x$T_0$8	70	37	33	1:0.9	0.23	38	32	1:0.8	0.51
$T_0$8x3125	89	39	50	1:1.3	1.36	44	45	1:0.9	0.01
2702x$T_0$8	35	18	17	1:0.9	0.03	15	20	1:1.3	0.71
$T_0$8x2702	62	33	29	1:0.9	0.26	28	34	1:1.2	0.58

Appendix Table 9. Number of T_1 plants expressing GUS, BAR (Basta tolerance), both GUS and BAR, and neither GUS nor BAR

GUS	BAR	Both	Neither
103	107	201	218

Appendix Table 10. Dry Matter Yield Trials (lb/A) ± Standard Error (SE).

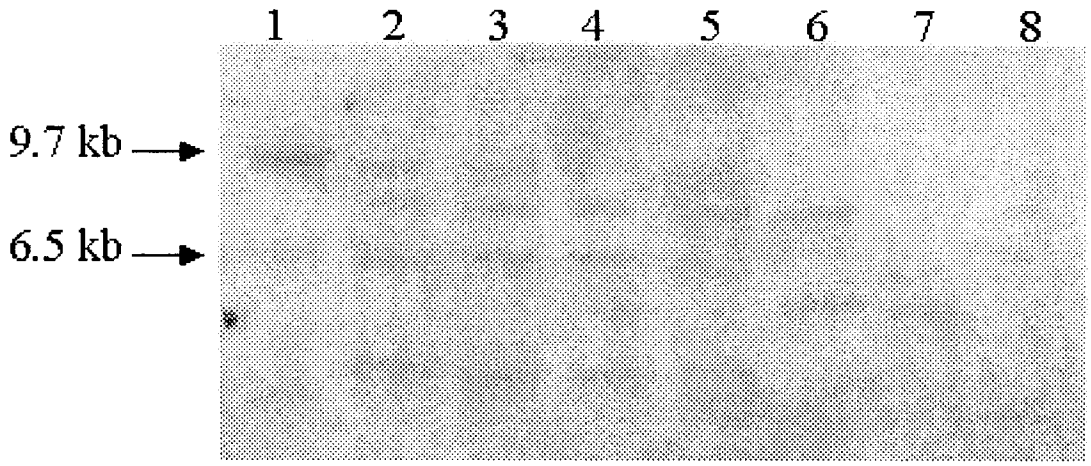
Knoxville Experiment Station

Entry	Alamo	TN2	TN4	TN20	SL93-3	SL94-1	NL94-1
Mean	17,222[a]	17,400	18,449*	17,554	16,754[a]	18,053	17,249
SE	698	679	433	661	914	565	1,314

