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ORIGINAL ARTICLE

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Light strongly promotes gene transfer from *Agrobacterium tumefaciens* to plant cells

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Abstract Light conditions during *Agrobacterium*-based plant transformation, the most routinely used method in plant genetic engineering, differ widely and, to our knowledge, have not been studied systematically in relation to transformation efficiency. Here, light effects were examined in two already optimized transformation procedures: coculture of *Agrobacterium tumefaciens* with callus from two genotypes of the crop plant *Phaseolus acutifolius* (tepary bean) and coculture of root segments from two ecotypes of *Arabidopsis thaliana*. Except for the light conditions during coculture, all steps followed established procedures. Coculture was done either under continuous darkness, under a commonly used photoperiod of 16 h light/8 h darkness or under continuous light. β-glucuronidase (GUS) production due to the transient expression of an intron-containing *uidA* gene in the binary vector was used to evaluate T-DNA transfer. In all situations, *uidA* expression correlated highly and positively with the light period used during coculture; it was inhibited severely by darkness and enhanced more under continuous light than under a 16 h light/8 h dark photoperiod. The promotive effect of light was observed with *Agrobacterium* strains harboring either a nopaline-, an octopine- or an agropine/succinamopine-type non-oncogenic helper Ti plasmid. The observed positive effect of light has obvious implications for developing and improving transient and stable transformation protocols, specifically those involving dark coculture conditions.

Keywords *Agrobacterium* · *Arabidopsis* · Coculture · Light · *Phaseolus* · Transformation

Abbreviations CaMV/p35S: cauliflower mosaic virus 35S promoter · GUS: β-glucuronidase · Ti: tumor-inducing · *uidA*: β-glucuronidase gene from *Escherichia coli*

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Introduction

Plant genetic transformation is a core research tool in modern plant biology and agricultural biotechnology. *Agrobacterium tumefaciens* remains the preferred method to genetically transform plants (Gheysen et al. 1998). In spite of progress in *Agrobacterium*-based technology, efficient transformation methods are still restricted to a few genotypes of a limited number of plant species. This is reflected in the fact that crop improvement through transgenesis involves usually a few elite and/or local varieties. Moreover, for most commercially important plants, currently available transformation procedures are too inefficient to be used in high-throughput applications. Improving transformation frequencies remains, therefore, one of the major challenges in plant transgene technology (Gheysen et al. 1998; Hansen and Wright 1999). Many environmental factors as well as plant and bacterial genes that affect DNA transfer from *Agrobacterium* to plants have been identified (Tzfira and Citovsky 2002, and references therein), and this forms the basis for improving transformation procedures.

In general, the light conditions during coculture vary considerably in different transformation procedures and have received little attention. In this study, we have conclusively identified light as an important factor that strongly promotes gene transfer from *Agrobacterium* to cells of *Arabidopsis thaliana* and *Phaseolus acutifolius*. Hence, light conditions should be considered more rigorously in the development of efficient *A. tumefaciens*- and *A. rhizogenes*-based transformation protocols. Because of the high impact of light on transformation, study of this phenomenon may help to unravel further the mechanism mediating this form of inter-kingdom gene transfer.

Materials and methods

Growth conditions and light treatments

Compositions of the media are summarized in Table 1. Two growth chambers at 22 ± 0.4°C under cool white light (35–40 μmol m^{-2} s^{-1}; #21 tungsten tubes, Osram, Munich, Germany) were used for all coculture studies. The growth chamber 1 had a photoperiod of 16 h light/8 h dark, while growth chamber 2 had continuous (24 h) light. Coculturing was carried out in transparent glass jars (8 cm diameter × 8.5 cm or 14.5 cm height) with autoclavable, transparent white plastic screw caps. A regime of continuous darkness (24 h) was created by covering the jars with a single layer of light-proof, gas-porous black cloth. All cultures of *Arabidopsis* were incubated in growth chamber 1, whereas those of *Phaseolus* (except during coculture) were kept in a growth chamber at 25°C under a photoperiod of 16 h light (40 μmol m^{-2} s^{-1}; #21 tubes, Osram) and 8 h dark. All culture containers throughout the experiments were sealed with white gas-porous tape (Urgopore, Chenoves, France).

