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Stable Reporter Gene Expression in Western White Pine (*Pinus monticola* Dougl. ex D. Don) by *Agrobacterium*-mediated Genetic Transformation

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ABSTRACT

An *Agrobacterium*-mediated transformation procedure was developed to transform the mature embryo from *Pinus monticola* (Dougl. ex D. Don) seeds with two binary vectors containing the reporter gene encoding the green fluorescent protein (GFP) or the β-glucuronidase protein (GUS), respectively. More than 1000 embryos from independent transformation events were tested for different western white pine seed families. Selection of kanamycin-resistant callus tissues showed that survival rates varied from 33 to 48% in different independent experiments. Transgenic callus tissues survived and continued to grow on the medium with kanamycin (25 μg/mL), whereas non-transgenic callus, regenerated from the embryos of the same seed family, died within 12 weeks. Integration and expression of the introduced reporter gene was confirmed in transgenic western white pine calli by GUS-staining analysis or microscopic observation of GFP fluorescence. Rates for stable reporter gene expression ranged from 2.9 to 6.5% for all embryos co-cultured with *Agrobacterium*. Our protocol has enabled the routine transformation of western white pine, a species that was previously difficult for gene manipulation. To our knowledge, this is the first report on genetic engineering of this conifer. Our results demonstrate that transgenic gene expression in western white pine is a feasible option for genetic improvement of this valuable conifer as well as for investigating its molecular interactions with the fungal pathogen *Cronartium ribicola* (J.C. Fisch.).

Keywords: genetic transformation, reporter gene, western white pine, zygotic embryo

INTRODUCTION

Western white pine (*Pinus monticola*, Dougl. ex D. Don) is one of the most important forest tree species in western North America. Its wild population has been dramatically decreased in the ecosystems since white pine blister rust (WPBR) was introduced to North America in the early 1900's. WPBR is caused by the fungal pathogen *Cronartium ribicola* (J.C. Fisch.). Selection for white pine with improved genetic resistance to *C. ribicola* has been performed for the last five decades in the USA and Canada. However, few resources for genetic resistance and other impediments such as the long life cycle make it difficult to restore this valuable species in forest plantations (Liu *et al*. 2004).

The disease resistance gene families and other plant gene families related to disease resistance have been characterized in western white pine (Liu and Ekramoddoullah 2003a; Liu *et al*. 2005b; Ekramoddoullah *et al*. 2006; Liu and Ekramoddoullah 2007, 2009). The next step toward understanding the molecular interaction between *P. monticola* and *C. ribicola* is to establish a genetic transformation system in this conifer species, which would allow functional genomics investigations of the resistance-related genes characterized thus far. Furthermore, the need to widen the spectrum of WPBR resistance is imperative and western white pine gene transformation would provide a feasible strategy by allowing the introduction of multiple resistance mechanisms into elite white pine seed orchards for durable host resistance against *C. ribicola* (Liu *et al*. 2004). Because the specific plant genotype greatly affects what techniques work for genetic transformation, development and further optimization of a technical protocol is a prerequisite for silvicultural improvement through genetic engineering in any forest species (Merkle *et al*. 2007).

Development of a technology for generation of transgenic plants expressing functional genes in a conifer pathosystem such as WPBR is a time consuming and labour intensive process. The objective of the present study was to explore transformation of *P. monticola* zygotic embryos using the gene transfer technology mediated by *Agrobacterium tumefaciens*. Plant transformation has been successfully carried out using various explants in a few conifer species (Walter 2004; Williams 2006). Transgenic conifer plants have been obtained through different techniques, including particle bombardment (Ellis *et al*. 1993) and *Agrobacterium*-mediated transformation (Levée *et al*. 1997).

