Transient gene expression in *Zoysia japonica* using *Agrobacterium tumefaciens*

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ABSTRACT

A protocol is presented for an efficient transient transformation of Japanese lawn grass (*Zoysia japonica* Steud. cv 'YK-EM2') using *Agrobacterium tumefaciens*. Factors such as infection period, co-cultivation period, use of 4-acetyl-2, 6-dimethoxyphenol (acetosyringone, AS) different *Agrobacterium* strains and hygromycin concentrations were evaluated. Of the five *A. tumefaciens* strains tested LBA 4404 (pIG-121) gave the best results (60% of the callus were GUS positive) during a 24 hour infection period. Four days (96 hr) of co-cultivation with *A. tumefaciens* strain LBA 4404 (pIG121Hm) was the most suitable for transformation compared to the other strains tested. AS actively induced the transfer of T-DNA from *Agrobacterium* to the infected plants. An AS 50mg·L^{-1} was optimum for successful transformation of *Zoysia*. Hygromycin had similar effects of increasing ß-glucuronidase (GUS)-transient activity. *A. tumefaciens* strain LBA 4404 (pIG-121) was the most efficient among the six strains tested in this study for GUS transient assay for a range of commercially important *Zoysia* cultivars. Days of infection, co-cultivation, concentration of AS and hygromycin can greatly influence the efficiency of transformation in *Zoysia*. These results open the way for the utilization of transgenic plants in breeding and improving *Zoysia* turfgrasses.

RESUMEN

En este artículo se presenta un protocolo de transformación transitoria del pasto japonés (*Zoysia japonica* Steud. cv 'YK-EM2') usando *Agrobacterium tumefaciens*. Se evaluaron el período de infección y de cocultivo, el uso de 4-acetyl-2, 6-dimetoxyfenol (acetosyringone, AS), el uso de diferentes cepas de *Agrobacterium* y diferentes concentraciones de hygromicina. De las cinco cepas de *A. tumefacienss* estudiadas, LBA 4404 (plG-121) dio los mejores resultados (60% de callos positivos en la prueba de GUS) durante un período de infección de 24 horas. Cuatro días (96 horas) de cocultivo con la cepa LBA4404 (pIG121Hm) de *A. tumefaciens* fue lo mas apropiado para la transformación en comparación con las otras cepas estudiadas. El AS indujo activamente la transferencia de T-DNA de *Agrobacterium* a las plantas infectadas. Una concentración de 50mg/L de AS fue la óptima para la transformación exitosa de Zoysia. El uso de hygromicina incrementó de forma similar la expresión transitoria de la ß-glucuronidasa (GUS). La cepa LBA 4404 (pIG-121) de *A. tumefaciens* fue la mas eficiente entre las 6 cepas estudiadas en lo referente al ensayo de expresión transitoria de GUS en varios cultivares de *Zoysia* importantes comercialmente. Los días de infección, co-cultivo y la concentración de AS e hygromicina pueden influenciar considerablemente la eficiencia de transformación en *Zoysia*. Estos resultados abren el camino para la utilización de plantas transgénicas para el mejoramiento genético del pasto ornamental *Zoysia*.

Additional index words: Acetosyringone, ß-glucuronidase, Japanese turf-grass, Transgenic plants and Plasmids
Abbreviations: GUS, ß-glucuronidase; AS, acetosyringone and cv, cultivar

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The grass *Zoysia japonica* is a warm-season turf grass, and is the most important turf grass in Japan. It is also widely used in Japan for animal grazing as well as for home lawns, golf courses, athletic fields and parks. It is well adapted to the temperate and subtropical regions of East Asia and the United States, where it is commonly used for home lawns, parks, golf courses, and roadsides (Watson, 1989). The attributes that make it a highly desirable low-maintenance turf grass are low nutritional requirements, and tolerance to temperature extremes and salinity. While it is generally tolerant to a large number of diseases and insects, it is susceptible to large brown patch (*Rhizoctonia solani*) and hunting billbug (*Sphenophorus venatus vestitus*). As the utilization area of this species increases significantly, the demand for genetically improved cultivars with both disease and insect resistance or with extended greening periods increases. However, to date, genetic and plant breeding research on this turf grass is minimal and few improved cultivars have been developed (Engelke and Murray 1989). No stable transgenic techniques for lawn grasses have been developed. 'YK- EM2', a leading variety of *Zoysia japonica* is susceptible to several pathogens. To alleviate these shortcomings, genetic engineering including a reliable transformation system is required. Several gene introduction methods such as the electroporation method and polyethylene glycol (PEG) have been developed that may have widespread use for this species.