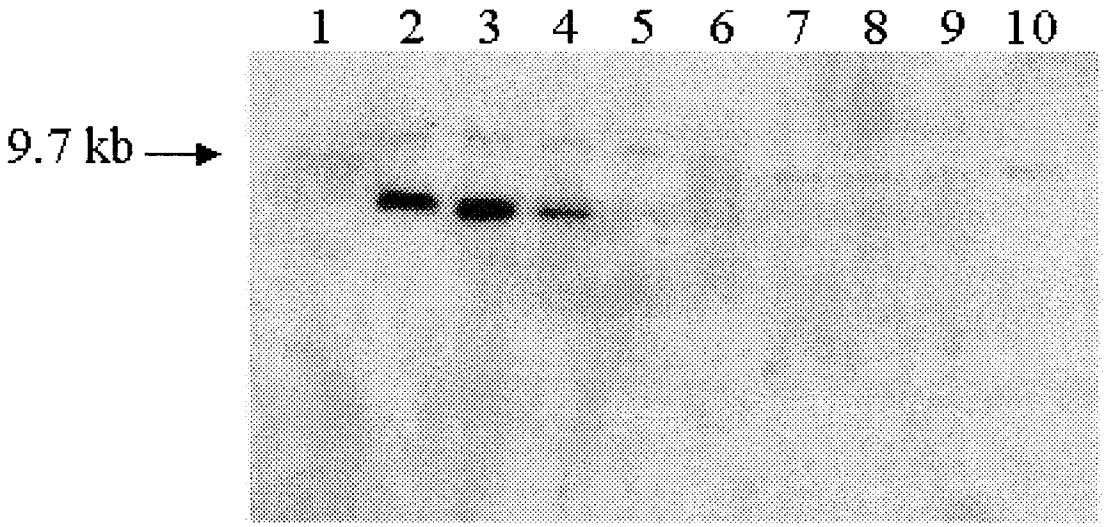
Highland Rim Experiment Station

Entry	Alamo	TN2	TN4	TN20	SL93-3	SL94-1	NL94-1
Mean	12,902	13,209	13,076	15,735*	12,537[a]	8,390[a]	15,488
SE	1,728	2,305	3,139	1,667	1,186	1,360	1,249

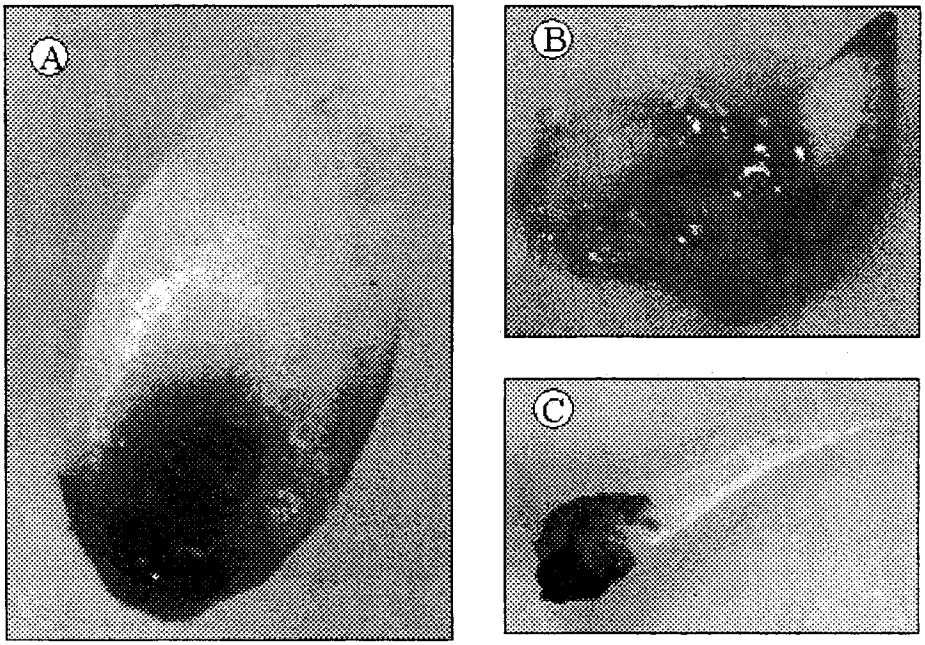
*Highest yielding entry
[a]Significantly different from highest yielding entry (±SE)