High transgene copy number has been identified as one of the major reasons for transgene silencing (Hansen and Wright 1999; Gelvin 2003; Walter 2004; Williams 2006). Compared to particle bombardment, *Agrobacterium*-mediated gene transfer usually results in a lower copy number of transgenes integrated into the plant genome. Transformation protocols with *Agrobacterium*-mediated techniques have been successfully established with various explant tissues for several conifer species, including eastern white pine (*Pinus strobus* L.) (Levée *et al*. 1999; Tang and Newton 2005), loblolly pine (*P. taeda*) (Wenck *et al*. 1999; Tang *et al*. 2001), European black pine (*P. nigra*) (López *et al*. 2000), maritime pine (*P. pinaster* Ait.) (Trontin *et al*. 2002; Tereso *et al*. 2006), Virginia pine (*P. Virginian* Mill.) (Tang and Newton 2004), Monterey pine (*P. radiata*) (Walter *et al*. 1998; Grant *et al*. 2004), chir pine (*P. roxbughii* Sarg.) (Parasharami *et al*. 2006), Norway spruce (*Picea abies*) (Wenck *et al*. 1999), black spruce (*Picea mariana*) (Tian *et al*. 2000), and white spruce (*Picea glauca*) (Le *et al*. 2001; Klimaszewska *et al*. 2001).

Embryogenic cell lines from immature zygotic embryos are widely used as explant material for conifer gene trans-

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formation (Levée *et al*. 1997, 1999; Wenck *et al*. 1999; Klimaszewska *et al*. 2001; Trontin *et al*. 2002; Tereso *et al*. 2006). Other explant tissues used for conifer gene transformation include cotyledons (Humara *et al*. 1999; Grant *et al*. 2004), zygotic embryos (Tang *et al*. 2001; Parasharami *et al*. 2006), and the shoot apex (Gould *et al*. 2002). However, no genetic transformation has been reported and only limited information about tissue culture and somatic embryogenesis is available in *P. monticola* (Percy *et al*. 2000). Here we report the development of *Agrobacterium*-mediated genetic transformation of western white pine.

MATERIALS AND METHODS

Plant materials

Western white pine used for genetic transformation included two seed families (No. 2384 and No. 5325) with variations of genetic resistance to *C. ribicola*, both seed lots were kindly provided by Dr. Rich Hunt (Canadian Forest Service, Natural Resources Canada). All plant growth regulators, antibiotics, medium chemicals, and acetosyringone were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Agrobacterium culture

Agrobacterium tumefaciens strain GV3101 was used in the present investigation with binary vector pBI121 (Clontech, Mountain View, CA, USA), or pCHF3GFP (kindly provided by Dr. S Huang, University of Missouri, USA) was used in the present investigation (**Fig. 1**). The T-DNA of binary vector pBI121 contains the *udiA* gene encoding β-glucuronidase (GUS), and the T-DNA of binary vector pCHF3GFP contains the *m-gfp5-ER* gene encoding green fluorescent protein (GFP). In both vectors, the reporter genes are driven by the CaMV 35S promoter, and the neomycin phosphotransferase II (*npt-II*) gene is driven by the NOS promoter for kanamycin resistance selection of transgenic plant tissues.

Plasmid DNA of binary constructs was introduced into *A. tumefaciens* competent cells with a freeze-thaw method as described previously (Liu and Ekramoddoullah 2003b). Single colonies of *Agrobacterium* were cultured overnight in liquid LB medium supplemented with gentamicin (50 mg/L), kanamycin (50 mg/L) and acetosyringone (40 mg/L) at 28°C until the culture reached an OD_{600} of 0.8~1.0. *Agrobacterium* cells were collected by centrifuging at 6,000 × *g* for 10 min and resuspended in liquid callus induction medium with 40 mg/L of acetosyringone at an OD_{600} of 0.4~0.6 for explant infection.

