Genetic Transformation of *Casuarina equisetifolia* by *Agrobacterium tumefaciens*

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Abstract Different seedlots of *Casuarina equisetifolia* and media compositions were studied to improve callus regeneration and genetic transformation. The explants included epicotyl fragments from 45-day-old seedlings and the biological vector to achieve gene transfer was based on the disarmed *Agrobacterium tumefaciens* strain C58C1 (pGV2260) containing the β-glucuronidase (GUS) or green fluorescent protein (GFP) reporter genes under the control of the constitutive 35S promoter. Following a selection on kanamycin, GUS or GFP expression was observed in *C. equisetifolia* calli and young shoots, thus suggesting a successful transfer of the reporter genes into the host plant.

1 Introduction

The family Casuarinaceae contains 4 genera and 96 species native from Australia to the islands of the Pacific and to Southeast Asia (Wilson and Johnson, 1989). Casuarinaceae includes important tree species with good potential for afforestation in subtropical and tropical areas (Midgley *et al*., 1983). *Casuarina equisetifolia* has been extensively cultivated in the inland and coastal areas of southern China (Zhong and Bai, 1996; Zhong and Zhang, 2003). Several *Casuarina* species have been *in vitro* regenerated, including *C. equisetifolia* (Duhoux *et al*., 1986), *C. glauca* (Le *et al*., 1996; Santi *et al*., 2003) and *Allocasuarina verticillata* (Cao *et al*., 1990; Franche *et al*., 1997). The natural susceptibility of members of the Casuarinaceae family to *Agrobacterium tumefaciens* has previously been used to develop gene transfer procedures for both *A. verticillata* (Franche *et al*., 1997) and *C. glauca* (Diouf *et al*., 1995; Franche *et al*., 1998; Smouni *et al*., 2002). A number of physiological and environmental factors, such as the presence of plant phenolic compounds, sugars, pH, temperature and osmoprotectant compounds, influence the induction of virulence genes and, consequently, the efficiency of T-DNA transfer from *A. tumefaciens* to the wounded plant cells. Previous experiments performed with *C. glauca* showed that optimal transformation rates were obtained when epicotyls from 45-day-old seedlings were co-cultivated for three days at pH 5.6 with the *A. tumefaciens* strain C58C1 (pGV2260) (Le *et al*., 1996).

In this study we report the induction of organogenic calli from epicotyls of *C. equisetifolia* and the genetic transformation of this actinorhizal species by *A. tumefaciens*. Calli and young shoots expressing the reporter genes GUS or GFP were obtained. These preliminary data pave the way for the genetic transformation of this environmentally important tree species.

2 Material and Methods

2.1 Seed source

The seeds of *C. equisetifolia* were collected in four locations: HA4, Huian Forest Farm, Fujian, China; HA3, Huian, Fujian, China; TH5, native forest in Thailand; and DS5, Dongshan, Fujian, China.

2.2 Disinfection and seedling production

Chemical scarification was carried out by soaking the seeds in concentrated H_2SO_4 (98%) for 2.5 min and subsequently rinsing with tap water for 30 min. The scarified seeds were then disinfected with 5% calcium hypochlorite for 35 min, rinsed 3 times with sterile distilled water, and germinated in Petri dishes containing H medium solidified with 8 g L^{-1} Difco-Bacto agar as previously described (Le *et al*., 1996).

2.3 Experimental design

Four experiments, referred as Exp. 1-4, were carried out in the laboratory of Institut de Recherche pour le Développement (IRD), Montpellier, France. Plant material was grown in a chamber at 27 ± 1℃ with a 16-h photoperiod (45-60 μmol m^{-2} s^{-1}), and growth media were renewed every 3 weeks.

The objectives of the experiments were the followings: Exp. 1: Callus induction on epicotyls from the four seedlots of *C. equisetifolia* grown on MSC (half-strength MS salts and NN vitamins) (Murashige and Skoog, 1962; Nitsch and Nitsch, 1969; Le *et al*., 1996), treatments were described in Table 1; Exp. 2: Effect of 6-benzylaminopurine (BA), naphthaleneacetic acid (NAA) and $AgNO_3$ on the organogenic potential of the calli induced from epicotyls, treatments were described in Table 2 with seedlot H 4; Exp. 3: Genetic transformation by *A. tumefaciens* of the four *C. equisetifolia* seedlots, treatments were described in Table 4; Exp. 4: Genetic transformation of *C. equisetifolia* seedlot HA4 by *A. tumefaciens* containing the binary vectors pBIN-35S-GUS-INT or pBIN-35S-GFP, treatments were described in Table 5.