Plant material

For *Arabidopsis thaliana* (L.) Heynh., seeds of two ecotypes (C24 and Columbia; Nottingham Arabidopsis Stock Centre, Nottingham, UK) were sterilized (Valvekens et al. 1988) and sown on GMA (Table 1). Uniformly grown seedlings were transplanted after 1 week onto the same fresh medium (30 seedlings per 15-cm plate with 100 ml of medium). Intact roots from 1-month-old plants were pre-cultured on CIMA for 3 days (Table 1). Roots from all plants were washed together in liquid CCMA (Table 1), blotted briefly on sterile tissue papers and cut into segments of 3–5 mm with a sharp scalpel. Equal amounts of root segments (2 g) were used per replicate jar for coculture. For *Phaseolus acutifolius*, greenhouse-grown plant-derived green nodular callus from a wild genotype (NI576) and in vitro germinated embryo-derived green nodular callus from a cultivated genotype (TB1) were induced and maintained for four passages as described before on CIM1 (Dillen et al. 1997) and CIM1/5 (Zambre et al. 1998) media, respectively (Table 1). Callus for *Agrobacterium* infection was used 5 days after culturing the fourth passage. Calli were chopped into small (3–4 mm) pieces, mixed together and distributed in jars for coculture. One gram of callus (60–65 pieces) for NI576 and 3 g for TB1 was used per replicate jar.

Bacteria, plasmids and coculture

All *Agrobacterium tumefaciens* strains had a C58C1Rif^R chromosomal background and contained the non-oncogenic Ti plasmids

Table 1 Composition of media. *A* Media used for *Arabidopsis* (C24, Columbia) and transgenic line KH15d2. *B Phaseolus acutifolius* (NI576), *P. acutifolius* (TB1) and transgenic line 8.1.22. All media were adjusted to pH before autoclaving (20 min); all hormones (except TDZ), acetosyringone, vitamins and antibiotics were added after autoclaving to cool (35–40°C) media; all chemicals were from Sigma-Aldrich. Agar was from Difco (Detroit, MI, USA), except for GMA (Gibco-BRL) [*MS* Murashige and Skoog salts and vitamins (Murashige and Skoog 1992), *B5* Gamborg salts and vitamins (Gamborg et al. 1968), *Mes* 2-(*N*-morpholino)ethanesulfonic acid, *2,4-D* 2,4-dichlorophenoxyacetic acid, *2ip* 6(γ,γ-dimethylallylamino)purine, *IAA* indole-3-acetic acid, *TDZ* thidiazuron, *GMA* germination medium for *Arabidopsis* (A), *CIMA* callus-inducing medium A, *CCMA* coculture medium A, *WMA* wash medium A, similar to CCMA but with cefotaximum and devoid of acetosyringone, *SIMA* shoot-inducing medium A, *CIM1* callus-inducing medium (1), *CCM1* coculture medium 1, *WMCCM1* wash medium 1, similar to CCM1 but with cefotaximum and devoid of acetosyringone, *CIM1CF* CIM1 with cefotaximum, *CIM1/5* CIM1 with fivefold lower phytohormone concentrations (1/5), *CCM1/5* coculture medium 1/5, *WMCCM1/5* wash medium 1/5, similar to CCM1/5 but with carbenicillin and devoid of acetosyringone, *CIM1/5CB* CIM1/5 with carbenicillin]