Transformation of mature zygotic embryos

Seeds were soaked in running water over night, and sterilized by treatment in 70% (v/v) ethanol for 1 min followed by incubation with 20% (v/v) bleach (5.25% $NaClO_3$, Javex) for 30 min. Following a distilled water rinse, embryos were dissected from seeds. After a three-day pre-culture on callus induction medium, those embryos showing good growth were selected and dipped in *Agrobacterium* suspension for a few seconds and co-cultured on the callus induction medium for three days. The media used for callus induction included BL (Brown and Lawrence 1968), DCR (Gupta and Durzan 1985), and PS (Tang and Newton 2005) basal media, supplemented with plant growth regulators at 5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/L kinetin, or 3.36 mg/L α-naphthalene acetic acid (NAA). Following co-culture of the explants with *Agrobacterium*, the infected embryos were washed with timentin (500 mg/L) for 1 min and rinsed with sterile water five times to remove *Agrobacterium*, then transferred onto fresh callus induction medium supplemented with timentin (500 mg/L) and kanamycin (15-25 mg/L). The medium was prepared as previously described (Peng *et al*. 2006) and changed every three weeks for subculture.

GUS histochemical assay

Transformed tissues were analyzed for β-glucuronidase expression using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt (X-Gluc, Clontech) as the substrate (Jefferson 1987). Transgenic tissue samples were vacuum infiltrated for a few minutes with 1 mM X-Glu in a GUS reaction buffer with 50 mM sodium phosphate, pH 7.0, 0.02% Triton X-100 and 0.5 mM each of $K_3[Fe(CN)_6]$ and $K4[Fe(CN)_6]$, and incubated at 37°C for 3-16 hrs. Subsequently, the tissues were cleared in 70% (v/v) ethanol (Liu *et al*. 2005a). To serve as control against any background GUS staining, non-transformed tissues were included in all staining experiments.

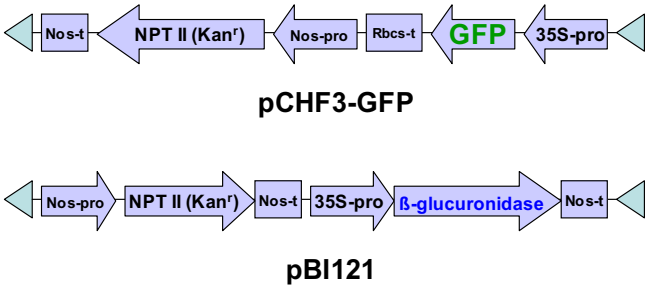

Fig. 1 Schematic representation of T-DNA regions of two constructs used for *P. monticola* transformation, pBI121 and pCHF3GFP. The reporter genes for GFP and β-glucuronidase were under control of the CaMV 35S promoter with the rbcs terminator or the nopaline synthase (Nos) terminator, respectively.

Observation of GFP gene expression

Transgenic callus at different selection stages was directly used for GFP fluorescence observation under a Zeiss epi-fluorescence microscope (Carl Zeiss Canada Ltd., Toronto, ON, Canada) with a FITC filter set comprising exciter filter (BP 450-490), chromatic beam splitter (FT 510), and barrier filter (LP 520). Transformation frequency was evaluated as the total number of callus lines with reporter gene expression per total number of *Agrobacterium*-inoculated zygotic embryos.

Statistical analysis

For quantitative analysis of kanamycin selection effect and callus growth, at least three biological repeats were performed for transformation experiments; each of them included at least hundred embryo explants. Samples were collected for analysis at different time points as indicated during culture process. One-way analysis of variation (ANOVA) was used to determine significance of data variations.