Although the development of transgenic turf grass plants has been reported for *Agrotis alba* (Asano and Ugaki, 1994), *A. palustris* (Hartman et al., 1994; Zhong et al., 1993), *Festuca rubra* (Spangenberg et al., 1994), *Festuca arundinacea* (Ha et al., 1992; Wang et al., 1992) and *Zoysia japonica* (Inocuma et al., 1998) by the PEG method, there are no reports on *Agrobacterium tumefaciens*-mediated gene transformation on *Zoysia japonica*.

Although a biotechnological approach could rapidly allow the development of cultivars with improved disease and insect resistance, cold tolerance, etc., there are few reports on the development of an *in vitro* cell culture system for *Zoysia japonica*, on which the approach depends (Inocuma et al., 1995). Plant regeneration through somatic embryogenesis is the major regeneration pathway in gramineous tissue culture (Vasil, 1988) and has several advantages for genetic manipulation because a large number of cells are competent to form transformed plants. This work describes an efficient *Agrobacterium*-based transient gene expression system for *Zoysia*.

MATERIALS AND METHODS

Zoysia cultivar and culture media. *Z. japonica* (cv. 'YK–EM2', from Yukijirushi, a Japanese Company) was obtained from Yukijirushi, Japan. Media used for tissue culture and transformations are listed in Table 1. Petri plates (9 cm

Table 1. Media used for tissue culture and transformation of *Zoysia japonica.*

Medium	Composition
MS-AZ	MS B5 vitamins (Chu et al., 1975), 30g•L^{-1} sucrose, 2, 4-D, 2mg•L^{-1}, 6BA 0.5mg•L^{-1}, Casamino acids 300mg•L^{-1}, Glycine 2mg•L^{-1}, Glucose 10g•L^{-1}, Gelrite 4g•L^{-1}, α-ketoglutaric acid 0.1g•L^{-1}, riboflavin 1mg•L^{-1}. pH. 5.8.
MS-AZ-AS	MS-AZ (Asano et al., 1996; Asano et al., 1996) supplemented with 50mg•L^{-1} acetosyringone.
AAM	(Hiei et al., 1994)
AB	(Chilton et al., 1974) 50mg•L^{-1} kanamycin, 50mg•L^{-1} hygromycin, 15 g•L^{-1} agar, pH 7.2.
HF	MS-AZ except 2, 4-D, and 6-BA are omitted.
AZ soln:	MS-AZ without gelrite.
MS-AZ CCH	MS-AZ with 500mg•L^{-1}carbenicillin, 100mg•L^{-1} cefotaxime and 50mg•L^{-1} Hygromycin.

Table 2. Effects of infection time, GUS incubation period, co-cultivation period, acetosyringone concentration, hygromycin concentration, carbenicillin concentration and OD on GUS activity of *Zoysia japonica*.

Treatments	GUS Results						
Time	2	4	8	16	24	48	96
Infection time (hr)	-	-	-	-	++	++	ND[z]
Co-cultivation period (day)	-	-	++	-	ND	ND	ND
GUS assay incubation time (hr)	ND	ND	ND	ND	+	++	++
Concentration (mg L^{-1})	0	10	20	50	100	200	400
AS Conc. (Infection)	-	-	+	++	++	ND	ND
AS Conc. (Co-culture)	-	-	++	++	+	-	ND
Hyg Conc.	-	-	-	++	+	+	-
Carbenicillin Conc.	-	ND	-	++	+	+	-
Concentration	0.02	0.2	2.0	2.5	3.0	3.5	4.0
O.D.$_{600}$	-	++	+	ND	ND	ND	ND

[z]ND: not determined.

AS: Acetosyringone, Hyg: Hygromycin, GUS: β-Glucuronidase

++: Significant difference between treatments at p=0.05. n=4

-, +: negative(-ve) and positive(+ve) result, respectively.

Table 3. Transformation efficiency of *Agrobacterium tumefaciens* strains in *Zoysia japonica*. Shown are the proportions of calli with blue inclusions in histological GUS positive and negative assay over all infected calli, transformants and percentage over all experiments.

A. tumefaciens strain	No. of calli[z]	GUS Negative (-ve)	GUS Positive (+ve)	GUS +ve (%)
pIG 121Hm LBA 4404	23	09	14[y]	60.86
pIG 121Hm EHA 101	25	16	09	36.00
pIG 121 HM C56C1	26	17	06	23.07
pBI 121 GUS, LBA 4404	28	22	06	21.42
pBE 2113 GUS, LBA 4404	24	17	07	29.16

[z]Calli from several plants were randomly selected for experiments with the different strains of *A. tumefaciens*. Experiments were replicated 3 times.
[y]Significantly different at p=0.05. n =4

diameter) were sealed with medical gas-permeable tape (Para film M, USA). Mature seeds were dehusked, washed with tap water, first sterilized with 2.0% sodium hypochlorite for 20 minutes and then again sterilized with 70% ethanol for 2 minutes. The seeds were then washed 3 times with sterilized deionised water and cultured on MS medium. The cultures were incubated in full light for one week and then in total darkness at 26º C for two weeks. At this time, the proliferated calli derived from the scutella were separated with a scalpel and compact calli (1-2 mm in diameter) were selected (Fig. 1a). Additional experiments were performed with this calli type including transformation.