Experiments were carried out in Petri dishes (10 cm in diameter) containing 12-32 epicotyl segments per petri dish, and epicotyl segments were around 1.2-1.5 cm in length. In the Exp. 3, 1688 epicotyl segments from the four *C. equisetifolia* seedlots were genetically transformed with C58C1 (pGV2260) containing pBIN-35S-GUS-INT (Table 4). Exp. 4 included 298 epicotyl segments cocultivated with C58C1(pGV2260; pBIN-35S-GFP5ER) and 354 explants genetically transformed with C58C1(pGV2260; pBIN-35S-GUS-INT) (Table 5). In the Exp. 3 and Exp. 4, selection of transgenic cells was achieved with the addition of 25 mg L^{-1} kanamycin in the nutrient medium for two weeks; kanamycin concentration was then raised to 50 mg L^{-1} as previously described (Le *et al*., 1996; Franche *et al*., 1997). The MSC nutrient medium consisted half-strength MS medium salts (Murashige and Skoog, 1962), NN medium (Nitsch and Nitsch, 1969) vitamins, and 30 g L^{-1} sucrose.

2.4 Data collection and treatment

All data were collected 2 months after culture. Calli with a diameter of at least 3 mm were counted Exp. 1 and Exp. 2. CN refers to "callus number" and the callus induction rate was calculated as follows: (%) = 100 × (segment number with at least 1 callus /total segments per replicate (Petri dish), in order to get standard and comparable data. Each replicate will get a figure to use for Analysis of Variance. Additional observations for Exp. 1 and Exp. 2 included TCS (top callus size, mm), BCS (basal callus size, mm), BN (bud number), RN (root number) and RL (root length, mm) and the CS (mean callus size per segment, mm) was calculated as follows $SQRT(TCS^2 + BCS^2)$. In Exp. 3 and Exp. 4, we observed indexes based on per Petri dish: KCN = kanamycin resistant callus number; ESN = epicotyl segment number with at least one callus; TSN = total segment number per Petri dish. The genetic transformation efficiency was determined as (GTE, %), with GTE_1 = (KCN/TSN) × 100%, and GTE_2 = (ESN/TSN) × 100%. All the count number data was exchanged by SQRT(x) (note: x means that CN, KCN, ESN or TSN in the experiments) and genetic transformation efficiency data was exchanged by ARSIN (SQRT (GTE/200))/3.1415926 × 2 × 90 before running GLM model for Analysis of Variation, and Duncan's Multiple Range (DMR) Test.

3 Results

3.1 *In vitro* culture

Significant differences were observed in callus induction among the four *C. equisetifolia* seedlots ($P < 0.01$) (Table 1). Seedlot HA4 gave the best results for callus induction with a rate of 97.8% and seedlot DS5 reacted poorly to callus induction with 10.7% of calli induced on the epicotyls. Seedlots HA3 and TH5 gave an intermediary response with respectively 48.8% and 54.2% of callus induction.

Table 1 Callus induction rate (%) on epicotyls fragments excised from four different seedlots in the Exp. 1

Design and treatment			Mean callus induction rate (%) at 2 months culture
Seedlot No.	Replicate (No. of PD)	Number of epicotyl segments	
HA4	6	101	$97.8^{a} \pm 1.4$
HA3	4	61	$48.8^{b} \pm 21.9$
TH5	9	185	$54.2^{b} \pm 12.3$
DS5	3	39	$10.7^{c} \pm 3.1$

Data were recorded two months after callus induction. PD = Petri dishes. Data was the means of 3-9 repetitions ± STD (Standard Error). Means with the same letter are not significantly different ($P < 0.01$), according to Duncan's Multiple Range Test.

3.2 Effect of BA, NAA and $AgNO_3$ on regeneration

Table 3 gives the details of the Duncan's Multiple Range Test for the 7 indexes. There were no significant differences in basal callus number and callus size among the 5 treatments R1-R5 described in Table 1 ($P < 0.05$ or $P < 0.01$); on the opposite, there were significant differences in top callus number, size, colour, and organogenic potential (bud number, root number and root length) among the 5 treatments ($P < 0.05$ or $P < 0.01$). Concerning the top callus, the R4 and R5 media was significantly more favourable to callus growth than the other media. In the R2 treatment,

induced calli exhibited a green colour that was a positive indication of the organogenic potential. The reported data suggest that the growth regulators concentrations and the addition of $AgNO_3$ in the R2 treatment could significantly improve buds regeneration from *C. equisetifolia* callus, whereas the R5 treatment induces rhizogenesis (increase in root number and root length). However, a major problem encountered in these experiments was the low efficiency of shoot differentiation and the poor shoot growth.

