Transformation of androgenic-derived *Festulolium* plants (*Lolium perenne* L. x *Festuca pratensis* Huds.) by *Agrobacterium tumefaciens*

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Keywords

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Abstract

Genetic transformation of androgenic-derived amphidiploid *Festulolium* plants (*Lolium perenne* L. x *Festuca pratensis* Huds., 2n=4x=28) by *Agrobacterium tumefaciens* has been achieved. Anther culture-induced calli of *Festulolium* "Bx351" were inoculated with *Agrobacterium tumefaciens* strain LBA4404 carrying pIG121-Hm encoding the hygromycin resistance (*hph*) and β-glucuronidase (*uidA*) genes under the control of a CaMV 35S promoter. Twenty-three putative transformants were obtained from the hygromycin selection, 19 of which (82.6%) showed GUS activity. The integration of transgenes was detected by using genomic DNA PCR analysis, RT-PCR analysis and Southern blot hybridization, respectively, which revealed that foreign gene was integrated into the genomes of dihaploid transformants (2n=2x=14). The haploid embryogenic system offers a stable means of transformation, as the introduced trait can be readily fixed through chromosome doubling.

Abbreviations:

2,4-D, 2,4-dichlorophenoxyacetic acid; CaMV, Cauliflower mosaic virus; GUS, β-glucuronidase; Kinetin, 6-furfurylaminopurine; PCR, Polymerase Chain Reaction; RT-PCR, reverse transcription-polymerase chain reaction

Introduction

Heterosis (hybrid vigor) is a common phenomenon in nature. Intergeneric hybrids between closely related *Lolium* and *Festuca* species are being used to broaden the gene pool and provide the plant breeder with options to combine complementary traits aimed at high quality but more robust grass varieties for the future (Humphreys et al. 2003). The complex of species has an enormous wealth of genetic variability and potentiality for genetic exchange, thus offering unique opportunities for the production of versatile hybrid varieties with new combinations of useful characteristics suited to modern grassland farming (Thomas et al. 2003). *Lolium* and *Festuca* species share valuable and complementary agronomic characteristics. *L. perenne* offers good regrowth and nutritive value and is a good species for grazing, and *F. pratensis* has greater cold tolerance (Humphreys et al. 1998). *Lolium* and *Festuca* species hybridize naturally, and, as hybrids, regularly exchange genes with high frequency. Recently, in Europe and Japan, *Festulolium* plants (*Lolium-Festuca* complex, intergeneric hybrids between *L. multiflorum*, *L. perenne* and *F. pratensis*, *F. arundinacea*) were developed as novel temperate forage grasses (Humphreys et al. 2003; Momotaz et al. 2004; Pašakinskienė et al. 1997; Zwierzykowski et al. 1999; Yamada et al. 2005).

Androgenesis is a well-established component of plant breeding methodology, and can make a significant contribution to cultivar development. The main advantage of doubled haploids is the reduction in the time needed to develop new cultivars. Doubled haploids are useful for increasing selection efficiency because additive genetic variation is more apparent. In genetic studies and genetic engineering, haploids can be used to detect linkage and gene interaction as well as to estimate genetic variance and the number

of genes for quantitative characteristics (Choo and Reinbergs 1987; Snape et al. 1984). The haploid embryogenic system offers an effective means of transformation, as the introduced trait can be readily fixed through chromosome doubling (Ferrie et al. 1995). Androgenesis was found to be an effective procedure for selecting *Lolium-Festuca* genotypes comprising gene combinations rarely or never recovered by conventional backcross breeding programs (Guo et al. 2005). In mutation studies, recessive and dominant traits are expressed and therefore can be easily selected using haploids (doubled haploids); therefore, the haploid system is an efficient tool system for mutation and *in vitro* selection studies (Ferrie et al. 1995; Swanson et al. 1989).

Genetic transformation methods have been used to introduce new traits into commercially important plants, thereby producing combinations of features which could not have been achieved by traditional breeding programs (Hansen and Wright 1999). The *Agrobacterium* system is attractive because of the ease of the protocol coupled with minimal equipment cost. Moreover, transgenic plants obtained by this method often contain single copies of insertions (Hansen and Wright 1999; Gelvin 1998). Initial attempts to transform monocotyledonous plants with *Agrobacterium* were largely unsuccessful due to the fact that monocotyledonous plants are not a natural host for *Agrobacterium.* Their cells produce different phenolic compounds in response to wounding and respond differently to the hormonal cues that induce tumor growth in dicots (Porter 1991; Puddephat 2003).

