

RESEARCH PAPER

Agrobacterium tumefaciens*-mediated transformation of *Cleome gynandra* L., a C₄ dicotyledon that is closely related to *Arabidopsis thaliana

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Abstract

In leaves of most C₄ plants, the biochemistry of photosynthesis is partitioned between mesophyll and bundle sheath cells. In addition, their cell biology and development also differs from that in C₃ plants. We have a poor understanding of the mechanisms that generate the cell-specific accumulation of proteins used in the C₄ pathway, and there are few genes that have been shown to be important for the cell biology and development of C₄ leaves. To facilitate functional analysis of C₄ photosynthesis, and to enable knowledge from *Arabidopsis thaliana* to be translated to C₄ species, an *Agrobacterium tumefaciens*-mediated transformation protocol was developed for the C₄ species *Cleome gynandra*. *A. tumefaciens*, harbouring the binary vector SLJ1006, was used to transfer the *uidA* gene under the control of the CaMV 35S promoter into *C. gynandra*. Co-incubation of hypocotyls or cotyledons with SLJ1006 allowed efficient transfer of DNA into *C. gynandra*, and media that allowed callus production and then shoot regeneration were identified. Stable transformants of *C. gynandra* with detectable amounts of β-glucuronidase (GUS) were produced at an efficiency of 14%. When driven by the CaMV 35S promoter, GUS was visible in all leaf cells, whereas *uidA* translationally fused to a *CgRbcS* gene generated GUS accumulation specifically in bundle sheath cells. This transformation procedure is the first for an NAD-ME type C₄ plant and should significantly accelerate the analysis of mechanisms underlying C₄ photosynthesis.

Key words: *Agrobacterium tumefaciens*, *Arabidopsis thaliana*, C₄ photosynthesis, *Cleome gynandra*, transformation.

Introduction

C₄ photosynthesis has evolved independently multiple times within the angiosperms and allows CO₂ to be concentrated around Ribulose Biphosphate Carboxylase Oxygenase (RuBisCO) (Sage, 2004). In tropical and subtropical regions the increased supply of CO₂ to RuBisCO represses its oxygenase reaction, increases the rate of photosynthesis, and allows higher rates of growth compared to C₃ species. In fact, the most productive native vegetation and domesticated crops all use the C₄ pathway (Brown, 1999) and, as a consequence, it has been proposed that placing characteristics of C₄ photosynthesis into crops such as rice could be

used to increase yields (Matsuoka *et al.*, 2001; Surridge, 2002; Hibberd *et al.*, 2008).

In most plants, the C₄ pathway involves photosynthesis proteins being compartmented between mesophyll (M) and bundle sheath (BS) cells (Hatch, 1987; Brown *et al.*, 2005), although single-celled C₄ photosynthesis has been reported in aquatic and terrestrial plants (Bowes and Salvucci, 1984; Reiskind *et al.*, 1989; Magnin *et al.*, 1997; Voznesenskaya *et al.*, 2001, 2002) as well as in a marine diatom (Reinfelder *et al.*, 2000). In the two-celled version of the C₄ pathway (Hatch, 1987), CO₂ enters M cells and is converted into

bicarbonate by carbonic anhydrase. Phosphoenolpyruvate carboxylase (PEPC) then combines HCO_3^- with phosphoenolpyruvate to generate the C_4 oxaloacetic acid (OAA), which is rapidly converted into either aspartate or malate. These C_4 acids then diffuse into BS cells through abundant plasmodesmata, where one of three separate C_4 acid decarboxylases releases CO_2 . In different lineages of C_4 plants, the three distinct decarboxylases known as NADP-dependent malic enzyme (NADP-ME), NAD-dependent malic enzyme (NAD-ME) and phosphoenolpyruvate carboxykinase (PEPCK) have been co-opted into this process. In many cases one of the enzymes is believed to act as the primary decarboxylase with another fulfilling a secondary role (Wingler *et al.*, 1999). The three carbon compound released after decarboxylation then diffuses back to the M, and in the case of pyruvate, rephosphorylation to PEP occurs, catalysed by pyruvate, orthophosphate dikinase (PPDK) (Hatch and Slack, 1968).