Component	A					B							
	GMA	CIMA	CCMA	WMA	SIMA	CIM1	CCM1	WMCCM1	CIM1CF	CIM1/5	CCM1/5	WMCCM1/5	CIM1/5CB
MS	+	–	–	–	–	+	+	+	+	+	+	+	+
B5	–	+	+	+	+	–	–	–	–	–	–	–	–
Sucrose (g/l)	20	–	20	20	–	20	20	20	20	20	20	20	20
Glucose (g/l)	–	20	1.98	1.98	20	–	1.98	1.98	–	–	1.98	1.98	–
Agar (g/l)	6	8	–	–	8	8	–	–	8	8	–	–	8
Mes (g/l)	–	0.5	3.9	3.9	0.5	–	3.9	3.9	–	–	3.9	3.9	–
2,4-D (mg/l)	–	0.5	0.5	0.5	–	–	–	–	–	–	–	–	–
Kinetin (mg/l)	–	0.05	0.05	0.05	–	–	–	–	–	–	–	–	–
Acetosyringone (μM)	–	100	100	–	–	–	200	–	–	–	200	–	–
2ip (mg/l)	–	–	–	–	5	–	–	–	–	–	–	–	–
IAA (mg/l)	–	–	–	–	0.15	0.25	0.25	0.25	0.25	0.05	0.05	0.05	0.05
TDZ (mg/l)	–	–	–	–	–	0.5	0.5	0.5	0.5	0.1	0.1	0.1	0.1
Cefotaximum (g/l)	–	–	–	0.5	0.5	–	–	0.5	0.5	–	–	–	–
Carbenicillin (g/l)	–	–	–	–	–	–	–	–	–	–	–	0.5	0.5
pH	5.7	5.7	5.5	5.5	5.7	5.7	5.5	5.5	5.7	5.7	5.5	5.5	5.7

pMP90, pGV2260 or pEHA101 (for details of Ti-plasmids, see Dillen et al. 1997). All strains contained the binary vector pTJK136 (Kapila et al. 1997), except the strain with pMP90, which contained pATARC3-B1b (Goossens et al. 1999a). Both binary vectors carried exactly the same p35S-*uidA* intron-3'*nos* cassette. Preparation of different bacterial strains for coculture was carried out as reported previously (Dillen et al. 1997). Bacteria were incubated in growth chamber #1 for 4 h for virulence induction and used for coculturing. Coculture media (CCM media; Table 1) with bacteria were poured as follows per jar: 70 ml at OD = 0.2 for roots, 60 ml at OD = 0.8 for NI576 callus (Dillen et al. 1997) and 200 ml at OD = 0.05 for TB1 callus (Zambre et al., unpublished data). The explants were added to this bacterial solution for coculture and the jars were incubated under different light regimes as described above for 2 and 7 days in the case of *P. acutifolius* (NI576 and TB1, respectively) and for 3 days in the case of *A. thaliana*. Cocultured jars were shaken manually once a day for a few seconds. After coculture, explants were washed with the appropriate wash media (Table 1), cultured on SIMA, CIM1CF and CIM1/5CB (Table 1), and incubated under 16 h light/8 h dark as described above. Cultured roots of *A. thaliana* and calli of *P. acutifolius* were subjected to GUS assays after 3 and 4 days of culture, respectively.

Evaluation of the effect of different light conditions on p35S-*uidA* expression

Two transgenic lines were used as controls to assess the effect of the various light conditions on p35S-directed expression of the *uidA* marker gene. A line of *A. thaliana*, ecotype C24 homozygous for a P35S-*uidA* construct (line number KH15d2; De Buck et al. 2001) and a line of *P. acutifolius*, genotype NI576 homozygous for a p35S-*uidA*-intron construct (line number 8.1.22; Goossens et al. 1999a, 2000) were employed. The lines were subjected to a culture procedure similar to that mentioned above, with or without coculture with *A. tumefaciens* [C58C1Rif^R(pMP90) with binary vector pATARC4-A; Goossens et al. 1999b]. The binary vector pATARC4-A is similar to pATARC3-B1b, but lacks the *uidA* gene.

uidA expression in *Agrobacterium*-treated tissues

Expression of the *uidA* gene was detected by using histochemical (Jefferson 1987) and fluorometric (with a detection limit above 0.5 units of GUS per mg of protein; De Buck et al. 2001) GUS assays. For histochemical assays, tissues were incubated in phosphate buffer (Dillen et al. 1997) at 37°C and the reaction was stopped as specified in the figures by addition of 70% ethanol. Chlorophyll from the callus was extracted into 70% ethanol. All experiments were repeated twice except that with line 8.1.22, which was performed once. All experiments had 2–4 replicates per treatment.