Bacterial strains. Plasmid pIG121-Hm (formerly pBIH1-IG; Akama et al., 1992) harbors an intron-GUS reporter gene that consists of the 35S promoter of cauliflower mosaic virus (CaMV), a modified intron of the castor bean catalase gene, and the GUS-coding region (Fig. 3A; Ohta et al., 1990). Active GUS protein encoded by this construct can only be synthesized in plant cells after gene transfer into plant nuclei. This plasmid expresses GUS activity at a level and pattern similar to that obtained with the original GUS reporter gene in tobacco but not in *A. tumefaciens* cells (Ohta et al., 1990). Disarmed *Agrobacterium tumefaciens* strains LBA4404 (Hoekema et al., 1983), EHA 101 (Hood et al., 1993) and C58C1 (Zambryski et al., 1983) containing binary plasmid pIG121 were compared as vector delivery systems. The binary plasmid pBE2113-GUS (E12 -GUS) (Fig. 4; Mitsuhara et al., 1996) which was derived from pE2113-GUS also used for transformation efficiency measurement. Prior to infection, the bacterial strains were incubated with the AB medium (Chilton et al., 1974). Approximately 4 hours before infection, the plant tissue and bacteria were pelleted by centrifugation for 10 minutes at 10,000g_n and resuspended in an AAM medium (Hiei et al., 1994) containing 50mg•L^{-1} acetosyringone (AS) according to an OD_{600}= (0.20). These were then placed in darkness for 24 h at 27º C.

Co-cultivation. The calli were transferred from AAM medium and kept in an MS-AZ-AS medium. The asctosyringone concentration was 20mg•L^{-1}. The plates were sealed with Para film. Co-cultivation was carried out in the dark at 27º C for 96 hr. The calli labeled, as controls were not infected with *Agrobacterium*. After co-cultivation, the infected calli were washed with sterilized water containing 250mg•L^{-1} carbenicillin, 200mg•L^{-1} cefotaxime and 50mg•L^{-1} hygromicin to kill the *Agrobacterium* and then transferred to MS-AZ HmCf (table 1) medium at 27º C.

Assay of GUS activity. Expression of GUS (ß-glucuronidase) activity in *Zoysia* cells was assayed as previously described by Jefferson (Jefferson et al., 1987). The segments of *Zoysia* tissue were inoculated in X-gluc solution containing 75mM phosphate buffer (pH 7.0), 0.4mg•mL X-gluc (N, N-dimethylformamide), 1.2% Triton X- 100, 40% methanol and 12.5mM potassium ferricyanide and ferro cyanide and then incubated overnight at 37º C. The number of blue spots were counted under a microscope and recorded.

Experimental design and data analysis: The effect of infection time, GUS incubation period, acetosyringone concentration, hygromycin concentration, carbenicillin concentration, OD and GUS assay in different *Agrobacterium* strains were repeated 3 times. Data were collected visually under a microscope. Data were analyzed by analyses of

Table 4. Effects of acetosyringone concentration (AS) of different strains of *A. tumefaciens* on inclusion of blue spots of *Zoysia japonica*.

	Number of blue spots			
Strain of *A. Tumefaciens*	0	1-10	10-20	>20
pIG 121 LBA 4404				
ASc 100	8	2	3	1
ASc 50	10	3	2	0
ASc 20	11	4	2	1
ASc 10	10	2	3	0
ASc 0	13	1	0	0
pIG 121 EHA-101				
ASc 100	12	1	1	0
ASc 50	12	2	1	0
ASc 20	10	3	1	0
ASc 10	8	1	0	0
ASc 0	12	1	0	0
pIG 121 C58C1				
ASc 100	14	2	0	0
ASc 50	12	1	0	0
ASc 20	13	1	0	0
ASc 10	10	0	0	0
ASc 0	14	0	0	0

ASc: Acetosyringone concentration (mg•L^{-1})