In recent years, successful *Agrobacterium*-mediated transformation of cereal crops has been reported, including transformation of wheat (Cheng et al. 1997; Cheng et al. 2003; Sahrawat et al. 2003), rice (Terada et al. 2004; Coca et al. 2004), maize (Shou et

al. 2004; Quan et al. 2004), barley (Murray et al. 2004; Horvath et al. 2003), sorghum (Jeoung et al. 2002; Zhao et al. 2000), rye (Popelka and Altpeter 2003), oats and pearl millet (Repellin et al. 2001). There have also been limited reports of the transformation of forage grasses, including tall fescue (*Festuca arundinacea* Schreb.), perennial ryegrass (*Lolium perenne* L.), Italian ryegrass (*Lolium multiflorum* Lam.) (Bettany et al. 2003; Wang and Ge 2005; Sato and Takamizo 2006; Bajaj et al. 2006), kleberg bluestem (*Dichanthium annulatum* Forsk.) (Dalton et al. 2003) and switchgrass (*Panicum virgatum* L.) (Somleva et al. 2002).

The objective of the current study was to develop a stable *Agrobacterium*-mediated transformation system for producing transgenic *Festulolium* plants (*Lolium perenne* L. x *Festuca pratensis* Huds.) that were derived from androgenic embryogenesis. GUS histochemical assay, genomic DNA PCR analysis, RT-PCR analysis and Southern blot hybridization were employed for the study of gene expression.

Materials and methods

Plant material

The amphidiploid *Festulolium* hybrid between *Lolium perenne* L. and *Festuca pratensis* Huds. “Bx351” (2n=4x=28) provided by Prof. M O Humphreys of the Institute of Grassland and Environmental Research, Aberystwyth, UK was investigated in this study. Plants were grown in soil in a greenhouse under controlled conditions including 25°C/18°C and a 16 h photoperiod. Seedlings were vernalised in a cold room (3°C, 8h

photo period, dim light) for 12 weeks, and the plants were then transferred into a greenhouse.

Androgenic cell culture and haploid callus induction

Spikes were harvested when the microspores were in the middle- to late-uninucleate developmental stage and stored at 4°C in the dark with the stalks in water for 3-10 days of cold treatment. Prior to excision of the anthers, the microspore developmental stage was checked microscopically. Spikes were surface sterilized with 2% sodium hypochlorite solution for 15 minutes, followed by a few rinses in sterile water. The excised anthers were plated in a Petri dish (diameter 90 mm) containing 45 ml Gelrite-solid medium. PG-96 (Guo et al. 1999; Guo and Pulli 2000) with 2.0 mgl^{-1} 2,4-D, 0.5 mgl^{-1} kinetin was used as the embryo (callus) induction medium. The Petri dishes were sealed with Parafilm, and incubated at 25°C in darkness. After 8-10 weeks of culture, calli (1-2 mm in size) were transferred to the solid 190-2 medium (Wang and Hu 1984) supplemented with 1.5 mgl^{-1} 2,4-D for callus growth. Fast-growing calli with a high potential for regeneration were used for transformation.

Agrobacterium strain and vector for transformation

A binary plasmid vector was introduced into a *Agrobacterium* strain by triparental mating (Bevan 1984). *Agrobacterium tumefaciens* strain LBA4404 was used in the present study. *Agrobacterium tumefaciens* strain LBA4404 line harbors plasmid pIG121Hm, carrying a neomycin phosphotransferase II gene (*nptII*) driven by the nos promoter, a hygromycin phosphotransferase gene (*hpt*) driven by the cauliflower mosaic virus (CaMV) 35S

promoter, and a b-glucuronidase gene (*uidA*) containing an intron from a castor bean catalase gene and driven by the CaMV 35S promoter (Fig. 1) (Ohta et al. 1990).

Agrobacterium containing the binary vector was cultured for two days at 28°C on a rotary shaker at 160 rpm in LB medium containing 50 mgl^{-1} kanamycin and 25 mgl^{-1} streptomycin (Hisano et al. 2004a). The *Agrobacterium* culture reached an OD_{600} of 0.5 with a spectrophotometer.