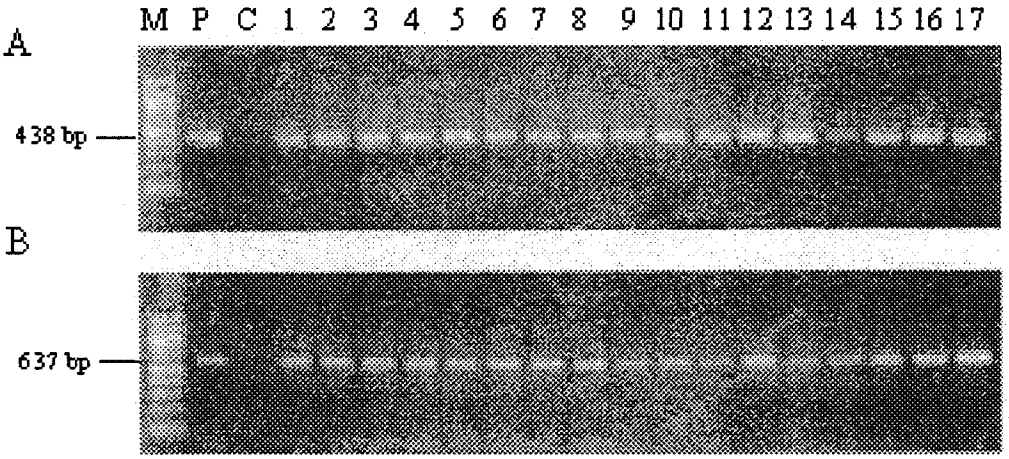
Appendix Fig. 1. Southern blot hybridization of six transgenic plants for the *bar* gene. Lane 1 pAHC25. Lanes 2 –7 transgenic plants 1 – 6. Lane 8 Alamo control. All DNA was digested with *Kpn* I, which linearizes the plasmid.



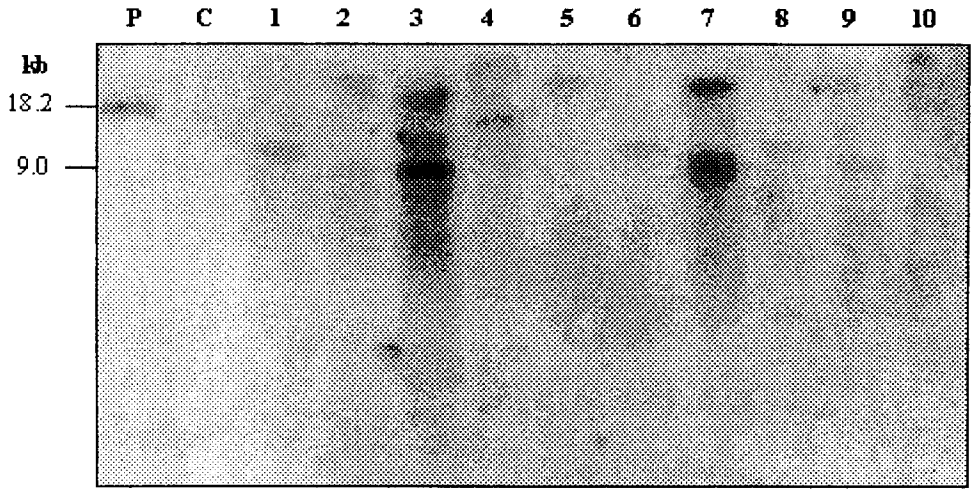
Appendix Fig. 2. Southern blot hybridization of seven transgenic plants for the *uid*A gene. Lanes 1 & 10 pAHC25. Lanes 2 – 8 transgenic plants 1 – 7. Lane 9 Alamo control plant. All DNA was digested with *Kpn* I.



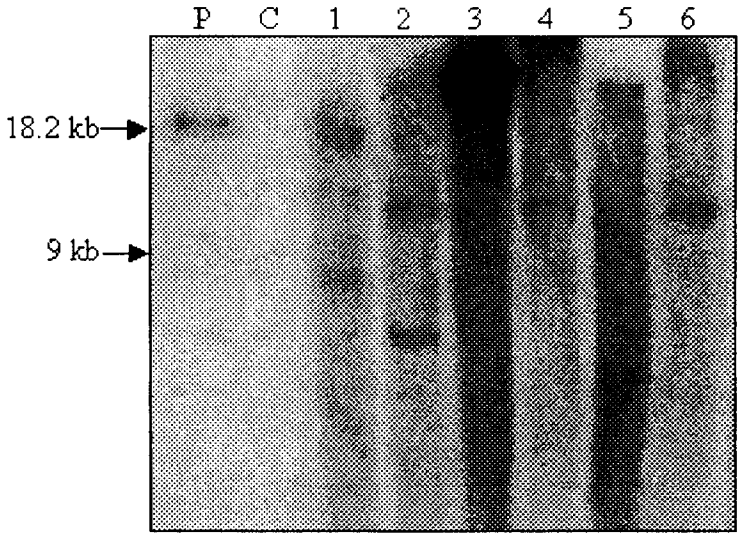
Appendix Fig. 3. GUS expression in Blackwell mature caryopses cocultivated with the *Agrobacterium tumefaciens* strain AGL 1 (pDM805). (A) GUS expression in an infected mature caryopsis after cocultivation with the bacterium. (B) Mature caryopsis derived callus expressing Gus after cocultivation. (C) GUS expression in callus formed from an infected mature caryopsis during selection with 10 mg L^{-1} bialaphos.