Results

The effect of light on T-DNA transfer from *A. tumefaciens* to plant cells was assessed by exposing *A. tumefaciens* and plant tissue cocultures to different light regimes. Two well-established plant transformation systems involving transformation- and regeneration-competent, but physiologically different plant tissues were used. In one system, chlorophyll-containing green callus from two genotypes of *P. acutifolius* (NI576 and TB1) were cocultured with *Agrobacterium*. The second involved chlorophyll-free root segments from two ecotypes of *A. thaliana* (C24 and Columbia). GUS production, resulting from the expression of an intron-containing *uidA* gene present as a transgene in the binary transformation vectors, was assayed to evaluate the gene transfer rate in plant tissues by histochemical (qualitative) and fluorometric (quantitative) methods. The different light regimes imposed during the coculture period were as follows: continuous darkness (hereafter referred to as darkness), a 16 h light/8 h dark cycle, and continuous light. All other steps of the transformation procedures were performed in a photoperiod of 16 h light/8 h dark.

In general, darkness during the coculture period severely inhibited T-DNA transfer compared with the two light regimes in both plant species (Fig. 1a, b). In the histochemical GUS assays, few and very weak spots of staining were observed after dark treatments in root tissues of both genotypes of *A. thaliana* (Fig. 1a), indicating gene transfer at a very low level. Histochemical GUS activity was monitored at different intervals and the spots observed in dark-treated tissues developed only upon longer incubation (after 24 h). A moderate increase in the number of GUS-positive sectors was observed in roots of both ecotypes of *A. thaliana* after a 16 h light/8 h dark cycle, compared with darkness. Furthermore, the number of GUS-positive sectors (Fig. 1a) increased dramatically in roots of both ecotypes of *A. thaliana* when tissues were exposed to continuous light during the coculture step. Results with callus from both genotypes of *P. acutifolius* were in general agreement with those obtained for *A. thaliana* roots. Here, the increase in GUS activity was even higher than in *A. thaliana* roots (Fig. 1b) when darkness was compared with a 16 h light/8 h dark cycle. In dark-treated callus, GUS spots appeared after an incubation of 12 h. However, *uidA* expression was not detected in the fluorometric assay in any of the dark-treated callus tissues (Table 2). In callus of the genotype NI576, continuous light increased *uidA* expression almost fourfold relative to that of the photoperiod 16 h light/8 h dark treatment, but in callus of the genotype TB1 it was similar under both light conditions (Table 2). When continuous light was compared to a light/dark cycle, a stimulating effect of light was also observed for T-DNA transfer in two breeding lines (Xan159 and G5477) of common bean, *P. vulgaris* (data not shown).

The above results were obtained with C58C1Rif^R (pMP90), an *Agrobacterium* strain containing a nopaline-type helper Ti plasmid. In order to see whether this promoting effect of light on *Agrobacterium*-mediated gene transfer was helper-plasmid specific, *A. tumefaciens* strains with the same chromosomal background but harboring an octopine-type (pGV2260) or an agropine/succinamopine-type (pEHA101) helper Ti plasmid were used for coculture with callus of TB1 under two light regimes. A similar pattern of increased GUS activity was observed with both *Agrobacterium* strains (Fig. 1b; zTable 2) after coculture under light/dark cycles compared with coculture in the dark.

In order to verify that the observed effects truly reflect T-DNA transfer efficiencies and not an influence of the light conditions on transcription of the p35S-uidA reporter gene or post-transcriptional events, we performed