Figure 1

Transformation

The transformation process can be divided into five steps: *Agrobacterium* infection, co-cultivation, carbenicillin treatment, hygromycin selection and green plant regeneration. Three days before *Agrobacterium tumefaciens* infection, the embryogenic calli (approximately three grams) were divided into 1- to 2-mm pieces and placed on 190-2 callus growth medium containing acetosyringone at 20 mgl^{-1}, four replications were carried out, totally approximately 12 grams embryogenic calli were employed in infection process. The calli were collected in a tea strainer and immersed in a bacterial suspension with acetosyringone at 40 and 100 mgl^{-1} in a Petri dish for 2-5 min with shaking. Excess liquid was removed by placing the calli on sterilized filter paper. Then the calli were co-cultivated with *Agrobacterium tumefaciens* for three days until growth of *Agrobacterium tumefaciens* was observed on the 190-2 co-cultivation medium containing 20 mgl^{-1} acetosyringone. After *Agrobacterium tumefaciens* infection, the calli were washed with

carbenicillin solution (500 mgl^{-1}) and transferred to 190-2 growth medium containing carbenicillin at 500 mgl^{-1} for seven to ten days to suppress bacterial growth. For selection, the calli were transferred to 190-2 growth medium containing hygromycin at 75 mgl^{-1} and carbenicillin at 250 mgl^{-1} for four weeks, and then subcultured in 190-2 growth medium only containing hygromycin at 75 mgl^{-1} for another four weeks. The processes of co-cultivation, carbenicillin treatment and hygromycin selection were performed at 25°C in the dark. For green plant regeneration, the hygromycin-resistant proliferating calli were transferred to 190-2 regeneration medium supplemented with 2,4-D at 1.5 mgl^{-1} and hygromycin at 75 mgl^{-1}. The calli were incubated at 25°C in a 16 h / 8 h day / night photoperiod with a light density of approximately 30 $\mu molm^{-2}s^{-1}$. After shoots (2-3cm) were developed, the plantlets were transferred to Magenta boxes containing solid 190-2 medium without growth regulators for root development.

Ploidy level estimation

The Partec CyStain UV precise P reagent kit was used out for nuclei extraction from leaf and for DNA staining (with DAPI, 4',6-Diamidino-2-phenylindole) of nuclear DNA in order to determine ploidy level by flow cytometry (Partec PAS, Münster, Germany). The detailed method for the nuclei extraction and DNA staining of nuclear DNA was described in the kit manual.

GUS histochemical analysis

The transgenic calli and plantlets selected by hygromycin-containing medium were tested using histochemical GUS staining. The tissue was rinsed once with sodium phosphate

buffer (pH 7.0) and then stained in 1 mM 5-bromo-4-chloro-3-indoyl glucuronide (X-gluc) in X-gluc buffer containing 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% Triton X-100 and 10 mM EDTA, pH 7.0. Samples were incubated overnight at 37°C and, if necessary, for one further day at 25°C. The tissues were cleared after staining by soaking in 95% ethanol. T-DNA delivery was assessed by counting the calli and plantlets that had at least one GUS focus.

Analysis of putative transformants using hygromycin

Tillers of putatively transformed plants along with the untransformed controls were dipped in 190-2 medium without sucrose but containing 50 mgl^{-1} hygromycin to test for hygromycin resistance. Damage was recorded after four days.

Extraction of DNA and PCR analysis

To confirm the integration of the transgenes in the transgenic *Festulolium* plants, PCR analysis was performed. Genomic DNA was isolated by homogenizing 100 mg of leaf tissue from putative transgenic plants by the CTAB method as described previously (Guo et al. 2003). The primers 5'-GTTACGTCCTGTAGAAACCC-3' and 5'-CTGTGACGCACAGTTCATAG-3' were designed to amplify the GUS gene. The amplification of the GUS gene using this pair of primers should result in a product of 0.7 kbp.

PCR amplification was performed in a total volume of 50 µl containing 500 ng genomic DNA as a template and 0.5 µM of each primer, 200 µM of dNTPs and 1 U rTaq^{TM} DNA polymerase (TaKaRa, Tokyo, Japan). Amplification was performed in a

GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Forster City, CA, USA) programmed for 30 cycles of 30 sec at 94°C, 30 sec at 56°C, 1 min at 72°C and a final elongation step at 72°C for 5 min. PCR products were separated on a 2% agarose gel and detected by staining with ethidium bromide.

