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Genetic Transformation of Wheat Mediated by *Agrobacterium tumefaciens*

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A rapid *Agrobacterium tumefaciens*-mediated transformation system for wheat was developed using freshly isolated immature embryos, precultured immature embryos, and embryogenic calli as explants. The explants were inoculated with a disarmed *A. tumefaciens* strain C58 (ABI) harboring the binary vector pMON18365 containing the β-glucuronidase gene with an intron, and a selectable marker, the neomycin phosphotransferase II gene. Various factors were found to influence the transfer-DNA delivery efficiency, such as explant tissue and surfactants present in the inoculation medium. The inoculated immature embryos or embryogenic calli were selected on G418-containing media. Transgenic plants were regenerated from all three types of explants. The total time required from inoculation to the establishment of plants in soil was 2.5 to 3 months. So far, more than 100 transgenic events have been produced. Almost all transformants were morphologically normal. Stable integration, expression, and inheritance of the transgenes were confirmed by molecular and genetic analysis. One to five copies of the transgene were integrated into the wheat genome without rearrangement. Approximately 35% of the transgenic plants received a single copy of the transgenes based on Southern analysis of 26 events. Transgenes in T_1 progeny segregated in a Mendelian fashion in most of the transgenic plants.

In the early 1980s, the era of plant transformation was initiated when *Agrobacterium tumefaciens*-mediated gene delivery was reported for the production of transgenic plants (De Block et al., 1984; Horsch et al., 1984, 1985). Initial successes were limited to the Solanaceae, tobacco in particular. This dramatically changed throughout the 1980s and into the 1990s, and it is now possible to transform a wide range of plants, including many agronomically important crops such as soybean, cotton, peanut, and pea (Hinchee et al., 1988; Umbeck et al., 1989; Schroeder et al., 1993; Cheng et al., 1996). Although *A. tumefaciens*-mediated transformation has significant advantages over naked DNA delivery, such as introduction of a few copies of genes into the plant genome, high co-expression of introduced genes, and easy manipulation in vitro, the *A. tumefaciens*-mediated transformation method for gene transfer has been limited to dicotyledonous plants (Songstad et al., 1995).

Several reports presented early attempts to transform the Gramineae with *A. tumefaciens*, including *A. tumefaciens*-mediated infection of plants with viral genomes (Grimsley et al., 1988; Raineri et al., 1990; Gould et al., 1991; Mooney et al., 1991; Chan et al., 1992, 1993; Schläppi and Hohn, 1992; Shen et al., 1993). Chan et al. (1993) first reported the production of transgenic rice plants by inoculating immature embryos with an *A. tumefaciens* strain and proved the transformation by molecular and genetic analysis. Recently, significant progress was made in *A. tumefaciens*-mediated transformation of rice and maize: a large number of transgenic plants were regenerated and characterized (Hiei et al., 1994; Ishida et al., 1996). Convincing and unambiguous data on transgene expression, gene segregation in the progeny, and DNA analysis were presented in these papers.

There have been limited studies on *A. tumefaciens*-mediated transformation of wheat (*Triticum aestivum* L.). Hess et al. (1990) pipetted *A. tumefaciens* into the spikelets of wheat, and several kanamycin-resistant grain progeny were obtained. However, the protocol was not reproducible and the Southern hybridization was not convincing in this study. Deng et al. (1990) infected the base of the leaf sheath and spike stem of wheat plants with several wild-type *A. tumefaciens* strains and opine-synthesizing tumors formed from these tissues. Mooney et al. (1991) infected the immature embryos of wheat with *A. tumefaciens* and a few kanamycin-resistant colonies were generated.

Here we present an *A. tumefaciens*-mediated transformation method for wheat using freshly isolated immature embryos, precultured immature embryos, and embryogenic calli as explants. We produced a large number of transgenic plants and demonstrated stable integration, expression, and inheritance of transgenes in wheat plants.

MATERIALS AND METHODS

Stock Plants and Explant Tissues

A spring wheat, *Triticum aestivum* cv Bobwhite, was used throughout this study. Stock plants were grown in an environmentally controlled growth chamber with a 16-h photoperiod at 800 μmol m^{-2} s^{-1} provided by high-intensity discharge lights (Sylvania, GTE Products Corp., Manchester, NH). The day/night temperatures were 18/16°C. Immature caryopses were collected from the plants 14 d after anthesis. Immature embryos were dissected aseptically and cultured on a semisolid or liquid CM4 medium (Zhou et al., 1995) with 100 mg L^{-1} ascorbic acid (CM4C).

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Abbreviations: MS, Murashige-Skoog; T-DNA, transfer-DNA.

The MS salts (Murashige and Skoog, 1962) in the CM4C medium were adjusted to full strength (the original amounts) or one-tenth-strength (Fry et al., 1987). The immature embryos were cultured on these media for 3 to 4 h (freshly isolated) or 1 to 6 d (precultured). Embryogenic calli were prepared by culturing the immature embryos on CM4C medium for 10 to 25 d. The callus pieces derived from immature embryos were inoculated with *A. tumefaciens* without being broken down (intact), or only the embryogenic callus sectors were selected and separated into small pieces (approximately 2 mm).

A. tumefaciens Strain, Plasmid, and Culture

Disarmed *A. tumefaciens* C58 (ABI) harboring binary vector pMON18365 (Fig. 1) was used for all the experiments. pMON18365 contains the GUS (*uid*A) gene with an intron and the NPT II gene as a selectable marker within the T-DNA region. Each gene was under the control of an enhanced 35S (E35S) promoter. Cultures of *A. tumefaciens* were initiated from glycerol stocks and grown overnight at 25 to 26°C with shaking (150 rpm) in liquid Luria-Bertani medium (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0) containing 50 mg L^{-1} kanamycin, streptomycin, spectinomycin, and 25 mg L^{-1} chloramphenicol with 200 μM acetosyringone, to mid-log phase (OD_{660} = 1–1.5). The *A. tumefaciens* cells were collected by centrifugation and resuspended in liquid inoculation medium (CM4C with one-tenth-strength MS salts and supplemented with 10 g L^{-1} Glc and 200 μM acetosyringone). The *A. tumefaciens* cell density was adjusted to give an A_{660} of 1 to 2 for inoculation.