RESULTS AND DISCUSSION

Selection of transgenic callus

Mature zygotic embryos were dissected from western white pine seeds and induced for callus production on one of the three basal media (BL, DCR, and PS) with supplement of plant growth regulators. Almost all of the embryos were able to produce callus tissues, and no significant differences were observed among three basal media. Following co-culture with *Agrobacterium* in transformation experiments, the embryos were transferred to fresh callus induction medium with kanamycin for selection of transgenic tissues. Little difference in callus growth was observed between embryo explants in the first two weeks following co-culture with *Agrobacterium* (**Fig. 2A**). However, after four weeks of kanamycin selection, measurement of callus sizes clearly revealed the selection effect of kanamycin on callus growth (**Fig. 2B**). At this selection stage, only 20.55 and 24.49% of total callus lines from pBI121 and pCHF3GFP, respectively showed the same or larger sizes than the average size of controls without kanamycin selection (**Fig. 3**). Average diameters of callus masses from *Agrobacterium*-mediated transformation with binary vector pBI121 and pCHF3GFP were 7.78 and 7.60 mm, respectively, significantly different (p<0.001) from callus masses without kanamycin selection,

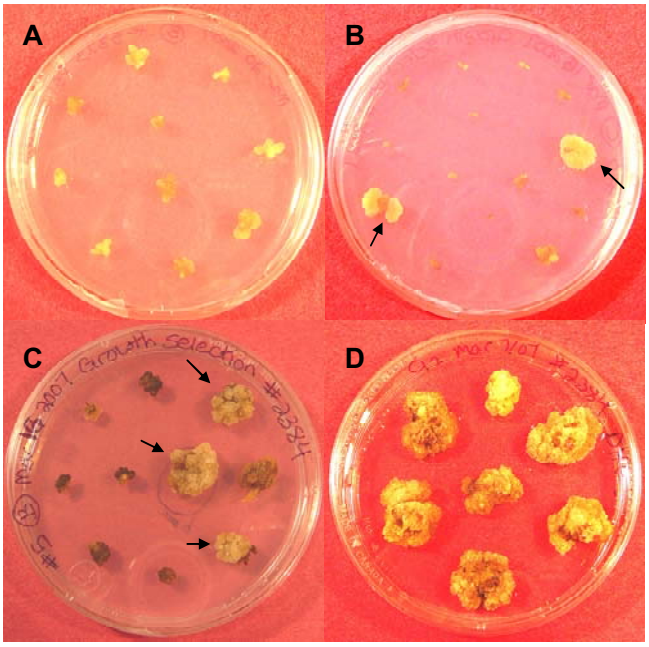

Fig. 2 Kanamycin selection of transgenic callus in western white pine. Callus was induced from mature zygotic embryos and grew on the callus-induction medium with kanamycin (25 mg/L): callus growth at the end of two weeks (**A**); at the end of four weeks (**B**); at end of eight weeks (**C**); and at end of 12 weeks of kanamycin selection (**D**). Arrows indicate kanamycin-resistant calli, other calli were killed by kanamycin toxicity and were removed after eight weeks selection.

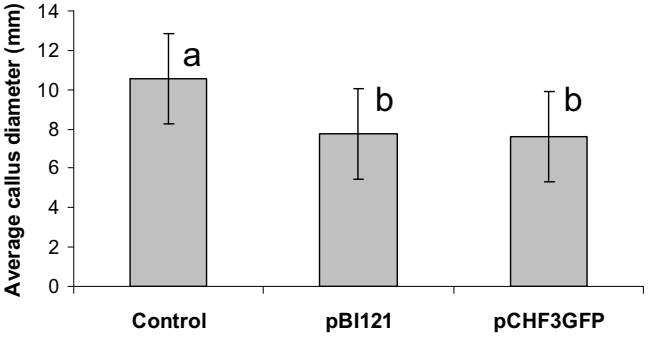

Fig. 4 Quantitative analysis of kanamycin selection effect on callus growth. Western white pine seed family No. 2384 was used for transformation. Experiments were repeated three times and each treatment consisted of 107-131 explants. Values represented the means ± STD (mm). Values followed by different letters are significantly different (p<0.001) by one-way analysis of variation (ANOVA).

which showed an average size of 10.53 mm (**Fig. 4**). This indicates that kanamycin at 25 mg/L was effective for selection of transgenic western white pine tissues. No significant difference was observed between vector pBI121 and pCHF3GFP, suggesting that both binary vectors with reporter genes (*udiA* and *m-gfp-ER*, respectively) were transformed into western white pine tissues with similar efficiencies.