RNA extraction and RT-PCR analysis

Total RNA was isolated by homogenizing 100 mg of leaf tissue from putative transgenic *Festulolium* plants and using the RNeasy® Plant Mini Kit (QIAGEN Science, Maryland, USA) according to the manufacturer's instructions. For RT-PCR analysis, the QIAGEN® OneStep RT-PCR Kit (Valencia, CA, USA) was used according to the manufacturer's instructions. RT-PCR was carried out in a total volume of 50 µl containing approximately 300 ng of RNA from transformed *Festulolium* plants and untransformed controls as templates together with 0.6 µM of each GUS specific primer (the same primers as in the PCR analysis), 400 µM of dNTPs and 2 µl QIAGEN OneStep RT-PCR Enzyme Mix.

Reverse transcription was performed in a thermal cycler programmed for 30 min at 50°C followed by an initial PCR activation step of 15 min at 95°C. HotStart Taq DNA polymerase is activated by this heating step. Omniscript and Sensiscript Reverse Transcriptases are inactivated, and the cDNA template is denatured. The following amplification was programmed for 30 cycles of 30 sec at 94°C, 30 sec at 56°C, 1 min at 72°C and a final elongation step at 72°C for 5 min. RT-PCR products were analyzed by electrophoresis on a 2% agarose gel.

Southern blot hybridization

Southern blot analysis was performed according to standard procedures (Sambrook et al., 1989). Plant genomic DNA was extracted from leaves according to the methods of CTAB (Guo et al. 2003). Twenty micrograms of DNA was digested with the restriction enzyme *Eco*RI that only cleaves once in the multiple cloning site located between *hph* gene and *gusA* gene in the binary vectors. DNA was transferred to nylon membranes (Hybond N+, Amersham). The hybridization probe (*hph*) was labeled with [α-^{32}P] dCTP.

Results and discussion

Induction of androgenic-derived calli with high regeneration competency

in the androgenic cell culture of *Festulolium* "Bx351" (*Lolium perenne* L. x *Festuca pratensis* Huds.), visible calli emerged at five to six weeks of anther culture in the PG-96 induction medium. Over 80% of inoculated anthers produced embryogenic calli with high potential for regeneration (Figure 2a). In some cases, green shoots were obtained directly from the calli in the induction medium when light was provided (Figure 2a, arrows showed the green shoots). These embryogenic calli with a high potential of regeneration were used as target tissues for *Agrobacterium tumefaciens* infection.

Figure 2

In the current *Festulolium* androgenesis study, more than 50% of dihaploid and tetraploid plants were male fertile or partially male fertile, and it was possible to produce progeny following hybridization with *L. perenne* or *F. pratensis*. In an androgenesis study of *L. multiflorum* x *F. pratensis,* genotypes with both male and female fertility were found in the dihaploid androgenic populations (Thomas et al. 2003).

Genetic transformation

A total of twenty-three putative transformants were regenerated from hygromycin containing selection medium (Figures 2b, 2d). Among twenty-three putative transformants, twenty-one were induced from *Agrobacterium tumefaciens* infection with acetosyringone at 40 mgl^{-1} and two of them were induced from *Agrobacterium tumefaciens* infection with acetosyringone at 100 mgl^{-1}. In this study, a relatively low level of acetosyringone at 40 mgl^{-1} showed benefits for transformation. Pre-induction or enhanced induction of the bacterial vir genes can be achieved through the use of acetosyringone and related compounds. Such approaches are only of value when the target tissue produces insufficient phenolic compounds to induce vir gene expression, which may also occur when short co-cultivation periods are used (Puddephat 2003). In previous work on *Agrobacterium tumefaciens*-mediated transformation of *Festuca arundinacea* and *Lolium multiflorum,* acetosyringone at 20 mgl^{-1} was used (Bettany et al. 2003). Callus pre-culture in an acetosyringone containing medium three days before transformation is an important step. The bacterial vir genes can be triggered by fast growing tissue and acetosyringone treatment. Increasing the level of tissue wounding, which increases the release of phenolic compounds, can stimulate bacterial virulence and provide greater access to transformable tissues (Puddephat 2003).