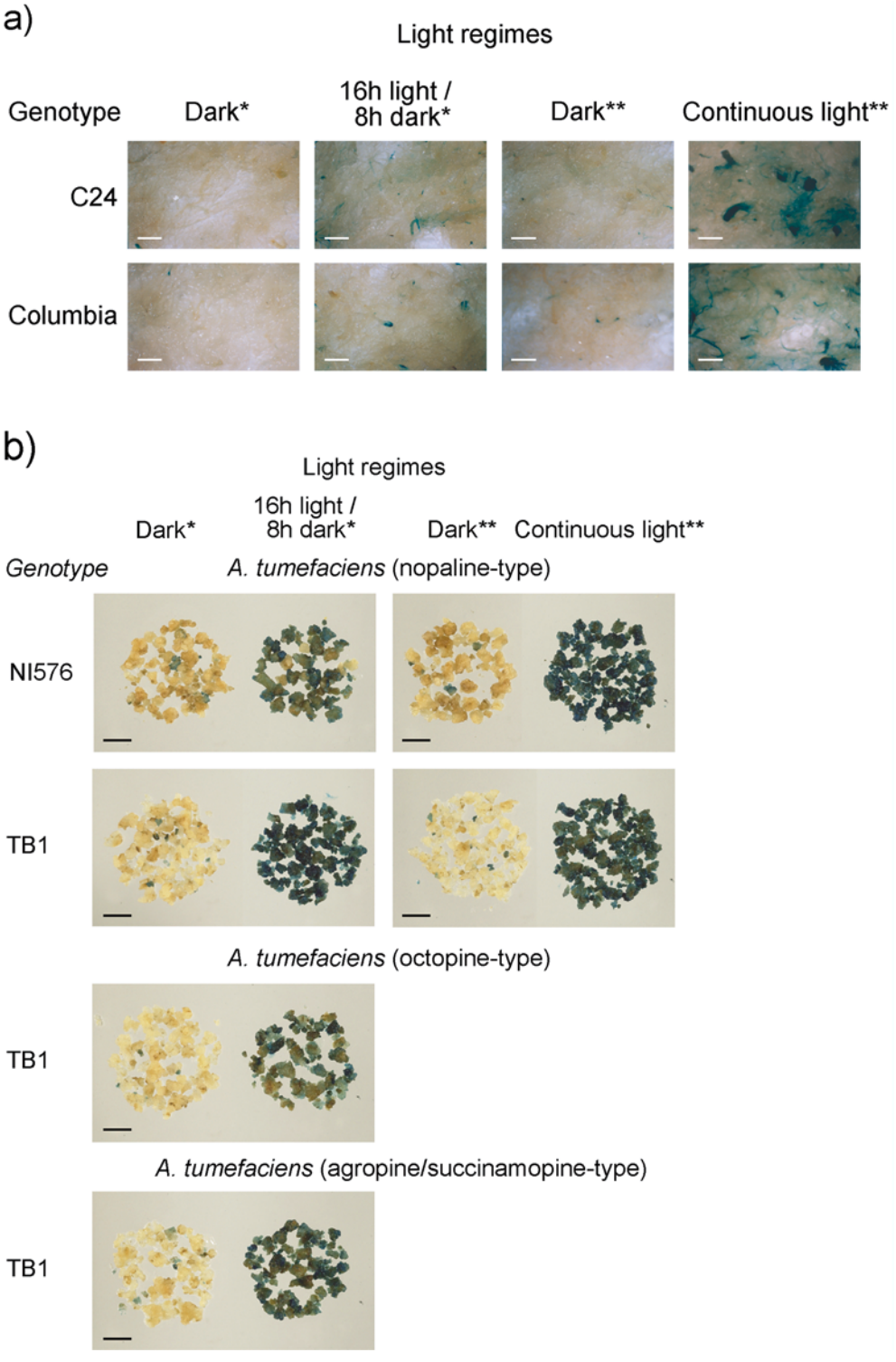

Fig. 1a, b Effect of two light regimes on *Agrobacterium tumefaciens*-mediated gene transfer as measured by histochemical GUS assays. **a** Roots of *Arabidopsis thaliana*. **b** Callus of *Phaseolus acutifolius*. *A. tumefaciens* nopaline-type strain was used for *A. thaliana* roots. The GUS staining reaction was stopped after 72 h for roots and 17 h for calli; *bars* 1.0 mm (a) and 4.0 mm (b) (* Tissues incubated in growth chamber #1, ** tissues incubated in growth chamber #2; see Materials and methods)

experiments using identical tissue culture conditions with transgenic homozygous lines of *A. thaliana* (KH15d2) and *P. acutifolius* (8.1.22) containing the *uidA* gene under the control of the CaMV 35S promoter. These control experiments included coculture with an *Agrobacterium* strain devoid of the *uidA* gene and a mock coculture omitting *Agrobacterium*. The results of fluorometric GUS assays showed no significant differences in *uidA* expression, regardless of the light conditions and the presence or absence of the bacteria during the coculture (Table 3). In most cases, dark-treated samples gave slightly higher GUS activity than light-treated ones (Table 3). Histochemical GUS staining from both lines, observed at different time intervals after