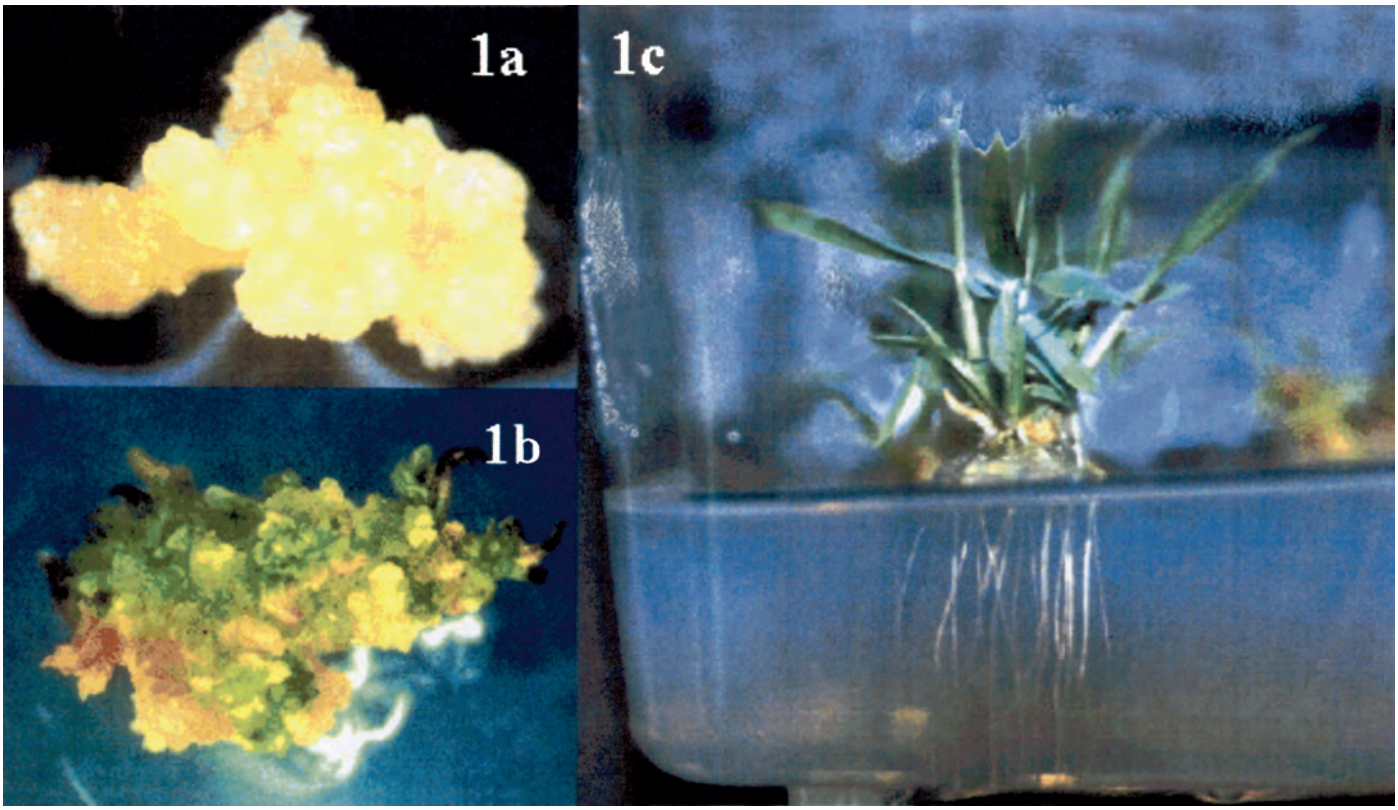

Fig. 1. Scutelum derived calli of *Zoysia japonica* cv. YK-EM2 had been co-cultivated with *A. tumefaciens* LBA 4404 and EHA101 (pIG121Hm) and transgenic cells and plants derived from the calli.
Fig. 1a-1c. Plant materials using the experiment (1a): Embryogenic calli in MS-AZ medium. (1b): Plant regeneration initiation in MS-HF medium. (1c): Plant regeneration: Established plant in MS-HF medium. The photograph was taken 6 weeks after selected cells had been transferred to regeneration medium.

variance (ANOVA). Parameters of the equations were compared by *t* tests.

RESULTS AND DISCUSSION

Table 2 shows the influence of the infection period on transformation of *Z. japonica* calli. Transformation was significantly higher at 24 and 48 hours after infection. Although prolonged infection periods of more than 24 hours have been successfully used for certain callus, 24-48 hours infection time has been regularly used in most transformation protocols, since longer infection periods frequently result in less effects. The transformation frequency reaching a maximum at 24-48 hr. Co-culture duration had no apparent effect on the area of transformed tissue per transformation event (data not shown). Although the calli infected for 16 days showed some GUS activity, the tissues were adversely affected during prolonged co-cultivation period. It is clear from the result that the optimum co-cultivation time with *Agrobacterium* was at least 8 days to obtain efficient expression of GUS. The influence of acetosyringone as a transformation enhancer of *Zoysia* is shown in table 2. The application of acetosyringone during co-cultivation increased the *Agrobacterium*-mediated transformation frequencies. Table 2 shows that acetosyringone in co-cultivation medium also increased the transformation frequency significantly at 20mg•L^{-1} acetosyringone. Acetosyringone also plays a significant role (50mg•L^{-1}) during the infection period.