Inoculation and Co-Cultivation

The immature embryos and embryogenic calli maintained on the CM4C medium as described above were transferred into an *A. tumefaciens* cell suspension in Petri dishes. A surfactant (0.01–0.075% [v/v] Silwet, Monsanto, St. Louis, MO) or pluronic F68 (0.01–0.2% [w/v] Sigma) was added to the inoculation medium in some experiments. The inoculation was conducted at 23 to 25°C for 3 h in the dark. After inoculation the *A. tumefaciens* cells were removed by vacuum or with a transfer pipette, and the explants were placed on semisolid or on a filter paper wetted with liquid CM4C with one-tenth-strength or full-strength MS salts and supplemented with 10 g L^{-1} Glc and 200 μM acetosyringone. The co-cultivation was performed at 24 to 26°C in the dark for 2 or 3 d.

Selection and Regeneration of Transgenic Plants

After co-culture the infected immature embryos and calli were cultured on the solid CM4C medium with 250 mg L^{-1} carbenicillin for 2 to 5 d without selection. *A. tumefaciens*-infected explants were then transferred to CM4C medium supplemented with 25 mg L^{-1} G418 and 250 mg L^{-1} carbenicillin for callus induction. Two weeks later, the explants were transferred to the first regeneration medium, MMS0.2C (consisting of MS salts and vitamins, 1.95 g L^{-1} Mes, 0.2 mg L^{-1} 2,4-D, 100 mg L^{-1} ascorbic acid, and 40 g L^{-1} maltose, solidified by 2 g L^{-1} gelrite) supplemented with 25 mg L^{-1} G418 and 250 mg L^{-1} carbenicillin.

At transfer to the regeneration medium, each piece of callus derived from one immature embryo or one piece of inoculated callus was divided into several small pieces (approximately 2 mm). In another 2 weeks, young shoots and viable callus tissues were transferred to the second regeneration medium, MMS0C, which contains the same components as MMS.2C with all antibiotics except 2,4-D included. When the shoots developed into about 3-cm or longer plantlets, they were transferred to larger culture vessels containing the second regeneration medium for further growth and selection. Leaf samples were taken from some of the plantlets for the GUS histochemical assay at this stage. Plants that were highly G418 resistant or GUS positive were transferred to soil. All of the plants derived from the same embryo or piece of callus were considered to be clones of a given event.

GUS Histochemical Assay

GUS activity was assayed histochemically in a 5-bromo-4-chloro-3-indolyl-β-glucuronic acid solution using the buffer described by Jefferson (1987) except that 20% methanol was added to eliminate the endogenous GUS activity.

Functional Assay of NPT II Genes

Paromomycin Spray

T_1 seeds harvested from each T_0 plant were planted in 2-inch pots grown under the same conditions as the stock plants as described above. When plants reached the 3-leaf stage, they were spayed with 2% (w/v) paromomycin (Sigma) plus 0.2% (v/v) Tween 20. One week later the plants were evaluated for paromomycin damage. The plants with a functional NPT II gene showed no bleached spots, whereas the plants without a functional NPT II gene exhibited bleached spots throughout. Paromomycin was used in this assay and the leaf-bleach assay as described in the following section because it is a similar aminoglycoside antibiotic to G418, and is more effective and less expensive for these assays.

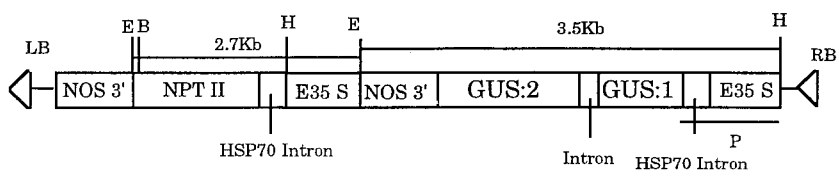

Figure 1. T-DNA regions of pMON 18365. RB, Right border; LB, left border; E35S, enhanced 35S promoter; HSP 70 intron, maize heat-shock protein 70 gene intron; NOS-3', 3' signal of nopaline synthase; NPT II, neomycin phosphotransferase II; H, *Hin*dIII; E, *Eco*RI; B, *Bam*HI; P, probe.

Leaf-Bleach Assay

After the T_0 plants were established in soil, leaf samples (5–7 mm long) were taken from the youngest fully expanded leaves and placed in a 24-well culture plate (Costar, Cambridge, MA). Each well was filled with 0.5 mL of a water solution composed of 300 mg L^{-1} paromomycin and 100 mg L^{-1} fungicide (Benlate, DuPont) or 100 mg L^{-1} fungicide only. Three leaf samples taken from the same leaf of each plant were placed in two wells containing paromomycin and fungicide and one well containing fungicide only, respectively. Leaf samples from the nontransformed cv Bobwhite plants at a similar developmental stage were used as a negative control. The samples were vacuum-infiltrated in a desiccator using an in-house vacuum system for 5 min and then the plates were sealed with Parafilm before being placed under light for 3 d. The leaf samples that were highly resistant to paromomycin remained green in most of the area except around the edges (<1 mm wide), indicating that the NPT II gene was functional. The leaf samples from the plants without the gene or with a nonfunctional gene were bleached completely by paromomycin (as were the negative controls) (Fig. 2D) or had only small patches of green areas.