Table 2 Media tested for callus induction and *in vitro* regeneration of *Casuarina equisetifolia.* seedlot H4 in the Exp. 2

Treatment	Time (weeks)		
	0	3	6
R1	BA0.5 + NAA 0.1	BA0.5 + NAA 0.1	BA0.5 + NAA 0.1
R2	BA0.5 + NAA0.01	BA0.5 + NAA 0.01	BA0.5 + NAA 0.01
R3	BA0.5 + NAA 0.1	BA0.5 + NAA 0.01	BA0.5 + NAA 0.01
R4	BA0.5 + NAA 0.1	BA0.5 + NAA 0.1 + $AgNO_3$ 3.0	BA0.5 + NAA 0.1
R5	BA0.5 + NAA 0.1	BA0.5 + NAA 0.01 + $AgNO_3$ 3.0	BA0.5 + NAA 0.01

Concentrations are indicated in mg L^{-1}.

Table 3 Effect of different concentrations of growth regulators and silver nitrate on the organogenic potential of *C. equisetifolia* seedlot H4 calli in the Exp. 2

Treatment	Duncan's Multiple Rang Test for callus, bud and root mean values at 2 months after treatment						
	MCN ± STD	TCS ± STD	BCS ± STD	CS ± STD	BN ± STD	RN ± STD	RL ± STD
R1	1.5^{a} ±0.1	0.9^{b} ±0.2	4.3^{a} ±0.3	4.5^{b} ±0.3	0.0^{b} ±0.0	0.3^{b} ±0.1	1.9^{b} ±0.9
R2	1.6^{a} ±0.1	1.4^{ab} ±0.3	4.3^{a} ±0.3	4.9^{ab} ±0.3	0.4^{a} ±0.2	0.4^{b} ±0.1	3.6^{b} ±0.9
R3	1.4^{a} ±0.1	1.2^{b} ±0.3	4.5^{a} ±0.3	4.9^{ab} ±0.3	0.0^{b} ±0.0	0.5^{b} ±0.1	3.4^{b} ±1.1
R4	1.7^{a} ±0.1	2.1^{a} ±0.3	4.5^{a} ±0.2	5.4^{a} ±0.3	0.0^{b} ±0.0	0.4^{b} ±0.1	2.5^{b} ±0.9
R5	1.5^{a} ±0.1	1.6^{ab} ±0.3	5.0^{a} ±0.3	5.7^{a} ±0.3	0.3^{ab} ±0.2	1.2^{a} ±0.3	14.8^{a} ±5.0
Sign. level	0.05	0.05	0.05	0.05	0.01	0.01	0.01

BA, NAA and $AgNO_3$ concentrations are provided in mg L^{-1}. Means with the same letter are not significantly different; Data was mean ± STD(Standard Error). The 8 indexes are CN (callus number), TCS (top callus size, mm), BCS(basal callus size, mm), BN(bud number), RN(root number), RL(root length, mm) and CS (mean callus size per segment, mm).

3.3 Genetic transformation by *A. tumefaciens* of four *C. equisetifolia* seedlots

For the Exp. 3, Table 4 shows the mean values of the data obtained after genetic transformation of the epicotyl fragments with C58C1(pGV2260; pBIN-pBIN-35S-GUS-INT). Positive controls (PC) included epicotyl fragments without contact with agrobacterium, grown on the organogenic medium MSC. Negative control (NC) included explants without contact with *Agrobacterium*, grown on the nutrient medium MSC containing the antibiotic kanamycin; no callus growth was expected in these conditions. Results in Table 4 indicate that there were significant differences in KCN, ESN, TSN and GTE among the seedlots HA3, HA4, TH5 and DS5, and among treatments ($P < 0.01$), but no significant differences among replicates.

Table 4 Genetic transformation efficiency by the strain C58C1 (pGV2260; pBIN19-35S-GUSINT) observed among the four *C. equisetifolia* seedlots in the Exp. 3

Seedlot	Treat.	Repl. (PD)	No. of segments	TSN	KCN	ESN	GTE_1, %	GTE_2, %
	GUS	18	466	15.3 ± 2.3	19.0 ± 4.6	14.25 ± 2.6	121.2 ± 14.3	92.58 ± 5.1
HA3	PC	4	61	25.9 ± 1.1	–	2.06 ± 0.32	–	–
	NC	2	34	17.0 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	GUS	26	548	16.8 ± 1.1	23.5 ± 1.1	16.3 ± 1.2	142.5 ± 10.5	97.0 ± 1.4
HA4	PC	6	101	21.1 ± 0.8	–	2.9 ± 0.3	–	–
	NC	2	45	22.5 ± 1.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	GUS	22	510	20.4 ± 1.4	12.5 ± 3.5	9.7 ± 2.5	63.2 ± 17.9	48.8 ± 12.3
TH5	PC	10	204	24.0 ± 0.9	–	2.1 ± 0.3	–	–
	NC	4	80	20.0 ± 0.7	0.3 ± 0.3	0.3 ± 0.3	1.3 ± 1.3	1.3 ± 1.3
	GUS	5	164	19.5 ± 3.5	2.0 ± 1.0	2.0 ± 1.00	9.7 ± 3.4	9.7 ± 3.4
DS5	PC	2	39	32.8 ± 4.8	–	0.0 ± 0.00	–	–
	NC	2	38	19.0 ± 1.0	0.0 ± 0.0	0.0 ± 0.00	0.0 ± 0.0	0.0 ± 0.0