Table 3 shows the efficiency of GUS+ activity of *Z. japonica* with different strains of *Agrobacterium*. It revealed that transformation efficiencies of the different *A. tumefaciens* strains were assessed as the proportion of blue inclusions in putatively transformed calli 8 days (192 hr) after transformation. The results of the transformation experiments summarized in Table 2 and Table 3 shows that the strain LBA 4404 had superior GUS positive expression infection efficiency. pIG 121-Hm (formerly pBIHI-IG; Akama et al., 1992) was almost twice as effective in transforming *Z. japonica* as the strain pBI 121-GUS (Jefferson et al. 1987) or twice pBE 2113-GUS (Mitsuhara et al., 1996). Callus cells were transformed at a very low rate, independent of the addition of acetosyringone or whether the calli were damaged or cut into small pieces. *Agrobacterium* strain LBA-4404 (pIG121-Hm) resulted in a large number of GUS-expressing cells (Fig. 1. 2a~2c). More than 60% of the calli showed GUS activity (Table 3).

Table 4 shows the effects of GUS activity with different concentration of acetosyringone and different strains of Agrobacterium tumefaciens. Table 4 also showed that AS 100 mg•L^{-1} may have the best result for producing a large number of spots in the strain LBA 4404.

Attempts to produce transgenic turf-grass plants by

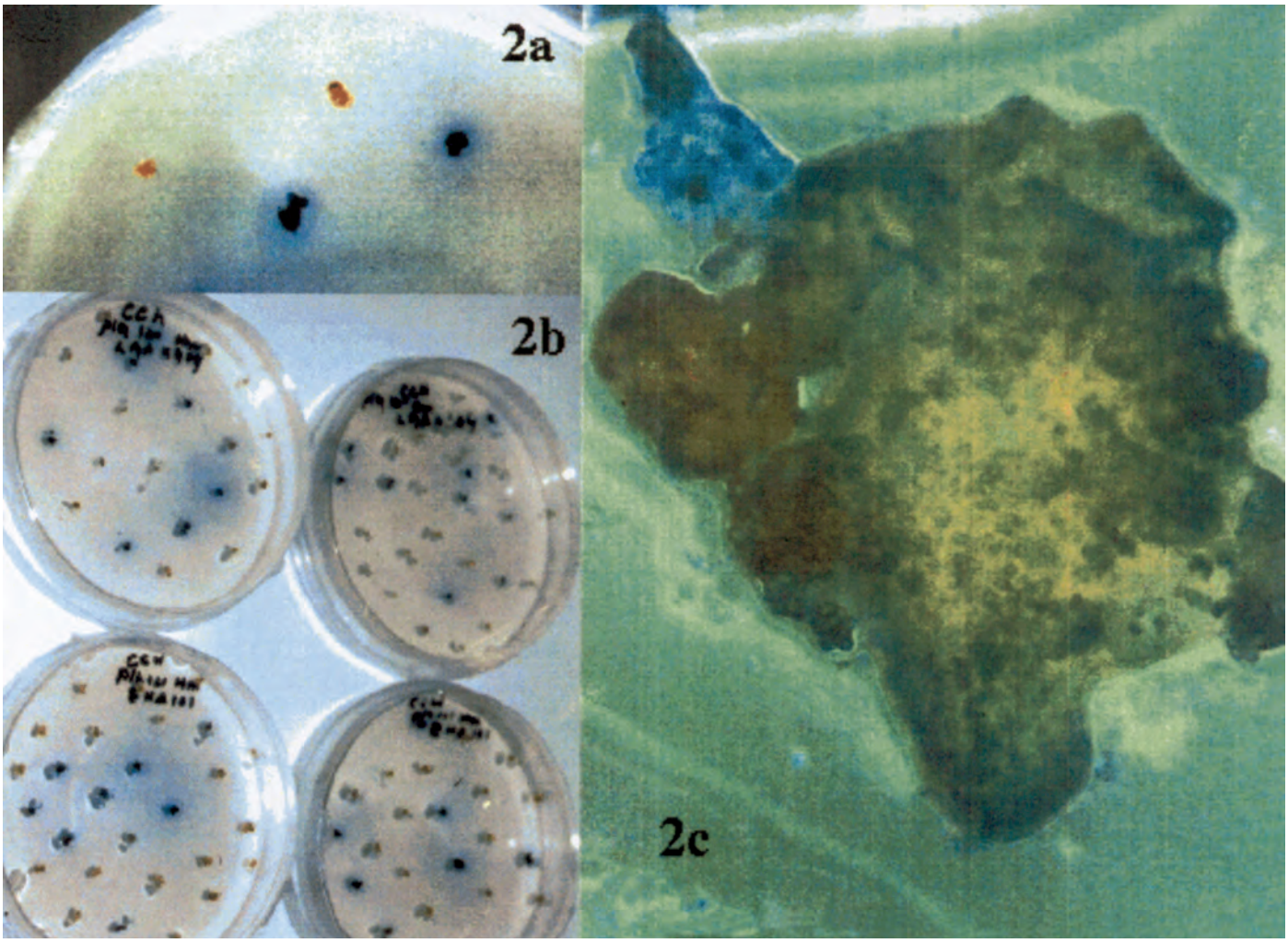

Fig. 2. Scutelum derived calli of *Zoysia japonica* cv. YK-EM2 had been co-cultivated with *A. tumefaciens* LBA 4404 and EHA101 (pIG121Hm) and transgenic cells and plants derived from the calli.
Fig. 2a-2c. Transient GUS assay (2a~2b): Upper two petriplates: Expression of GUS after infection with LBA 4404 (pIG121Hm) in MS-AZ-CCH medium. The calli were stained with 5-bromo-4-chloro-3-indolyl glucuronide (X-Glue) after 8 days of co-cultivation. Lower two petriplates: expression of GUS after infection with EHA101 (pIG121Hm) in MS-AZ-CCH medium. (2c): Spots of transient GUS expression on *Zoysia* calli (LBA4404).