DNA Analysis

Genomic DNA was isolated from leaf tissue of T_0 plants and T_1 progeny following the method of Roger and Bendich (1985). An equal amount of *Eco*RI-digested genomic DNA (15 μg per lane) was separated on an agarose gel, blotted onto a membrane, and probed with a ^{32}P-labeled fragment containing the enhanced 35S promoter and the 5′ intron of the heat-shock protein 70 gene from maize following the manufacturer's protocol for the GeneScreen Plus membrane (DuPont).

Progeny Analysis

The segregation of the GUS and NPT II genes in the progeny of T_1 or reciprocal crosses was determined by one of the following methods: (a) paromomycin spray on the T_1 seedlings and GUS histochemical assay on leaf tissue, as described above; (b) leaf-bleach assay on the T_1 seedlings at the two-leaf stage; and (c) GUS histochemical assay on the immature (17 d after anthesis or older) and mature seeds harvested from the T_0 plants. The immature seeds were sterilized in 10% (v/v) bleach (containing 5.25% sodium hypochlorite) for 15 min followed by three rinses with sterile water. The mature seeds were soaked in the water for several hours and then sterilized in 20% bleach for 40 min. Finally, the seeds were washed in sterile water three times for 30 min each. Each seed was longitudinally cut into two uneven parts. The embryo from the large part was isolated and cultured on the MMS0C medium for germination. The seedlings were eventually transferred to soil. The small part and the large part without the embryo were used for the GUS histochemical assay. The T_1 seeds with or without the functional GUS gene could be determined based on the GUS activity in the embryo and endosperm tissues (Fig. 2H). The plants in soil were also assayed by leaf-bleach assay for the NPT II activity and GUS histochemical assay at different stages. The data were then analyzed by the χ^2 test to determine the number of the functional GUS or NPT II gene loci.

RESULTS

Factors Influencing the Efficiency of T-DNA Delivery

Various factors influencing the efficiency of T-DNA delivery were evaluated in the preliminary experiments. These factors include different explant types, *A. tumefaciens* cell density for inoculation, inoculation and co-culture time period, co-culture medium, surfactants in the inoculation medium, and induction agents in the inoculation and co-culture media. Leaf tissue from young seedlings, immature inflorescences, freshly isolated immature embryos, or precultured immature embryos, embryogenic callus derived from immature embryos, and cells in suspension cultures derived from wheat cv Mustang were inoculated and co-cultured with *A. tumefaciens* ABI:pMON18365.

GUS expression was detected in all of the tissues after either 2 or 3 d of co-culture and a 2-d delay of selection. Highly efficient T-DNA delivery was observed on both freshly isolated immature embryos and precultured immature embryos when surfactant (Silwet) was present in the inoculation medium (Fig. 2, A and B). The GUS spots were present across all of the scutellum surface of freshly isolated immature embryos, whereas most of the GUS spots were localized on the areas starting to form callus in the precultured immature embryos. Leaf sections, when vacuum infiltration was applied during inoculation, showed high GUS activity. The suspension cells exhibited the highest-efficiency T-DNA delivery even without the addition of surfactant in the inoculation medium. Therefore, the suspension cells were chosen as a model system to optimize the transformation parameters for wheat (M. Cheng, J.E. Fry, C.M. Hironaka, and T.W. Conner, unpublished data).

A moderate number of GUS spots were observed on the embryogenic callus, whereas the spots were usually larger than in the immature embryos. Higher *A. tumefaciens* cell density and a longer time for inoculation and co-culture usually yielded more efficient T-DNA delivery on various tissues or cells, but more cell damage was observed. The salt strength in the inoculation medium was also found to influence the T-DNA delivery. For example, when one-tenth-strength MS salts were used for the inoculation and co-culture medium, transient GUS expression was significantly higher on the freshly isolated immature embryos than when the full-strength MS salts were used.

Another significant factor influencing T-DNA delivery was the presence of a surfactant in the inoculation medium. Two types of surfactants were evaluated based on T-DNA delivery efficiency with different wheat tissues. Both Silwet and pluronic F68 were found to have a significant positive effect on the transient GUS expression on different explants, especially on the immature embryos (Table I; Fig. 2A). Silwet at 0.01% started to enhance the transient GUS