According to previous reports, the effective concentration of kanamycin for selection of *Agrobacterium*-transformed conifer tissues depends on conifer species and explant type. In loblolly pine apex and meristem-based transformation, a sub-lethal kanamycin concentration (25 mg/L) was chosen for cell propagation in the transgenic sector without destroying the shoot structure (Gould *et al*. 2002). Kanamycin at 50 mg/L was effective for selection of transgenic larch embryonal tissues (Levée *et al*. 1997). In eastern white pine transformation using embryogenic clones, 25 mg/L kanamycin was sufficient to prevent untransformed cells from proliferation within three to four weeks (Levée *et al*. 1999). In western white pine, we found that transgenic callus tissues survived and continued to grow on medium with kanamycin (25 mg/L), whereas non-transgenic callus, regenerated from embryos of the same seed family, darkened and died within 8 to 12 weeks (**Fig. 2C, 2D**). The survival rates of callus lines varied from 33 to 48% among western white pine families.

Reporter *udiA* gene expression in transgenic callus

PCR analysis is a routine method to verify foreign gene integration in transgenic tissues. Elimination of *Agrobacterium* usually depends on the types and concentrations of antibiotics used in plant gene transformation (Levée *et al*. 1997; Gould *et al*. 2002). Even with optimized antibiotic conditions, *Agrobacterium* could not be completely removed after several rounds of subculturing. Compared to Southern-blot analysis of genomic DNA, PCR analysis may produce

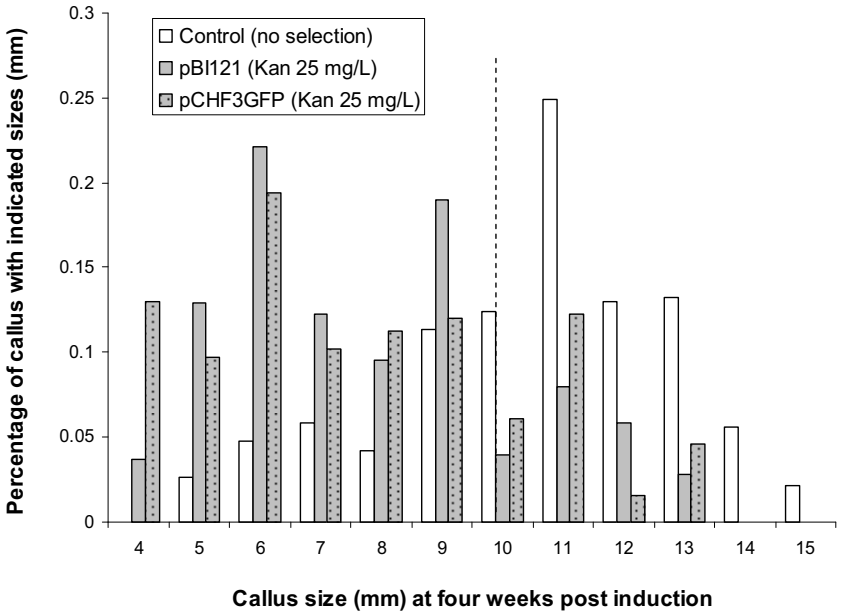

Fig. 3 Percentage of *P. monticola* callus with indicated sizes (diameter, mm) at the end of four weeks post co-culture with *Agrobacterium*. Both pBI121 and pCHF3GFP were used for plant transformation. Western white pine calli grew on the induction media containing kanamycin for transgenic selection, or on the callus induction medium without kanamycin for the control. The average size of control calli was marked by a dash line.