various researchers worldwide were reviewed by Inocuma (Inocuma et al., 1998) concluding that turf-grass species are recalcitrant plants for transformation. This is the first report of *Zoysia* plant being successfully infected with *Agrobacterium*. In our protocol, transformation through *Agrobacterium*, the low efficiency of the infection is a disadvantage, which must be overcome. In our first attempts, we tried to improve the concentration by using acetosyringone and hygromycin, which have been employed to obtain transgenic plants. In this study, acetosyringone was used at a final concentration ranging between 0 to 100 mg•L^{-1}. We found that resistant calli only emerged by the application of higher concentrations of acetosyringone. Application of 50 mg•L^{-1} promoted *Zoysia* calli differentiation and positive GUS callus. These results indicated that the application of acetosyringone to the medium was essential for *Zoysia* grass callus transformation for both infection and co-culture.

We observed that there was no valid information regarding hygromycin effect on the GUS transient assay of *Zoysia* grass. In our study, GUS transient blue spots were observed mainly attached to hygromycin containing medium. Subsequently, it was determined that long-term exposure to GUS reaction medium did produce spots. We conclude that hygromycine certainly affects GUS transient expression for *Zoysia* grass. It may be possible that this effect is caused by plasmid activity (increasing number per cell or expression activity) that is directly connected by the hygromycin resistant gene. Further studies are needed to establish the best hygromycin concentration.

The transient GUS assay reported here shows that the *Agrobacterium* mediated *Zoysia* grass transformation are efficient and reproducible. A binary vector, pIG 121 Hm was used, which was derived from one of the most common binary vector, pBI121 (Ohta et al., 1990). Previous studies reported that a super-binary vector, in which a DNA fragment from the virulence region was introduced into a binary vector, was more

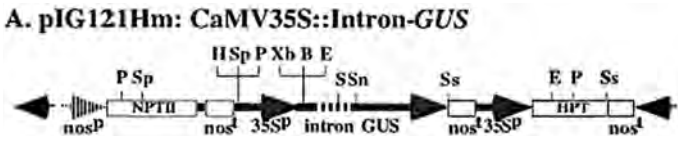

Fig. 3. Structure of binary vectors chimeric genes. CaMV 35S promoter-a modified intron of the castor bean catalase-GUS chimeric gene (CaMV 35S::Intron-GUS in pIG121-Hm) (Ohta et al., 1990)