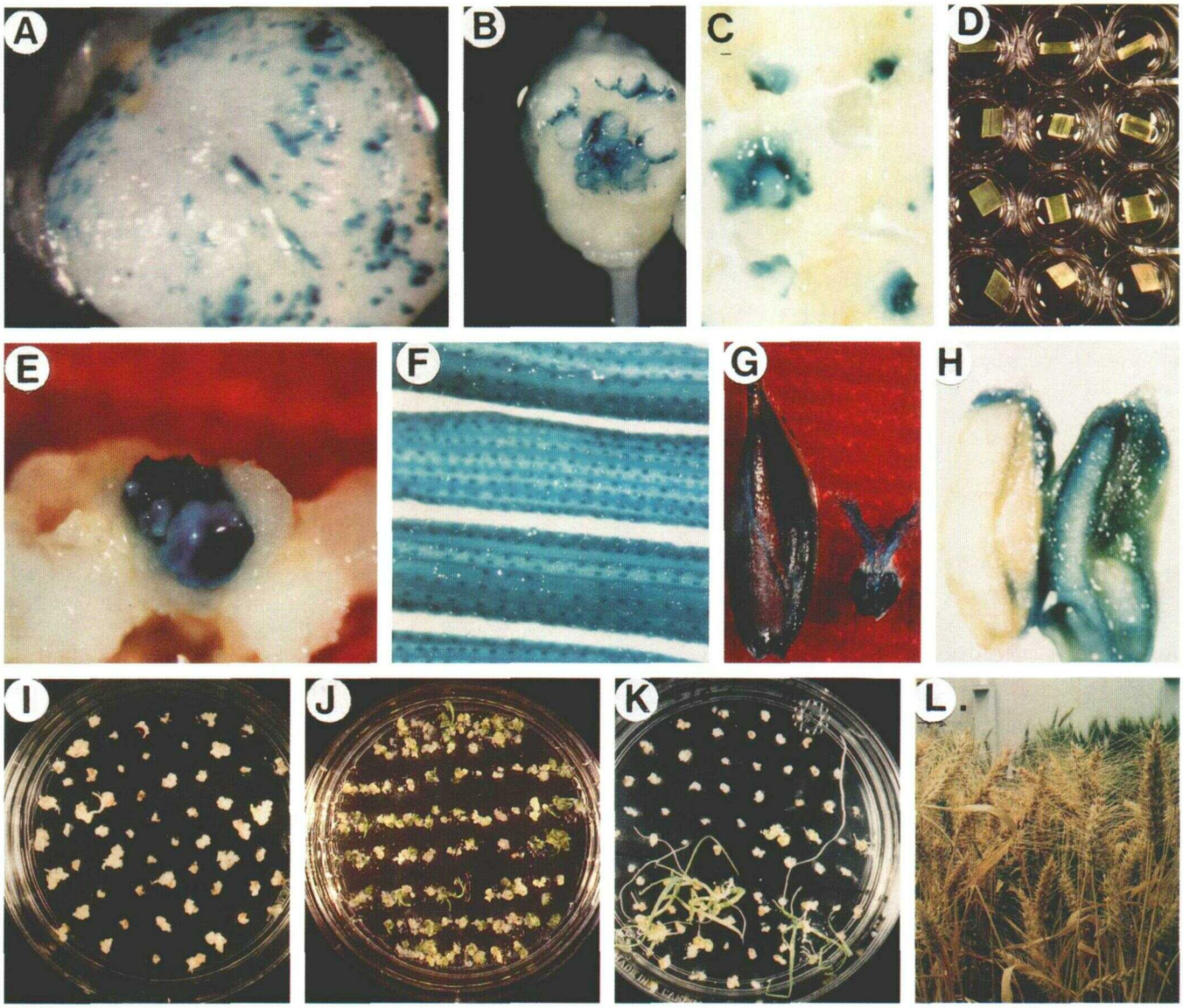

Figure 2. Transient GUS expression in *A. tumefaciens*-infected explants, stable GUS expression in various tissues from transgenic plants, and steps in the regeneration of transgenic plants. A, Transient GUS expression in a freshly isolated immature embryo 4 d after inoculation. B, Transient GUS expression in a precultured immature embryo 3 d after inoculation. C, Transient GUS expression in embryogenic calli 5 d after inoculation. D, Leaf-bleach assay. The wells of the first column (left) contained 100 mg L^{-1} fungicide-water solution and the remaining wells contained the 300 mg L^{-1} paromomycin and 100 mg L^{-1} fungicide-water solution. The first three rows of wells included leaf samples from three transgenic events with functional NPT II activity. The last column (bottom) was a leaf sample from a nontransgenic plant as a control. E, GUS expression in stably transformed, embryo-like tissue. *A. tumefaciens*-infected freshly isolated immature embryo was cultured on G418-containing CM4C medium for 3 weeks. F, GUS expression on young leaf tissue from a transgenic plant. G, GUS expression in a young ovary and glume tissues of a transgenic plant. H, Segregation of the GUS expression in T_1 seeds assayed at 20 d after anthesis from a GUS-positive T_0 plant. Some seeds showed GUS activity in both the pericarp (the maternal tissue) and the aleurone layer (right), and others had GUS activity only in the pericarp (left). I, Callus induction on G418-containing CM4C medium. Inoculated immature embryos were cultured on selective callus-induction medium CM4C for 2 weeks. J, Shoot regeneration from embryogenic calli after 2 weeks of culture on first-regeneration medium MMS.2C containing G418. K, Plantlet regeneration after the embryogenic calli or shoots were cultured on second-regeneration medium MMS0C containing G418 for 2 weeks. L, Transgenic T_0 plants set seeds in a growth chamber.

expression on the scutellum side of the embryos. The concentration of Silwet at 0.05% gave the highest transient GUS expression, approximately 19-fold higher than the control. However, when the concentration of Silwet was greater than 0.05%, most of the immature embryos could not survive. Based on this result, 0.01 to 0.02% Silwet was used routinely in our stable transformation experiments. Pluronic F68 at 0.01 to 0.05% had the same effect as Silwet

Table I. *Effect of surfactant when present in the inoculation medium on transient GUS expression in freshly isolated immature embryos (IE)*

Concentration of Surfactant (Silwet)	IEs with GUS Spots	GUS Spots/IE
% (v/v)	% of total	
0.00	11/34 (34)	7.8
0.01	15/19 (79)	17
0.05	13/13 (100)	149
0.1	8/8 (100)	111
0.5	4/4 (100)	140

on the transient GUS expression on the immature embryo explants. Although both Silwet and pluronic F68 enhanced the efficiency of T-DNA delivery on the precultured immature embryos and embryogenic calli, they were not as significant as in the immature embryos. Silwet and pluronic F68 at 0.02% increased the transient GUS expression approximately 4-fold in the embryogenic calli compared with the control. An average of 30 blue spots was observed on each embryogenic callus (14 d old, intact) (Fig. 2C).

The presence of induction agents such as acetosyringone and Glc in the inoculation and co-culture media was crucial for efficient T-DNA delivery on some of the explants. For example, when acetosyringone and Glc were absent in the inoculation and co-culture media, the T-DNA delivery efficiency was significantly reduced in the freshly isolated immature embryos.