The transformation efficiency (the number of the regenerated plantlets resistant to hygromycin based on the total number of calli infected by *Agraobacterium*) was 4.2% for the low level of acetosyringone at 40 mgl^{-1}. The successful production of transgenic plants has four essential requirements, namely suitable vectors, selectable markers and efficient techniques for both the transformation and regeneration of whole plants (Puddephat 2003). In many systems, cells that are competent for transformation may not necessarily participate in regeneration. The utilization of *Agrobacterium* for the transformation of monocots has required advances in plant tissue culture technologies. The selection of appropriate starting material for *Agrobacterium*-mediated transformation of monocotyledonous plants is an important determinant of success. A tissue culture stage is required in most current transformation protocols to ultimately recover plants. Indeed, it is the totipotency of plant cells that underlines most plant transformation systems. Generally, transgenic plants are regenerated from cell culture via two methods, somatic embryogenesis and organogenesis (Hansen and Wright 1999).

In the present study, we report the successful *Agrobacterium tumefaciens*-mediated transformation in *Festulolium* amphidiploid plants (*Lolium perenne* L. x *Festuca pratensis* Huds.) using androgenic-derived haploid callus. Microspores from *Lolium x Festuca* hybrids may contain a large number of possible desirable gene combinations from the genomes of both species, such as extreme freezing tolerance and drought resistance (Guo et al. 2005; Zare et al. 1999; Zare et al. 2002). The androgenic haploid embryogenic system offers an effective means of transformation, as the introduced trait can be readily fixed through chromosome doubling. Normally, the transgenic plants obtained from microspores are homozygous. However, in transformation study of barley using particle bombardment with the *V st1* gene,

independent segregation of the transgenes was observed in the T_0 barley plant MS53/2. Therefore, the integration must have occurred after spontaneous chromosome doubling followed by the regeneration of a hemizygous plant (Leckband and Lörz 1998). Corresponding results were shown by a fluorescence in situ hybridization study (Pedersen et al. 1997). Androgenic-derived calli or plants have been used as target material for gene transformation in a range of crops. In *Agrobacterium rhizogenes*-mediated transformation of *Brassica oleracea*, significantly increased numbers of transformants were obtained from double haploid lines compared to the parental F_1 cultivar (Cogan et al. 2001). Using doubled-haploid tissues as target materials, successful transformations were achieved by the *Agrobacterium*-mediated method in tobacco, cabbage and wheat (Liu et al. 2003; Tsukazaki et al. 2002; Brisibe et al. 2002).

Transient expression of the GUS gene

The GUS assay confirmed the presence of GUS in transgenic calli and shoots (Figure 2c) in nineteen out of twenty-three regenerants (82.6%), although the intensity and distribution of blue staining varied among the tissues. The transgenic calli and young shoots showed the highest intensity of coloring whereas the GUS staining was relatively weak in adult leaves. Control calli and leaves did not show intrinsic GUS-like activity.

PCR analysis

To screen putative transformants, PCR was carried out using the GUS gene as a marker. In the genomic DNA of twenty-three putative transformants, the GUS gene was detected in nine of them. All nine of these plants, namely transgenic plant No. 1, 2, 3, 6, 7, 11, 15,

19 and 23, showed transient expression of the GUS gene by GUS histochemical analysis, whereas no GUS gene amplified bands were observed for the other fourteen plants (Figure 3). The GUS gene expressions varied at different levels (Figure 3). Thus the GUS gene was successfully integrated into the genome of nine out of the twenty-three putative transformants (39.1%) *Festulolium* plants.

Figure 3

RT-PCR analysis

Although PCR analysis is a convenient means of initially screening for transformants, it does not confirm the stable integration of transgenes because positive detection by PCR might reflect the survival of *Agrobacterium tumefaciens* in the host plant tissue or the existence of a non-integrated Ti plasmid (Tsugawa et al. 2004). Therefore, RT-PCR analysis was carried out using GUS gene-specific primers. All nine PCR positive transformants were detected by RT-PCR (Figure 4).

Figure 4

The use of *Agrobacterium* vectors for genetic transformation confers advantages over direct DNA delivery techniques. These advantages include a high frequency of stable genomic integration and a single/low copy number of the intact transgene (Sahrawat et al. 2003). In our previous transgenic perennial ryegrass transformation study using a particle bombardment-mediated method, wheat fructosyltransferase *wft*1 and *wft2*

gene expression was probably obstructed by the presence of multi-copies of the transgene which may induce homology-dependent gene silencing (Hisano et al., 2004b). The advantages of *Agrobacterium*-mediated transformation have encouraged researchers to develop efficient protocols for the genetic transformation of economically important cereals (and forage grasses) employing *Agrobacterium* as a vehicle of delivery of foreign DNA (Sahrawat et al. 2003).