GUS: ß-glucuronidase reporter gene; PC: postive control; NC: negative control. TSN = total mean segment number per petri dish; KCN = Km resistant callus number; ESN = epicotyl segment number with at least one callus; and calculated the genetic transformation efficiency (GTE,%), GTE_1 = (KCN/TSN) × 100%, GTE_2 = (ESN/TSN) × 100%. Data was mean ± STD.

3.4 GFP and GUS gene expression in the seedlot HA4

As reported in Table 5 in the Exp. 4, the efficiency of genetic transformation was similar using the *A. tumefaciens* strain containing either the binary vector pBIN-35S-GUS-INT or the vector pBIN-pBIN-35S-GFP. GFP expression was observed under UV light in the kanamycin resistant calli and blue indogo colour was seen in the antibiotic resistant calli and young shoots obtained after the genetic transformation with the vector containing the ß-glucuronidase gene. No callus growth was observed for the negative control.

Table 5 Genetic transformation of the seedlot HA4 by the *A. tumefaciens* strains C58C1 (pGV2260; pBIN19-35S-GUSINT) and C58C1 (pGV2260; pBIN19-35S-GFP) in the Exp. 4

Genes	Repl. (PD)	No. of segments	TSN	KCN	ESN	GTE_1, %	GTE_2, %
PC	4	61	15.3 ± 0.6	–	14.8 ± 0.6	–	–
GFP	14	298	21.3 ± 0.7	5.1 ± 0.6	4.8 ± 0.5	24.4^{a} ± 2.7	22.7^{a} ± 2.4
GUS	18	354	19.7 ± 0.9	3.2 ± 0.4	3.1 ± 0.4	16.5^{a} ± 2.0	16.0^{a} ± 2.0
NC	2	45	22.5 ± 1.5	0.0 ± 0.0	0.0 ± 0.0	0.0^{b} ± 0.0	0.0^{b} ± 0.0

PC: postive control; GFP = green fluorescent protein reporter gene; GUS: β-glucuronidase reporter gene; NC: negative control. TSN = total mean segment number per petri dish; KCN = Km resistant callus number; ESN = epicotyl segment number with at least one callus; and calculated genetic transformation efficiency (GTE,%), GTE_1 = (KCN/TSN) × 100%, GTE_2 = (ESN/TSN) × 100%. Data was mean ± STD.

4 Discussion

In this study, we reported the successful callus induction on epicotyls of *C. equisetifolia*, and we obtained GUS and GFP gene expression in calli and young shoots after genetic transformation with *A. tumefaciens*. In other Casuarina species, the use hormones has proven to be effective for plant regeneration (Duhoux *et al*., 1986; Le *et al*., 1996; Parthiban *et al*., 1996; Surendran *et al*., 1996).

In our study, the type, concentration, and combination of hormones were shown to play an important role in stimulating regeneration in *C. equisetifolia*. Data from the literature demonstrate that plant growth regulators can greatly affect callus formation and subsequent bud regeneration (Shen *et al*., 2009). In our study, adventitious bud regeneration was improved with the addition of the $AgNO_3$; this finding was consistent with previous reports on regeneration of other plants (Aditi *et al*., 2008). We also established that the genotype was important for *C. equisetifolia* callus induction. This is in agreement with numerous reports indicating that the genotype is one of the key factors for plant regeneration (Liu and Pijut, 2008).

Following genetic transformation with the strain C58C1 (pGV2260), we obtained potentially transformed calli and young shoots of *C. equisetifolia* that were kanamycin resistant and expressed the reporter genes GFP and GUS under the control of the 35S promoter. To further establish the gene transfer into *C. equisetifolia*, these preliminary data have to be confirmed by a molecular analysis based on Southern hybridization. In addition, *in vitro* culture procedures still have to be improved in order to obtain rooted transgenic plants of *C. equisetifolia*. The accumulation of phenolic compounds in the calli is probably one of the major factors limting the organogenic potential of *C. equisetifolia*.

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