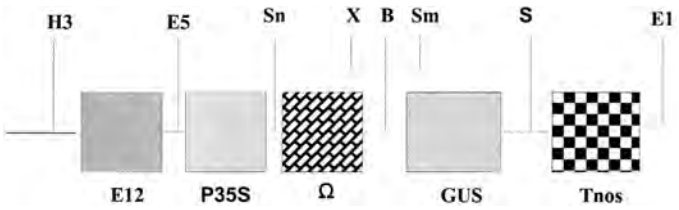

Fig. 4. Structure of pBE2113-GUS/pBE2113-GUS. E12: 5 -upstream sequence of CaMV 35S promoter (-419 to -90) X 2. Ω : 5 -untranslated sequence of TMV. GUS: protein-coding region of gene for ß-glucuronidase. Tnos: polyadenylation signal of the gene for nopaline synthase in the Ti plasmid. H3, *Hin*dIII; E5, *Eco*RV; Sn, *Sna*BI; X, *X*baI; B, *Bam*HI; Sm, *Sma*I; Sc, *Sac*I; E1, *Eco*RI (Mitsuhara et al., 1996).

effective for transforming rice and that difficult cultivars could more easily be transformed by using the super-binary vector (Hiei et al., 1994). Our data indicate that transient gene expression could be achieved in *Zoysia* by using an ordinary vector.

The expression of the GUS-intron gene as detected by GUS activity staining was a very reliable indicator of single cell transformation, since the intron in the GUS-intron sequence can only be spliced in the eukaryotic plant genome and cannot be effectively spliced in *Agrobacterium* (Vancanneyt et al., 1990). In most cells, GUS staining was very strong and intrinsic GUS activity was not seen in non-transgenic cells (1a, 1b). The vector has the intron in 5' end of GUS genes of pIG121 Hm was reported to increase the level of GUS activity 80-90 fold as compared with the intronless plasmid (Tanaka et al., 1990). The intron GUS gene used in this study was a convenient marker gene for *Zoysia* transformation.

The data indicate that the tendency of the frequency of transient GUS assay to AS concentration are the same among the three strains used in our experiment and this shows that the AS concentration is one of the limiting factors for *Agrobacterium* infection in *Zoysia* grass. LBA 4044 is the most effective strain to infect, in terms of transient GUS assay. The advantage of *Agrobacterium*-mediated gene transfer over other methods that can be used for the transformation of higher plants include the high efficiency of transformation, the transfer of pieces of DNA with defined ends, the transfer of relatively large segments of DNA, and the absence of a requirement for protoplast-culture techniques. Therefore, this type of gene transfer is normally the method of choice when more than one method is available. The ability of *Agrobacterium* to transfer monocotyledons like *Zoysia* has been a subject of serious debate for sometime, since these plants are not natural host hosts of this bacterium.

Our final objective is to develop a technique for the introduction of foreign genes in *Zoysia* grass cells and the production of transgenic *Zoysia* grass plants for practical gene introduction. *Zoysia* grass can be regenerated from protoplasts by means of the polyethylene glycol (PEG) method (Inocuma et al., 1998) and seeds in *in vitro* tissue culture. To produce a whole transgenic *Zoysia* grass plant, it is necessary to combine 2 techniques for foreign gene introduction and regeneration from transgenic cells. We have shown that acetosyringone and hygromycin application were effective in the production of transgenic Japanese lawn grass cells. The results of this report will be applied in the following study to produce whole transgenic Japanese lawn grasses.

In summary, we have developed a simple method for the production of transgenic plants in *Zoysia* grass. The protocol was modified from that reported for *Japonica* rice. We are now transferring some useful genes in *Zoysia* cultivars by using this approach. It may be possible to transform other recalcitrant cultivars of *Zoysia* and to other monocots by employing the methods described in this study. In conclusion, the *Zoysia* grass transformation method used here is effective and enabling repeated transformation of the same individual plant genotype. In this study, we demonstrated that the use of acetosyringone is effective for *Agrobacterium* gene transformation to *Zoysia* and that *Agrobacterium*-mediated transformation is more efficient than other attempted transformation methods (*i.e.* bombardment etc.) and enables the production of transformed *Zoysia* calli.

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LITERATURE CITED

Akama, K., H. Shiraishi, S. Ohta, K. Nakamura, K. Okada and Y. Shimura. 1992. Efficient transformation of *Arabidopsis thaliana*: comparison of the efficiencies with various organs, plant ecotype and *Agrobacterium* strains. Plant Cell Rep. 12:7-11.

Asano, Y. and M. Ugaki. 1994. Transgenic plants of *Agrotis alba* obtained by electroporation-mediated direct gene transfer into protoplasts. Plant Cell Rep. 13:243-246.

Asano, Y., H. Katsumoto, C. Inocuma, S. Kaneko, Y. Ito and L. Fuji. 1996. Cytokinin and thiamine requirements and simulative effects of riboflavin and ∝-ketoglutaric acid on embryogenic callus induction from seeds of *Zoysia japonica* sp. J. Plant Physiol. 149:413-417.