Southern blot hybridization

Hybridization signals corresponding to bands of different molecular weight were observed in the southern hybridization. The results demonstrated that the transgene was integrated in the genome of the independently androgenic-derived *Festulolium* transformed plants (Figure 5).

Figure 5

Analysis of transgenic plants

Hygromycin resistance tests were employed for the transformed plants (Nos. 1, 3, 15, 19, 23) along with the untransformed controls at a hygromycin concentration of 50 mgl^{-1}. In transformed plants, growth was normal, and no distinct damage occurred to the plantlets. In untransformed and control plants, there was necrosis and wilting of the leaves, growth and development of the plantlets were obviously restrained.

Ploidy level was analyzed by a flow cytometer with DAPI staining. Among the twenty-three putative transformants, eighteen plantlets (78.3%) were dihaploids (2n = 2x = 14), while the others had a gametic chromosome number. All nine transformants

confirmed by PCR and RT-PCR analysis were dihaploids. The morphological difference between haploids and dihaploids is obvious, the haploid with narrow leaves is weak and cannot survive for long. Some morphological characters such as growth vigor and shape of the leaves were measured, the morphology of transformed dihaploid plants seemed to be normal and there was no difference compared with wild type plants.

Conclusions

In this study, we described the *Agrobacterium tumefaciens*-mediated transformation of the novel temperate forage grass *Festulolium* (*Lolium perenne* L. x *Festuca pratensis* Huds.). Integration of transgenes was detected by using genomic DNA PCR analysis, RT-PCR analysis, Southern blot hybridization as well as GUS histochemical assay. These results indicate that the *Agrobacterium tumefaciens*-mediated transformation is stable for transferring foreign genes into *Festulolium* plants. Combined with intergeneric hybridization and double haploid breeding methods, the transformation process explored in the current study will speed up the breeding process of this novel forage grass.

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Figure legends

Figure 1. Linear map of the T-DNA region of plasmid pIG121-Hm. RB: right border; LB: left border; NPT II: neomycin phosphotransferase; GUS: β-glucuronidase; HPT: hygromycin phosphotransferase; PNOS: nopaline synthase promoter; P35S: CaMV 35S promoter; T: nopaline synthase terminator; I: intron.

Figure 2. Regeneration of androgenic-derived transformed calli and histochemical detection of GUS activity in transgenic plants of Festulolium "Bx351" (*Lolium perenne* L. x *Festuca pratensis* Huds.) (a) Embryogenic calli with high regeneration potential induced via anther culture of *Festulolium* "Bx351". Bar: 5 mm. (b) Shoots regeneration from calli of *Agrobacterium*-transformed Festulolium "Bx351". Bar: 12 mm. (c) Distribution of GUS histochemical assay in shoots and calli of *Festulolium* "Bx351". Bar: 2 mm. (d) Transgenic *Festulolium* "Bx351" plants established in a growth chamber.

Figure 3. Electrophoretic patterns of the amplified products of PCR analysis of transgenic plants of *Festulolium* "Bx 351". PCR with GUS gene-specific primers was performed. Transgenic plant Nos. 1, 2, 3, 6, 7, 11, 15, 19, 23 showed the GUS gene amplified products at varied levels. Lane NC 1, negative control without template DNA; Lane NC 2, negative control using non-transformed plantlet. M, molecular markers (fragments of 1 kbp, 0.9 kbp, 0.8 kbp, 0.7 kbp, 0.6 kbp, from top to bottom).

Figure 4. Detection of transgenic plants of *Festulolium* "Bx 351" by RT-PCR analysis. PCR with GUS gene-specific primers was performed. Lane NC, negative control using non-transformed plantlet. M, molecular markers (fragments of 1 kbp, 0.9 kbp, 0.8 kbp, 0.7 kbp, 0.6 kbp, 0.5 kbp, from top to bottom).

Figure 5. Southern hybridization analysis of a DNA blot containing *Eco*RI digested genomic DNA from regenerated *Festulolium* "Bx 351" plants and hybridized with *hph* probe which was〔α-^{32}P〕dCTP-labeled. DNA size markers are shown on the right.