Regeneration of Transgenic Wheat Plants from Various Explants

A. tumefaciens-infected immature embryos and embryogenic calli were cultured on callus-induction medium CM4C with G418 for selection. Two weeks after callus induction, approximately 30 to 80% of the immature embryos formed embryogenic callus (Fig. 2I), whereas the inoculated embryogenic calli proliferated further on this medium. The GUS assay on some of the explants at this stage showed that the transformed, embryo-like tissue had developed from some of the inoculated explants (Fig. 2E). Developed calli were then broken down into small pieces, and transferred to the first regeneration medium for further selection. Multiple green shoots (most of them were not transformed) regenerated rapidly from the embryogenic calli (Fig. 2J). After 2 weeks of selection on the first regeneration medium, all of the viable shoots and callus tissues were transferred to the second regeneration medium, MMS0C with G418, for further selection (Fig. 2K). On this medium the most likely transformed shoots showed high resistance to G418, whereas most of the non-transformed shoots were not able to grow rapidly. Finally, highly resistant plantlets were transferred to larger culture vessels for further growth and selection. The transformed plants usually grew vigorously and formed strong root systems on the G418-containing MMSOC medium. The plants that survived the selection were moved to soil when they were approximately 10 to 15 cm in length.

Identification of Transgenic Plants and Transformation Efficiency

Most of the transgenic plants were identified by the GUS assay on the leaf tissues while the plantlets grew in the regeneration medium. After they were moved to soil, different tissues were collected at various stages for additional histochemical GUS assay. Leaf samples were also collected after the plants survived in soil for the leaf-bleach assay. Most of the plants had visible GUS activity in different tissues (Fig. 2, F–H), although the younger leaf tissue had higher activity than the older tissue, and young floral tissue had higher activity than the leaf tissue. However, a few of the plants that showed no visible GUS activity in leaf tissue had relatively high GUS activity in young floral tissues such as young ovary, stigma, glume, and lemma.

All of the plants showing GUS expression also had NPT II activity determined by the leaf-bleach assay except one that showed high-NPT II activity but no detectable GUS activity in any of the tissues (Table IV, event 21). The co-expression of GUS and NPT II genes in the plants produced via *A. tumefaciens*-mediated transformation was over 98% (49/50, Table IV) in our study. In contrast, the co-expression of the gene of interest (including the GUS gene) and the NPT II gene in the plants generated using the biolistic method, with either co-bombardment or 2 genes in the same construct, was from 42 to 62% in our laboratory, based on the analysis of 343 events with 4 different genes of interest. Therefore, the co-expression of two genes in the transgenic plants was significantly higher with the *A. tumefaciens*-mediated transformation than with the biolistic method.

Transgenic plants produced from all three kinds of explants are summarized in Table II. The transformation efficiencies for the freshly isolated immature embryos, precultured immature embryos, and embryogenic calli were 1.12% ± 0.79 ($\bar{X}$% ± SE), 1.56% ± 1.19, and 1.55% ± 1.08, respectively, no significant difference in the transformation efficiency was shown among the three explant types, although it varied among experiments. Transgenic plants could be regenerated from all three explants; however, several experiments with all three explants actually failed to produce any transgenic plants. These experiments were not included in Table II. The freshly isolated immature embryos always showed efficient transient GUS expression when the surfactants were present in the inoculation medium, but they could not recover well after inoculation and co-cultivation.

Although many different media and co-culture conditions were attempted, the majority of the inoculated immature embryos failed to form embryogenic calli or formed very limited calli on the scutellum surface. Precultured embryos usually showed good transient GUS expression on areas starting to form callus, and also exhibited better culture response. Among these three explants, embryogenic callus cultured for more than 10 d in the callus-induction medium showed the best culture response. Usually, 100% of the explants continued to proliferate on the callus-induction medium with the selection agent present.

Table II. *Summary of transformation results using three kinds of explants*

Experiment	Explant[a]	Explants (A)	Transgenic Events (B)	Transformation Efficiency (B/A)
		no.		%
1	FIIE	160	1	0.6
2	FIIE	250	3	1.2
3	FIIE	700	1	0.14
4	FIIE	124	1	0.8
5	FIIE	140	2	1.4
6	FIIE	38	1	2.6
7	PCIE (1 d)	23	1	4.3
8	PCIE (3 d)	98	1	1.0
9	PCIE (3 d)	104	2	1.9
10	PCIE (3 d)	36	1	2.8
11	PCIE (5 d)	97	1	1.0
12	PCIE (6 d)	40	1	2.5
13	EC (10 d)	239	1	0.4
14	EC (10 d)	232	1	0.4
15	EC (14 d)	47	1	2.1
16	EC (15 d)	110	3	2.7
17	EC (17 d)	50	1	2.0
18	EC (21 d)	73	2	2.7
19	EC (25 d)	308	1	0.3

[a] FIIE, Freshly isolated immature embryo; PCIE, precultured immature embryo; EC, embryogenic callus. The number of days of the immature embryos cultured on callus induction medium (CM4C) prior to inoculation is given in parentheses.

Characterization of the T_0 Plants

Plants identified as transgenic were grown in a growth chamber and evaluated for morphology and fertility. More than 100 events were established in soil and examined (Fig. 2L). All of the plants were fertile or partially fertile. The majority (about 80%) of the transformed plants produced as many seeds as the seed-derived control plants.

DNA was extracted from leaf tissue of 26 T_0 plants derived from independent events, and digested with *Eco*RI and hybridized with a probe consisting of E35S and the maize HSP 70 intron sequence (Fig. 3). DNA from nontransformed plants used as a negative control showed no hybridization to the probe. Since the T-DNA of pMON18365 had two *Eco*RI sites, the 2.7-kb band represented the internal fragment with the NPT II gene cassette without nos 3′ (Fig. 1). All 26 transgenic events had the 2.7-kb band (Fig. 3; Table III).