Chilton, M.D., T.C. Currier, S.K. Farrand, A. J. Bendich, M.P. Gordon and E.W. Nester. 1974. *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown call tumors. Proceed. Natl. Acad. Sci., USA. 71: 3672-3676.

Chu, C.C., C.S. Wang, C.C. San, C. Hsu, K.C. Yin and C.Y. Chu. 1975. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci. Sin. 18, 659-668.

Engelke, M. C. and J.J. Murray. 1989. *Zoysia* grass breeding and cultivar development. Proceed. of the 6th Intl. Turfgrass Conf.: 423-425.

Ha, S.B., F.S. Wu and T.K. Thorne. 1992. Transgenic turf-type tall fescue (*Festuca arundinacea* Schreb.) plants regenerated from protoplast. Plant Cell Rep. 11: 601-604.

Hartman, C.L., L. Lee, P.R. Day and N.E. Tumer. 1994. Herbicide resistant turf grass (*Agrotis palustris* Huds.) by biolistic transformation. Bio Tech. 12:919-923.

Hiei, Y., S. Ohta, T. Komari and T. Kumashiro. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the t-DNA. Plant J. 6(2):271-282.

Hoekema, A., P.R. Hirch, P.J. Hooykaas and R.A. Schilperoort. 1983. A binary plant vector strategy based on separation of *vir* and T-region of the *A. tumeficiens* Ti plasmid. Nature 303:179-180.

Hood, E.E., S.B. Glevin, L.S. Metchers and A. Hoekema. 1993. New *Agrobacterium* helper plasmids for gene transfer to plants. Trangenic Res. 2:208-218.

Inocuma, C., K. Sugiura, N. Imaizumi and C. Cho. 1998. Transgenic Japanese lawn grass (*Zoysia japonica* Steud.) plants regenerated from protoplasts. Plant Cell Rep. 17:334-338.

Inocuma, C.K., K. Sugiura, C. Cho, R. Okawara and S. Kaneko. 1995. Plant regeneration from protoplasts of Japanese lawn grass. Plant Cell Rep. 13:527-539.

Jefferson, R.A., T.A. Kavanagh and M.W. Bevan. 1987. GUS fusions: 3-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901-3907.

Mitsuhara, I., M. Ugaki, H. Hirochika, M. Ohshima, T. Murakami, Y. Gotoh, Y. Katayose, S. Nakamura, R. Honkura, S. Nishimiya, K. Ueno, A. Mochizuki, H. Tanimoto, H. Tsugawa, Y. Otsuki and Y. Ohashi. 1996. Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. Plant and Cell Physiol. 37:49-59.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant 15:473-496.

Ohta, S., S. Mita, T. Hattori and K. Nakamura. 1990. Construction and expression in tobacco of a ß-glucuronidase (GUS) reporter gene containing an intron within the coding sequence. Plant Cell Physiol. 31: 805-813.

Spangenberg, G., Z.Y. Wang, J. Nagel and I. Potrykus. 1994. Protoplast culture and generation of transgenic plants in red fescue (*Festuca rubra* L.). Plant Sci. 97:83-94.

Tanaka, A., S. Mita, J. Kyozuka, K. Shimamoton and K. Nakamura. 1990. Enhancement of foreign gene expression by a dicot intron in rice but not in tobacco is correlated with an increased level of mRNA and an efficient splicing of the intron. Nucleic Acids Res. 18:6767-6770.

Vancanneyt, G., R. Schmidt, A. O'Conor-Sanchez, L. Willimitzer, M. Rocho-Sosa. 1990. Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*- mediated plant transformation. Mol. Gen. Genet. 220:245-250.

Vasil, I.K. 1988. Progress in the regeneration and genetic manipulation of cereal crops. Bio Tech. 6:397-402.

Wang, Z., T. Takamizo, V.A. Iglesias, M. Osusky, J. Nagel, I. Potrykus and G. Spangengberg. 1992. Transgenic plants of tall fescue (*Festuca arundinacea* Schreb.) obtained by direct gene transfer to protoplast. Bio Tech. 10(6): 691-696.

Watson, J.R. 1989. The USGA research committee activities with particular reference to the utilization, problems and perspective of *Zoysia* grass in the USA. Proc. 6th Int Turfgrass Res. Conf.: 437-440.

Zambryski, P., H. Joos, C. Genetello, J. Leemansm, V. Montagu and J. Scheel. 1983. Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. Embo J. 2: 2143-2150.

Zhong, H., M.G. Bolyard, C. Srinivasan and M.B. Sticklen. 1993. Transgenic plants of turfgrass (*Agrotis pallustris* Huds.) from microprojectile bombardment of embryogenic callus. Plant Cell Rep. 13:1-6.