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Figure 1

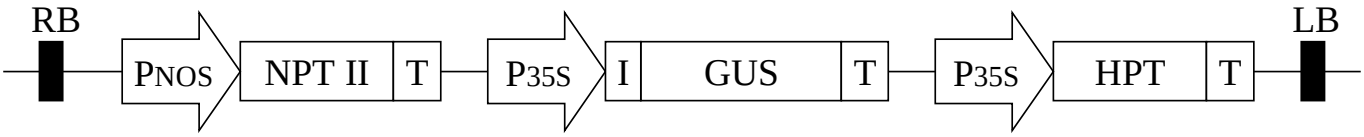

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Figure 2

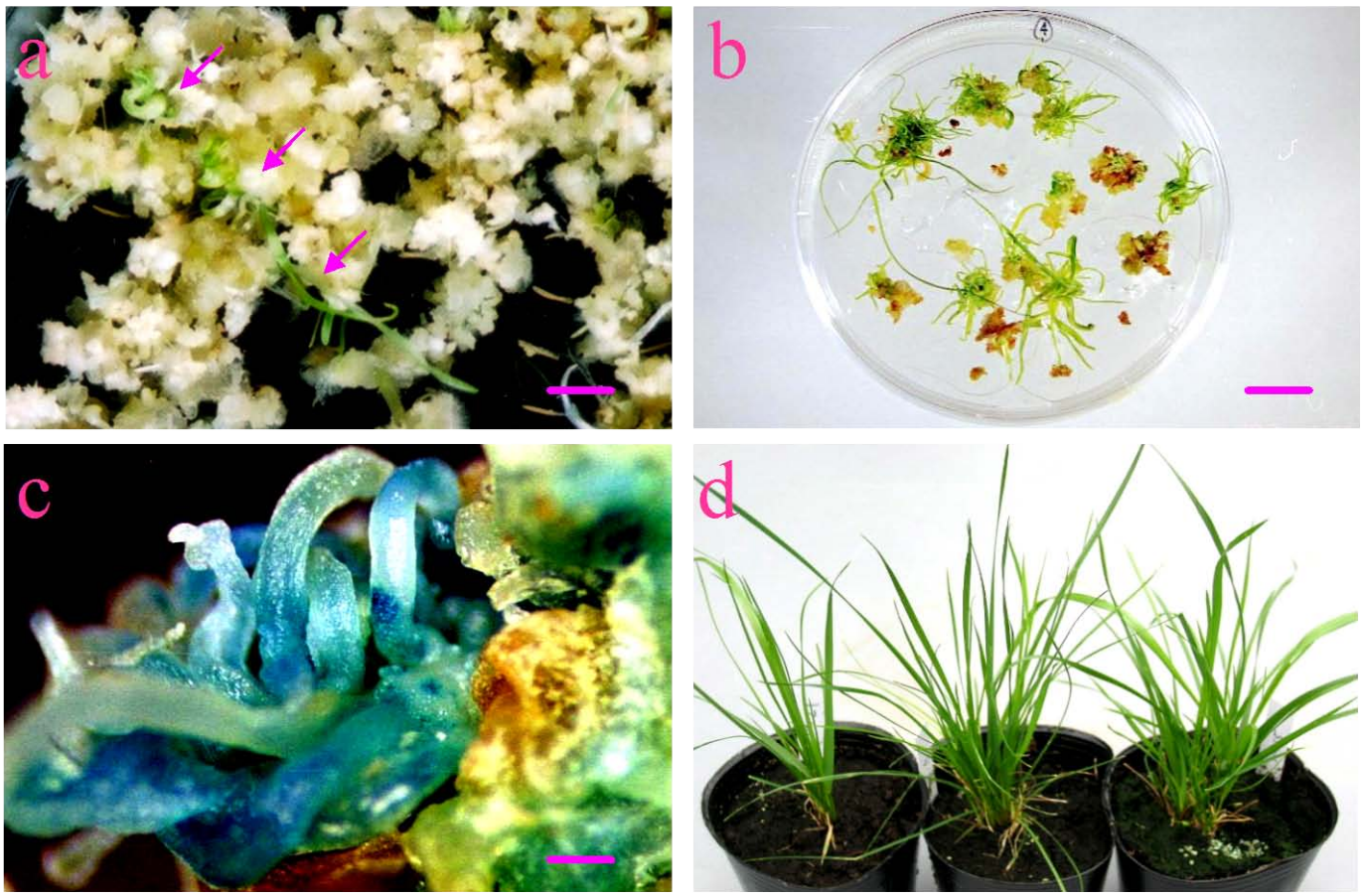

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