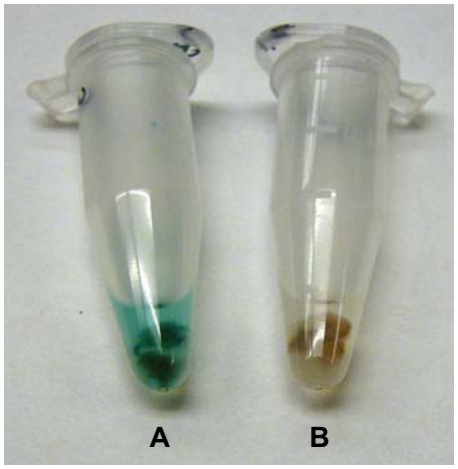

Fig. 5 Expression of the *udiA* gene in western white pine callus. *Agrobacterium*-mediated transformation was performed with binary vector pBI121. (**A**) Stable *udiA* expression in callus was revealed by the GUS histochemical assay after eight weeks post co-cultivation; (**B**) control callus from uninfected embryo showed no *udiA* expression.

false-positive results for the identification of transgene integration in plant genomes (Grant *et al*. 2004). Therefore, PCR analysis is more often used in the initial screening of transgenes in most plant transformation studies. In the present study, the transformation frequency was considered as the number of kanamycin-resistant or transformed lines with obvious reporter gene expression per initial embryos co-cultured with *Agrobacterium* (**Table 1**). To evaluate stable transformation efficiency of reporter genes in western white pine tissues, we analyzed the expressed foreign proteins directly to verify reporter gene integration and expression at the end of eight weeks kanamycin selection. The GUS histochemical staining assay showed that positive transgenic lines accounted for 4.5 to 6.5% of total embryos subject to *Agrobacterium* co-culture (**Fig. 5; Table 1**). Our finding is similar to the *Agrobacterium*-mediated transformation efficiency reported in *P. radiata* at about 1.5% (Grant *et al*. 2004) and in hybrid larch (*Larix kaempferi* × *L. decidua*) at 2-4% (Levée *et al*. 1997).

There is potential for expression of genes in bacteria from plant promoters. For example, strong expression of the *lux* gene was found in the *A. tumefaciens* strain with CaMV 35S promoter (Jacob *et al*. 2002). To avoid GUS signal possibly from *Agrobacterium* cells, a further observation of *P. monticola* callus tissues under microscopy showed cytosolic localization of expressed GUS protein inside western white pine cells (data not shown).

Reporter *m-gfp-ER* gene expression in transgenic callus

A high transient expression frequency usually does not lead to high stable transformation frequency. The transit *udiA* gene expression rate decreased from 31.8% at day 1 to 0% at day 15 post reporter gene transformation in embryogenic cultures of eastern white pine (Zipf *et al*. 2001). Therefore, we monitored stable GFP expression by fluorescence microscopy at two and eight weeks post co-culture of *Agrobacterium* with kanamycin selection (**Table 1**). After two weeks of kanamycin selection, the GFP expression rate was 23%, much higher than that after eight weeks of kanamycin selection (**Table 1**). After eight weeks selection, the *m-gfp-ER* gene expression rates were 2.9 to 5.9%, similar to the results of those transgenic lines with pBI121 (**Table 1**). We also observed un-even GFP fluorescence especially at the early stage of two weeks kanamycin selection (**Fig. 6**). This could be explained by the existence of different proportions of transformed to non-transformed cells. Variations in GFP fluorescence intensity indicated the expression levels of the *m-gfp-ER* gene were different among kanamycin-resistant callus lines, which may result from the position effect of the T-DNA insertion (Matzke *et al*. 1998). A high copy number also possibly leads to transgene silencing (Hansen and Wright 1999; Gelvin 2003).

Table 1 Reporter gene expression rates of western white pine callus lines post co-culture of *Agrobacterium* with kanamycin selection.