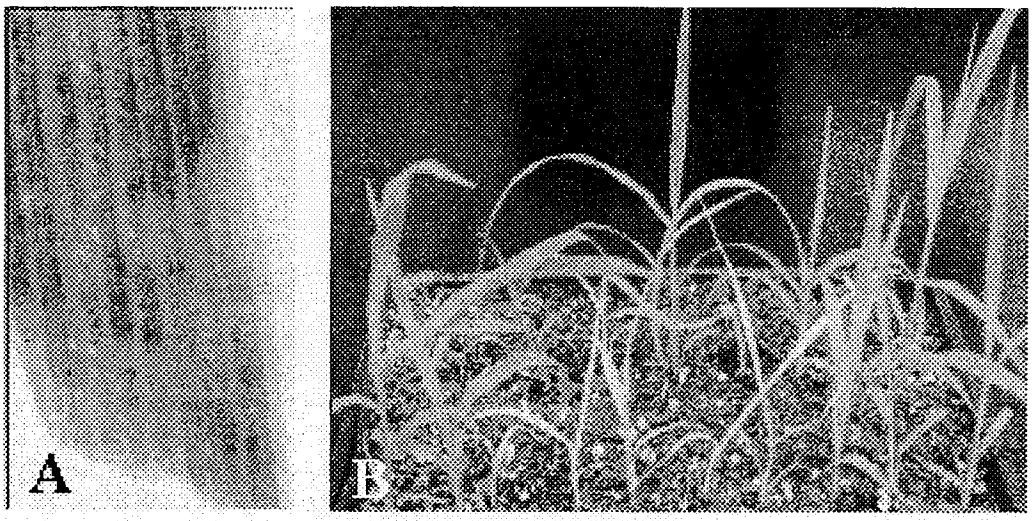
Appendix Fig. 4. Polymerase chain reaction (PCR) analysis of T_0 plants. Genomic DNA and plasmid DNA were amplified with AmpliTaqâ DNA Polymerase Stoffel Fragment (Perkin Elmer). Two sets of primers were used for detection of the *bar* gene (A) and the *gus* gene (B). Lames: M 100 kb ladder, P plasmid DNA, C DNA from a nontransformed plant, 1-17 DNA from T_0 plants.



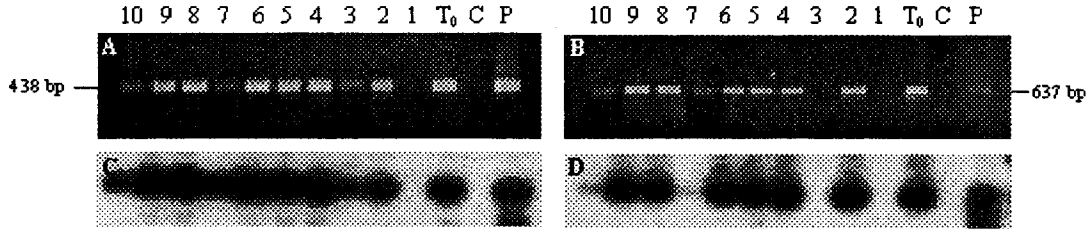
Appendix Fig. 5. Blot of *Spe*I-digested DNA from T_0 switchgrass plants presenting different copy numbers of the T-DNA after hybridization with the [^{32}P]-labeled *gus* probe. Lane: P, plasmid pDM805; C, a control Alamo plant; 1-10, transgenic plants. Sizes of the plasmid pDM805 (18.2 kb) and the T-DNA (9 kb) are also indicated.