Because the third *Eco*RI site must be derived from the wheat genome, the number of hybridizing bands around or greater than 3.5 kb reflected the number of copies of the integrated gene (GUS) in the plants unless repeats of multiple copies of the T-DNA had been integrated. All of the detected bands except the 2.7-kb band represented the fragments of more than 3.5 kb. The mobilities of the bands differed from plant to plant, indicating independent events and random integration. The copy number of the integrated gene (GUS) varied from 1 to 5 (Fig. 2; Table III). A single copy of the transgene (GUS) was carried by 35% of the plants (9/26), and 50% (13/26) contained two or three copies. Only 15% of the plants (4/26) carried four to five copies of the transgene.

Inheritance of Transgenes

The selfed and backcrossed progeny were evaluated for resistance to paromomycin and GUS expression in the T_1 seeds or T_1 plants. The segregation patterns of 50 events are shown in Table IV. Paromomycin-resistant and -sensitive seedlings or GUS-positive and -negative seeds or plants were clearly distinguishable by spraying the paromomycin on the seedlings or by histochemical GUS assay. A segregation ratio of 3:1 was observed for 22 out of 50 (44%) independent events, indicating a single functional GUS or NPT II gene locus. Twenty-two percent of the events (11/50) had two or more functional loci. Thirty-two percent (16/50) of the events had a non-Mendelian segregation pattern; that is the GUS or NPT II gene segregated at a 1:1 ratio, or the number of GUS-negative or paromomycin-sensitive plants was greater than the number of GUS-positive and paromomycin-resistant plants.

Six out of eight events (nos. 5, 15, 16, 17, 25, and 28) containing a single copy of the GUS gene based on Southern analysis showed a 3:1 segregation ratio of GUS-positive plants to GUS-negative T_1 plants. If more than one copy of

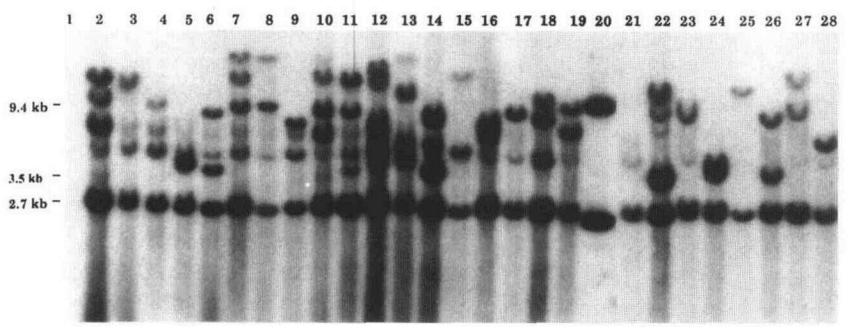

Figure 3. Southern analysis of T_0 transgenic events. DNA samples (15 μg) from 26 T_0 transgenic events (lanes 2–19 and 21–28; the lane number is the same as the event number) and one nontransformed wheat plant (lane 1) were digested with *Eco*RI, and the resulting fragments were resolved by electrophoresis and transferred to a membrane. The membrane was hybridized with a ^{32}P-labeled DNA probe corresponding to E35S promoter and the 5′ intron of maize HSP 70 gene. In lane 20, 5 ng of pMON18365 DNA digested with *Eco*RI was loaded as a positive control. The positions and lengths, in kilobars, of the molecular size markers are indicated.

Table III. *Copy number and functional loci of the GUS gene in transgenic events*

Events	Copy No. (GUS gene)	2.7-kb *Eco*RI	Functional Loci[a]
2	3	+	NA
3	2	+	?
4	3	+	1
5	1	+	1
6	2	+	1
7	4	+	2
8	2	+	1
9	2	+	1
10	3	+	?
11	4	+	?
12	5	+	2 or more
13	3	+	1
14	3	+	?
15	1	+	1
16	1	+	1
17	1	+	1
18	3	+	3
19	2	+	1
21	1	+	?
22	4–5	+	3 or more
23	1	+	?
24	1	+	NA
25	1	+	1
26	2	+	?
27	2	+	NA
28	1	+	1

[a] NA, Not analyzed; ?, the functional loci could not determined based on the segregation data because of the non-Mendelian segregation fashion in those events.

the gene was inserted in the plant genome, the estimated functional loci based on the segregation data were less than the copy number measured by Southern analysis in almost all of the cases (nos. 4, 6, 7, 8, 9, 13, and 19). The consistency of functional loci and the real copy number was observed in only one event (no. 18), which contained three functional loci and three copies of the gene.

The segregation ratios in the T_1 progeny from the reciprocal crosses of T_0 events 13, 28, 29, 30, and 60 are summarized in Table V. Events 13 and 28 had the 3:1 segregation ratio in the selfed progeny, whereas the progeny from the reciprocal crosses had a 1:1 segregation ratio. This result indicates that the transgenes were able to pass to the progeny through both male and female gametes.

The T_1 progeny from T_0 plants 18 and 28, which gave segregation patterns of 63:1 and 3:1 for GUS expression, respectively, were analyzed by Southern hybridization (Fig. 4). The T_0 plant 18 had three inserts, and two of the T_1 plants had exactly the same bands as their parent (lanes 3 and 4). Two other T_1 plants (lanes 5 and 6) had a band with the same size, and another band with a distinct size, indicating that the DNA coding the GUS gene segregated in the progeny. These results suggest at least two independent inserts in event 18. Because T_0 event 18 gave a segregation pattern of 63:1 for GUS expression, all three inserts should be independent. The T_0 event 28 had one insert (lane 7), and two of the T_1 plants (lanes 9 and 10) had the same pattern as their parent. One GUS-negative T_1 plant (lane 8) from T_0 event 18, which was selected as an example, did not show any hybridization signal.