Family ID	Vector	Explant (n)	Rates at two weeks (%)	Rate at eight weeks (%)
#5325	pBI121	66	---	4.5
	pCHF3GFP	67	23	5.9
#2384	pBI121	61	---	6.5
	pCHF3GFP	69	---	2.9
Average		65.75	---	4.95

Compared with other reporter genes such as *udiA*, the *m-gfp-ER* reporter gene has several advantages. It needs no substrate for detection and shows no toxic effect to living cells. Most importantly it can be observed in living cells, which is very convenient for transgenic selection at an early stage without disturbing the growing plant tissues. The selected western white pine transgenic callus lines with *m-gfp-ER* expression were able to continue propagating over multiple subcultures as long as 12 weeks in our experiments, allowing the potential to generate transgenic plantlets from differentiation of these tissues. Our future research will focus on regenerating western white pine seedlings from different callus lines containing the reporter genes.

Our main objective was to establish a protocol for stable gene expression in western white pine cells with high repeatability and efficiency in various seed families. Transgenic tissues, obtained through infection of mature zygotic embryos with *Agrobacterium*, demonstrate the usefulness of the present protocol for introducing genes associated with important economic traits, such as improved disease resistance, into the *P. monticola* genome. The zygotic mature embryo was chosen as explant for genetic transformation of western white pine because there is no season requirement

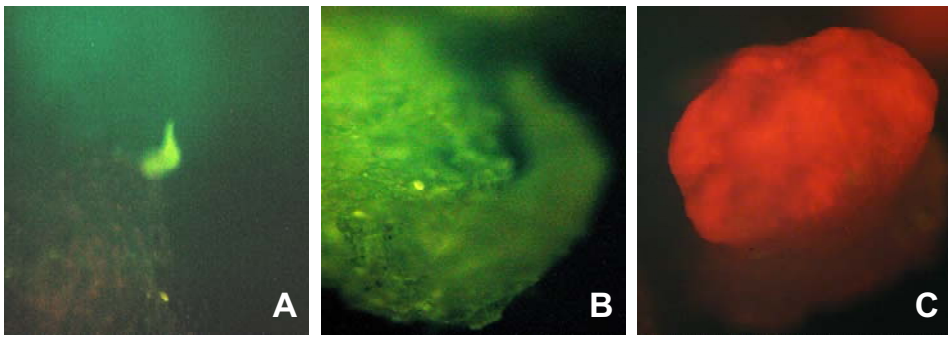

Fig. 6 Fluorescence microscopy of callus grown on medium with kanamycin selection. (**A**) Stable GFP expression in kanamycin-resistant callus at a local part of one callus line; (**B**) GFP fluorescence observed in the tissues throughout the whole callus line; (**C**) Red fluorescence in nontransformed callus from control experiment without *Agrobacterium* co-culture.

as compared with initiation of somatic embyogenic cell line from premature embryos. Due to strong family effect, it was necessary to select fewer, more responsive families for the development of somatic embyogenic cell line in western whit pine (Percy *et al*. 2000). In western white pine breeding program, our strategy is to pyramid multiple resistance mechanisms into one elite seed orchard for more durable resistance against *C. ribicola* infection (Liu *et al*. 2004). The protocol developed in the present study makes it feasible for the genetic improvement of western white pine by genetic engineering.

Expression of β-glucuronidase and GFP in callus demonstrates the efficacy of this system for expression of any other useful foreign protein in western white pine. The protocol reported here would be implemented in research programs investigating white pine interactions with *C. ribicola* at the molecular level. Western white pine genes encoding candidate proteins related to disease resistance activity, such as anti-microbial peptides, chitinases, pathogenesis-related family 10 (PR10) proteins, and putative disease resistance proteins will be over-expressed or knocked down in white pine to verify their functions in host defence against rust infection. A further differentiation of transgenic callus will lead to creation of transgenic conifer trees resistant to *C. ribicola*.

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