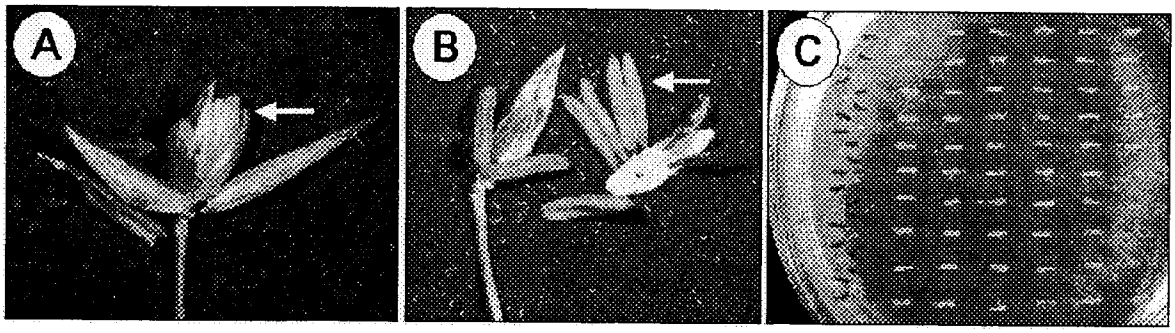
Appendix Fig. 6. Southern blot analysis of T_0 plants. Genomic DNA was digested with *Hind*III (without a restriction site in the plasmid pDM805). Resultant fragments were separated by gel electrophoresis, transferred onto a Hybond N membrane and hybridized with the *gus* gene probe. A 637-bp fragment generated by PCR was used as a template for synthesis of the [^{32}P]-labeled *gus* probe by random primer labeling. Lane: **P** undigested plasmid DNA; **C a** control Alamo plant; **1-6** T_0 plants. Sizes of the plasmid pDM805 (18.2 kb) and the T-DNA (9 kb) are indicated.



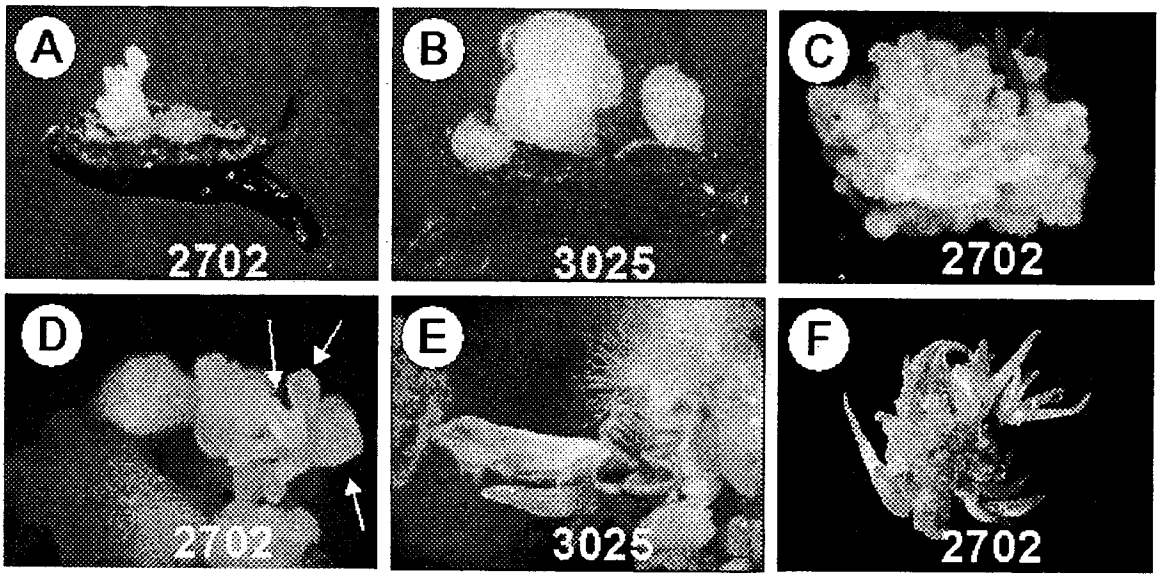
Appendix Fig. 7. (A) GUS activity in leaf tissue. (B) Response to Basta of T_1 plants derived from a controlled cross between the T_0 plant 19 and a nontransgenic plant Alamo 3125. The transgene inheritance analysis showed the expected ratio of 1:1.