Mendelian segregation for paromomycin resistance and GUS expression was also observed in the T_2 progeny plants 28 and 49 as an example. Two-thirds of the GUS-positive T_1

Table IV. *Segregation of the NPT II and GUS genes in the T_1 progeny*

Events	T_1 Plants Assayed by Paromomycin Spray			T_1 Plants Assayed for GUS Activity		
	Resistant (R)	Sensitive (S)	R/S	Positive (+)	Negative (−)	+/−
3	14	17	1:1	14	7	1:1
4	20	11	3:1	20	11	3:1
5	24	11	3:1	24	11	3:1
6	28	6	3:1	28	6	3:1
7	33	1	15:1	33	1	15:1
8	29	6	3:1	29	6	3:1
9	26	6	3:1	26	6	3:1
10	14	17	1:1	14	17	1:1
11	11	23	1:2	11	23	1:2
12	32	0	32:0	32	0	32:0
13	29	7	3:1	29	7	3:1
14	12	22	1:2	12	22	1:2
15	30	5	3:1	30	5	3:1
16	21	9	3:1	21	9	3:1
17	32	4	3:1	32	4	3:1
18	52	9	3:1	59	1	63:1
19				78	17	3:1
21	30	8	3:1	0	40	0:40
22				74	0	74:0
23				28	32	1:1
25				74	27	3:1
26				2	98	1:49
28	24	9	3:1	24	9	3:1
29	37	1	15:1	37	1	15:1
30	34	3	15:1	34	3	15:1
31	35	0	15:0	35	0	15:0
32	24	10	3:1	24	10	3:1
33	32	2	15:1	32	2	15:1
34	26	6	3:1	26	6	3:1
35	27	8	3:1	27	8	3:1
36	3	30	1:10	3	30	1:10
37	6	21	1:3	6	21	1:3
38	8	13	1:2	8	13	1:2
39	1	34	1:34	1	34	1:34
41	8	15	1:1	8	15	1:1
42	28	6	3:1	28	6	3:1
43	33	0	15:0	33	0	15:0
44	13	1	15:1	13	1	15:1
45	18	5	3:1	18	5	3:1
46	20	0	15:1	20	0	15:1
48	7	5	1:1	7	5	1:1
49	12	2	3:1	12	2	3:1
50	6	20	1:3	6	20	1:3
51	25	6	1:3	25	6	1:3
52	22	14	1:1	22	14	1:1
53	28	8	3:1	28	8	3:1
54	10	10	1:1	10	10	1:1
55	21	9	3:1	21	9	3:1
56				22	9	3:1
57	44	13	3:1	47	13	3:1

Table V. *Segregation of the NPTII gene in the progeny from the reciprocal crosses*

Crosses	Resistant Plants (R)	Sensitive Plants (S)	R:S
	no.		
No. 13 selfing	26	6	3:1
No. 13 X BW	8	3	1:1
BW X 13	20	13	1:1
No. 28 selfing	49	20	3:1
No. 28 X BW	11	15	1:1
BW X 28	5	8	1:1
No. 29 selfing	67	4	15:1
No. 29 X BW	9	2	3:1
BW X 29	10	4	3:1
No. 30 selfing	69	3	15:1
No. 30 X BW	22	9	3:1
BW X 30	16	4	3:1
No. 60 selfing	41	17	3:1
No. 60 X BW	16	12	1:1
BW selfing	0	76	NA

[a] BW, cv Bobwhite.

plants from both T_0 plants produced GUS-positive and GUS-negative T_2 at a ratio of 3:1. The T_2 progeny of the remaining one-third were exclusively GUS positive. The T_2 progeny of GUS-negative T_1 plants from both plants maintained the same expression pattern. These results suggested that the T_1 generation segregated into both homozygotic and heterozygotic plants, and that the transgenes were stably passed to their progeny in a Mendelian fashion.

DISCUSSION

We are reporting a rapid transformation method for wheat via *A. tumefaciens*. Our results showed strong evidence that the T-DNA was stably integrated into the wheat genome and transmitted to the progeny. Over 100 independent transformants have been regenerated, and one-half of them were characterized. This study and the studies on rice and maize transformation mediated by *A. tumefaciens* (Hiei et al., 1994; Ishida et al., 1996) have provided strong support that monocotyledons can be transformed as dicotyledons using *A. tumefaciens* by manipulating various factors such as explant tissues, inoculation, and co-culture conditions, as well as the *A. tumefaciens* strain and the combination of the *A. tumefaciens* strain and plasmid.

All of the studies of *A. tumefaciens*-mediated transformation of maize or rice used two strains, A281 or its derivative, EHA101 (Hood et al., 1986), and LBA4404 (Chan et al., 1993; Hiei et al., 1994; Aldemita and Hodges, 1996; Ishida et al., 1996; Rashid et al., 1996). The performance of the so-called "super-virulent" strain has been emphasized in some of the reports. Successful transformation of maize using *A. tumefaciens* was reported only when the "super-binary" vector was used. In the present study a nopaline *A. tumefaciens* strain C58 carrying the "ordinary" binary vector was used for infecting various explants, and this strain appeared to work efficiently. Stable transformants could be obtained from nonregenerable wheat suspension-cultured cells, and from regenerable immature embryos and embryogenic calli. Transgenic plants were successfully produced from all of the regenerable explants used.

Various factors influenced the T-DNA delivery and stable transformation efficiency. Inoculation and co-culture conditions can be varied so as to favor the plant cell survival. Different tissues or cells exhibited various abilities to survive after *A. tumefaciens* infection. For example, precultured immature embryos, embryogenic calli, and suspension cells, which were cultured for a period of time prior to inoculation, showed better survival than the freshly isolated immature embryos. Therefore, higher *A. tumefaciens* cell densities, higher concentrations of the surfactant, and longer amounts of time may be used for inoculating these explants. Acetosyringone and Glc can be added to the inoculation and co-culture media, particularly when using the freshly isolated immature embryos.