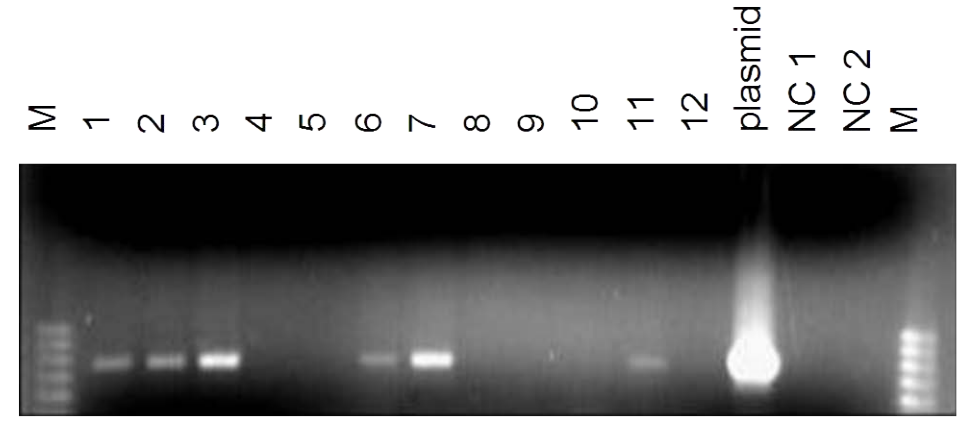
Figure 3



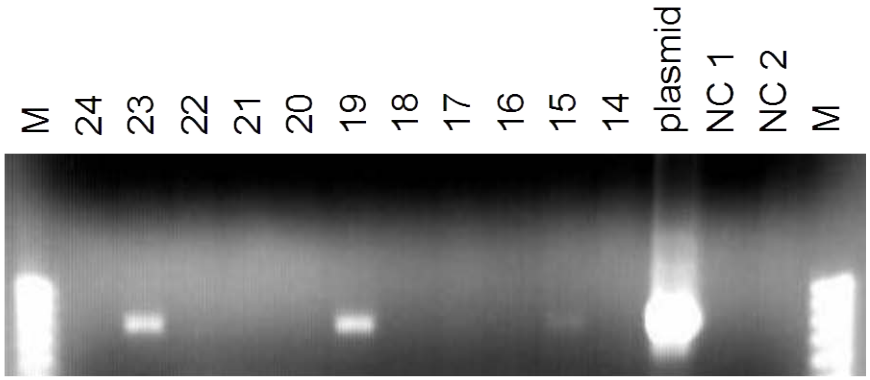


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Figure 4

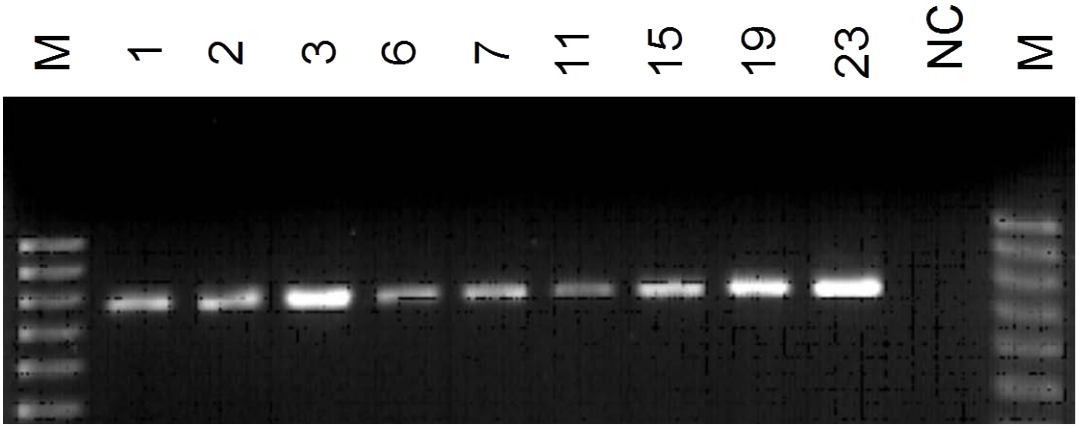

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Figure 5.