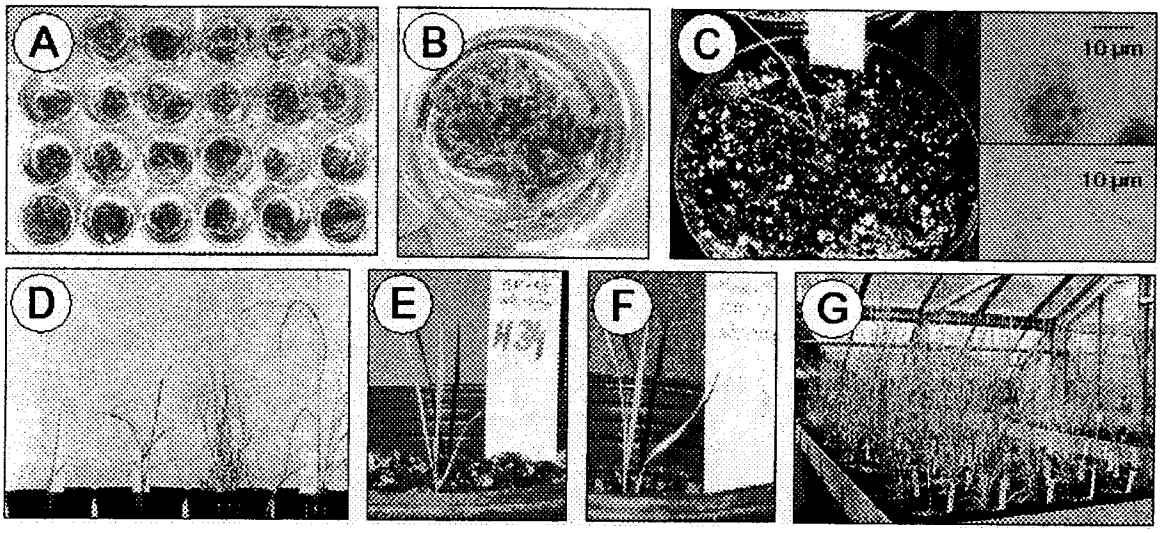
Appendix Fig. 8. Polymerase chain reaction (PCR) analysis of T_1 switchgrass progeny showing the 438 and 637 bp fragments corresponding to the bar (A) and gus (B) genes. Southern blot analysis of the PCR gels using [^{32}P]-labeled probes for the bar (C) and gus (D) genes. Lane: P, plasmid pDM805; C, a control Alamo plant; T_0, the female parent plant 35; 1, a T_1 plant which showed no transgene expression; 2-10, Basta tolerant T_1 plants.