The T-DNA delivery efficiency was significantly decreased when acetosyringone was absent. The similar observation was also noticed in rice and maize transformation (Hiei et al., 1994; Ishida et al. 1996). However, in our study with wheat suspension-cultured cells, exogenous induction agents such as acetosyringone and Glc were not necessary for the stable transformation (M. Cheng, J.E. Fry, C.M. Hironaka, and T.W. Conner, unpublished data). These results suggested that different tissues or cell types may have different competence for *A. tumefaciens* infection. Based on our results, the acetosyringone and Glc were recommended to be included in the inoculation and co-culture media for the stable transformation of the regenerable explants.

Surfactant present in the inoculation medium was one of the important factors noticed in this study. Two surfactants, Silwet and pluronic F68, proved to have a positive effect on the T-DNA delivery. The possible explanation for the effect the surfactants on enhancing T-DNA delivery might be the surface-tension-free cells favoring the *A. tumefaciens* attachment. We also tested other surfactants such

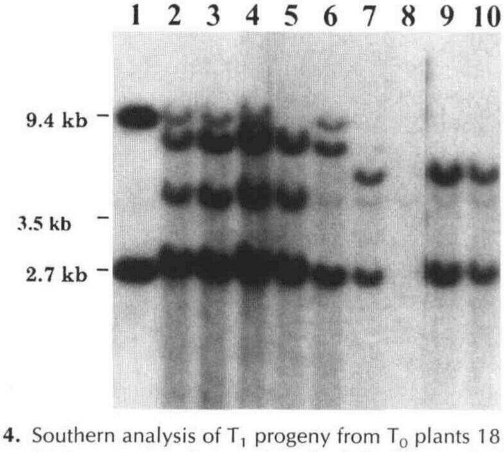

Figure 4. Southern analysis of T_1 progeny from T_0 plants 18 and 28. Southern blots were made as described in Figure 3. Lane 1, pMON18365 DNA as a positive control. Lane 2, DNA samples from T_0 plant 18. Lanes 3 through 6, DNA samples from T_1 progeny of T_0 plant 18. Lane 7, DNA sample from T_0 plant 28. Lane 8, DNA sample from GUS-negative T_1 plant of T_0 plant 28. Lanes 9 and 10, DNA samples from T_1 plants of T_0 plant 28. The positions and lengths, in kilobases, of the molecular size markers are indicated.

as Tween 20 and Triton X, which appeared to be too toxic to the wheat tissues even when only a small amount was added to the inoculation medium.

This transformation system was efficient and required only 2.5 to 3 months from inoculation to transfer of the plants to soil. The transformation efficiency was as high as 4%. Most of the published studies on wheat transformation by the biolistic method showed that it took a fairly long time for tissue culture and regeneration (from 12–28 weeks) (Vasil et al., 1993; Weeks et al., 1993; Becker et al., 1994; Nehra et al., 1994; Zhou et al., 1995; Ortiz, et al., 1996), and the transformation efficiency was from 0.1 to 5.7%. Altpeter et al. (1996) reported a protocol for accelerated production of transgenic wheat by particle bombardment in which 8 to 9 weeks were required to produce transgenic plants after the initiation of culture and the transformation efficiency was up to 2%. Using the same regeneration and selection protocol presented in this paper, up to 20% transformation efficiency can be achived in our laboratory through the biolistic approach. We think that once the inoculation and co-culture conditions are further optimized to obtain efficient T-DNA delivery with conditions favoring plant cell recovery, the transformation efficiency may be improved to as high as with the biolistic method.

Southern analysis showed different hybridization patterns among all of the tested T_0 transformants, indicating that T-DNAs were randomly integrated into the wheat genome. The T-DNA fragments that hybridized to the probe consisting of the E35S promoter and the maize HSP70 intron clearly did not derive from the vectors in the free *A. tumefaciens* cells that might exist in the plants regenerated from inoculated explants; otherwise, there would have been two bands, as in the control lane. Based on the samples tested, approximately 35% of the plants have single inserts, which was close to that observed in rice (32%) (Hiei et al., 1994), but significantly lower than that in maize (60–70%) (Ishida et al., 1996). The differences could be due to the plant species, explant types, or other factors such as *A. tumefaciens* strain and plasmid. The number of events with a single insert produced using the *A. tumefaciens*-mediated transformation was significantly higher than that with the biolistic method. Using similar constructs, the same cultivar and regeneration and selection protocol, 77 events were produced via the biolistic method in our laboratory. Only 17% (13/77) plants contained single copies of transgenes (data not shown).

The genetic analysis of T_1 and T_2 progeny also provided strong evidence of the incorporation of T-DNA into the wheat genome. The NPT II and GUS genes were inherited to the T_1 and T_2 generations in a Mendelian fashion in most of the events. The data from the Southern analysis of the T_1 generation supported the genetic data in most of the cases, although non-Mendelian segregation patterns were observed occasionally. Similar results were also reported in rice and maize (Hiei et al., 1994; Ishida et al., 1996) and in dicot species transformed by *A. tumefaciens* (Hobbs et al., 1990; Ulian et al., 1994). Gene silence and nondetectable gene expression level in the transgenic plants might be partially responsible for causing the abnormal segregation patterns.

In summary, we have developed a method for rapid production of transgenic plants via *A. tumefaciens* from three kinds of explants of wheat. The transformation efficiency was 0.14 to 4.3% based on the experiments that produced the transgenic plants. The transformed plants appeared to be morphologically identical to nontransformed, growth chamber-grown control plants. In most of the cases, the transformed genes behaved as dominant loci exhibiting normal Mendelian segregation. Therefore, an *A. tumefaciens*-mediated transformation system is now available as an alternative routine method for genetic transformation of wheat.

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