Table 2 Effect of two light regimes on *A. tumefaciens*-mediated gene transfer to callus of *P. acutifolius* measured by fluorometric GUS assays. * Tissues incubated in growth chamber #1, ** tissues incubated in growth chamber #2. Values are the means of three replicates ± SE, except for the octopine and agropine/succinamopine strains in a light/dark regime, which is the mean of two replicates (– not performed)

A. tumefaciens strain	Genotype	GUS activity (U GUS/mg protein)			
		Dark*	16 h light/8 h dark	Dark**	Continuous light
Nopaline	NI576	< 0.5	2.16 ± 0.19	< 0.5	7.81 ± 1.83
	TB1	< 0.5	11.65 ± 0.89	< 0.5	11.59 ± 3.53
Octopine	TB1	< 0.5	2.70	–	–
Agropine/succinamopine	TB1	< 0.5	2.89	–	–

Table 3 Effect of two light regimes in the presence or absence of *A. tumefaciens* on *uidA* gene expression in transgenic *P. acutifolius* [genotype NI576 (8.1.22), homozygous for p35S-*uidA*-intron] and *A. thaliana* [ecotype C24 (KH15d2), homozygous for p35S-*uidA* gene]. * Tissues incubated in growth chamber #1, ** tissues incubated in growth chamber #2. Values are the means of three to four replicates ± SE (– not performed)

Plant	*A. tumefaciens*	GUS activity (U GUS/mg protein)			
		Dark*	16 h light/8 h dark	Dark**	Continuous light
P. acutifolius	Present	1.83 ± 0.37	1.64 ± 0.02	1.60 ± 0.21	1.42 ± 0.02
	Absent	1.76 ± 0.12	1.40 ± 0.06	1.39 ± 0.20	1.44 ± 0.12
A. thaliana	Present	4690 ± 466	4803 ± 749	4712 ± 448	4068 ± 68
	Absent	4768 ± 276	4318 ± 461	–	–

the start of the GUS assay, were consistent with the results of the fluorometric GUS assay (data not shown).

Discussion

Agrobacterium-mediated transformation of green nodular callus from a crop plant, *P. acutifolius,* and root segments from the model plant *A. thaliana* were used to establish the effect of light on gene transfer from *Agrobacterium* to plant cells. We assessed T-DNA transfer frequencies with the *uidA* gene driven by a constitutive p35S promoter and its expression dynamics to assess . Control experiments showed that p35S-directed expression was not affected by the various light conditions used. This is consistent with numerous previous results showing that this promoter is insensitive to light conditions (e.g. Frohnmeyer et al. 1994; Cooke and Webb 1997; Yamamoto et al. 1997; Kurata et al. 1998). Some authors showed that light suppressed gene expression driven by p35S (Ellis et al. 1991; Schnurr and Guerra 1999; Zuker et al. 1999). This may be due to improved stability of *uidA* mRNA in the dark (Bovy et al. 1995; Dickey et al. 1992).

Our results indicate a reproducible, strong and positive correlation of T-DNA transfer with light. In both plant species and both genotypes of each species, coculture in darkness highly inhibited gene transfer. Coculture under a photoperiod of 16 h light/8 h dark increased transient *uidA* expression moderately in both genotypes of *Arabidopsis* and drastically in both genotypes of *P. acutifolius*. Exposure to continuous light enhanced *uidA* expression considerably over 16 h light/8 h dark cycles in both genotypes of *A. thaliana* and in one of the two genotypes of *P. acutifolius* (NI576). In genotype TB1 of *P. acutifolius*, GUS activity was not affected. This may be related to the fact that a prolonged coculture period (7 days) was used for genotype TB1 but not for genotype NI576, as this proved deleterious for the latter. The longer coculture period substantially increased transient *uidA* expression compared with coculture for 48 h under identical experimental conditions (Zambre et al., unpublished data). The positive effect of longer coculture may have masked an effect of continuous light on genotype TB1. However, in all cases, it is clear that exposure to light during coculture was essential for efficient T-DNA transfer, regardless of explant type, plant species or genotype, as well as of *Agrobacterium vir* plasmids or coculture periods. This suggests a general promotive role of light in *Agrobacterium*-mediated T-DNA transfer to plant cells.