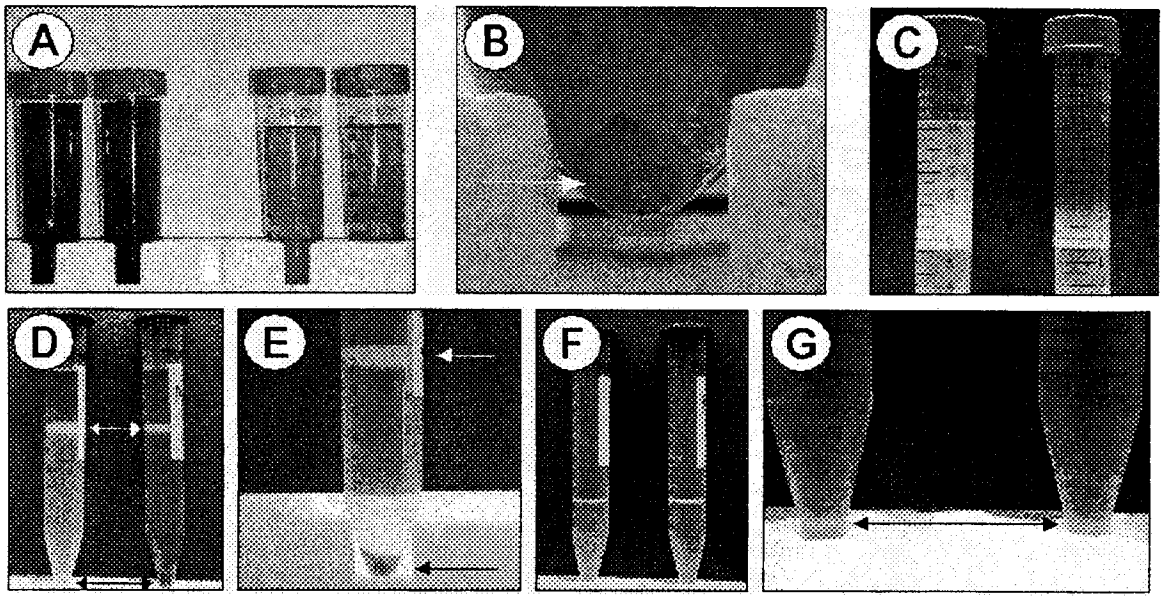
Appendix Fig. 9 A–C. Anther culture of switchgrass. (A - B) Spikelets have two florets with the upper being perfect (white arrow) and the lower staminate (red arrow). (C) Anthers plated from both florets, perfect – the first three anthers (left) in each row, and staminate – the second three anthers (right) in the same row. Each petri dish contained 10 rows. Ovaries from the perfect florets were also plated to the left of each corresponding anther row.



Appendix Fig. 10 A–F. Callus/embryo-like structure development. (A - B) Embryo-like structures and callus tissue emerging from inside of anther after 4-12 months of culture. (C) High embryogenic response with numerous structures developed. (D) Somatic embryos (arrows) produced from anther. (E) Shoot regeneration. (F) Albino shoot.



Appendix Fig. 11 A–G. Maintenance of anther-derived cultures of two Alamo genotypes (2702 and 3025) plated onto medium in 1999. (A) Cultures in a multiwell plate. (B) Higher magnification of a well showing shoot regeneration. (C) Haploid plant established in soil with 9 chromosome pairs in bivalent associations at diakinesis (top) and 18 individual chromosomes in anaphase I (bottom). (D) Plants in pots. Note the two plants on the right are turning yellow. (E) Seven-month-old plant in pot exemplifying slow growth. (F) The same plant – at 11 months age. (G) Greenhouse bench containing anther-derived plants. About 200 plants were successfully established.



Appendix Fig. 12 A–G. Microspore isolation. (A) Filtrate with microspores in tubes. (B) Pellet (arrow) collected after centrifugation at 90 g. (C) Resuspended pellet layered over maltose solution. (D) Viable microspores collected in a band at the maltose/mannitol gradient interface (white arrows). Debris collected at the tube bottom is indicated by the black arrows. (E) Higher magnification of (D). (F) Microspores after being resuspended and washed in mannitol. (G) Pellet collected after centrifugation at 60 g. This pellet was again resuspended in induction medium and analyzed with a hemocytometer to adjust the proper density of microspore culture.

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