While optimizing transformation of the cultivated *P. acutifolius* genotype TB1 (Zambre et al., unpublished data), the effect on stable transformation frequencies was found to be correlated with the transient transformation frequencies. Whereas 12 g of callus cocultured under 16 h light/8 h dark generated five independent transgenic plants, no transgenic plants were recovered from an equal amount of callus cocultured in continuous darkness (Zambre et al., unpublished data).

There are few comparative data on this subject. Damgaard and Rasmussen (1991) reported increased transformation rates in *Brassica* plants with *A. rhizogenes* under continuous illumination. However, continuous light was applied during growth of the *Brassica* plants from which explants were taken and after the

coculture. Hence, it is not possible to judge in which phase light conditions were crucial for increased transformation. Escudero and Hohn (1997) reported inhibition of T-DNA transfer in intact tobacco seedlings sprayed with *A. tumefaciens* and kept in the dark. This inhibition was attributed to stomatal closure, the stomata being the most likely infection route in this particular transformation system. In carnation, constant darkness during coculture was reported to increase transformation (Zuker et al. 1999). However, the explants had been stored at 4°C for a month before coculture and it is not clear whether the differences in transformation response were due to such a cold pretreatment.

T-DNA transfer by *Agrobacterium* is a tightly regulated process and multiple factors from both plant and bacterial cells are simultaneously required for the transformation process (Tzfira and Citovsky 2002 and references therein). Any of these factors could be affected in response to different light conditions. From the bacterial side, the efficiency of T-DNA transfer depends largely on how efficiently *vir* genes are induced. In this respect, light has been shown to enhance the amount of coniferyl alcohol, the phenolic *vir* gene inducer from the orchid *Dendrobium* (Nan et al. 1997). Nevertheless, in our experiments, the promotive effect of light on gene transfer is probably not mediated by increased production of the *vir* gene inducers because (1) the explants were extensively wounded, which releases *vir* gene inducers; (2) the bacteria were pre-induced with acetosyringone before coculture; and (3) the coculture media were optimized for *vir* gene induction with respect to pH and the concentration of monosaccharides and acetosyringone (Table 1). Also, bacterial concentrations did not vary significantly at the end of the coculture in different treatments (data not shown). Most probably, T-DNA transfer frequencies are influenced by light not at the level of the bacteria but through plant cell competence for *Agrobacterium* attachment or T-DNA uptake. As shown with the *Arabidopsis* root explant transformation system, the competence of plant cells for T-DNA transfer is indeed a limiting factor (De Buck et al. 2000). Different light conditions may affect physiological factors that, in turn, influence competence for T-DNA transfer. These factors include plant hormone levels, cell proliferation and cell cycle stage (Sangwan et al. 1991, 1992; Schläppi and Hohn 1992; De Kathen and Jacobsen 1995; Villemont et al. 1997). Whether the promotive effects of light described here are mediated by any of these physiological factors remains to be determined. Transient expression of a CaMV p35S-*uidA* gene delivered to embryos or seedlings of *Picea* by particle bombardment was not affected by light conditions (Ellis et al. 1991; Gray-Mitsumune et al. 1996). Thus, the stimulatory effect of light may be specific for *Agrobacterium*-mediated T-DNA delivery.

Many *Agrobacterium*-based plant transformation protocols for crop and model plant species use dark coculture conditions without specifying the effect on transformation rate. To our knowledge, an advantage of dark conditions for the plant cells and/or the bacteria has not been clearly demonstrated. Incubation in darkness seems to improve the morphogenic capacity of callus or explants (Mohamed et al. 1992; Compton 1999), essentially by preserving endogenous light-sensitive hormones (Pádua et al. 1998; Compton 1999) or by preventing accumulation of phenolic compounds (Bhojwani and Razdan 1983; Arezki et al. 2001). Based on our results, the above transformation procedures may be improved by incubating cocultures in the light, but keeping the original dark incubation conditions before and after coculture in order to protect the productivity of cocultured material. Changing light conditions during *Agrobacterium*-plant cell coculture is indeed an easy, economical and practically feasible approach for enhancing T-DNA transfer.

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