Despite the basics of the C_4 pathway having been defined for decades, there still is a relatively poor understanding of the mechanisms that generate the accumulation of proteins used in C_4 photosynthesis in the M or BS cells (Brown *et al.*, 2005). For example, although an element known as the mesophyll enhancing module1 (MEM1) has been identified in the PPC promoter of *Flaveria bidentis* (Gowik *et al.*, 2004; Akyildiz *et al.*, 2007) and gel-retardation assays showed that a region about 200 bp upstream of the maize PPDK gene generates mesophyll specific expression of GUS (Matsuoka and Numazawa, 1991), in neither case have *trans*-factors responsible for generating M-specific expression been isolated. In the C_4 plants studied to date, *RbcS* seems to be regulated at multiple levels; including transcriptional (Viret *et al.*, 1994; Purcell *et al.*, 1995), post-transcriptional, and translational elongation (Berry *et al.*, 1985, 1986, 1987, 1990) but again, no *trans*-factors have been isolated. For many of the other genes encoding enzymes (e.g. carbonic anhydrase, malate dehydrogenase, phosphoenolpyruvate carboxykinase) that have been recruited into the C_4 pathway, there is no information on mechanisms generating cell-specific expression.

Our understanding of the genetic basis associated with the alterations in cell biology and development of a C_4 leaf is also poor. For example, there have been no genes identified to date that control the expansion, proliferation or polarized positioning of chloroplasts in BS cells, the increased plasmodesmatal connectivity between M and BS cells, nor Kranz anatomy itself. This is despite the fact that genes have been isolated in *Arabidopsis thaliana* that are involved in many of these processes. For example, the *Min*, *FtsZ*, and *GCL* genes are known to control chloroplast division (Colletti *et al.*, 2000; Vitha *et al.*, 2001; Maple *et al.*, 2004), CHUP1 is involved in controlling chloroplast movement (Oikawa *et al.*, 2003) and glucan 1,3- β -glucosidases are implicated in plasmodesmatal connectivity (Levy *et al.*, 2007). To date, it has not been possible to determine whether alterations to any of these genes are associated with the modifications to C_4 cell biology. This is partly because transformation systems for the main NADP-ME-type

C_4 models, maize and *Flaveria*, are relatively difficult (Ishida *et al.*, 1996; Chitty *et al.*, 1999). To our knowledge, there are no transformation systems available for NAD-ME or PEPCK-type C_4 species, and the phylogenetic distance of transformable C_4 species from *A. thaliana* also means that direct comparison with the most widely studied model plant is unlikely to be meaningful.

The most closely related genus to *A. thaliana* that is known to contain C_4 species is *Cleome* L. (Hall *et al.*, 2002; Brown *et al.*, 2005). *Cleome* belongs to the NAD-ME C_4 subtype and contains C_3 species and some that appear to be intermediate between the two forms of photosynthesis as well as *bone fide* C_4 species (Marshall *et al.*, 2007; Voznesenskaya *et al.*, 2007). Although new sequencing technologies will generate insight into C_4 photosynthesis, functional analysis will still be needed to validate candidates. An efficient transformation system in a C_4 plant that is closely related to *A. thaliana*, would possibly help address areas where our knowledge of mechanisms underlying C_4 photosynthesis is poor because it would allow gene reporter studies and candidate genes to be mis-expressed and knocked down. It would also allow meaningful comparative analysis of genes and proteins recruited into C_4 photosynthesis in both *A. thaliana* and a closely related C_4 plant. This will help to determine how gene expression and protein function have altered during the evolution of the C_4 pathway. Therefore, a variety of approaches were tested to allow the stable transfer of genes into *C. gynandra* L., and a simple method is reported here that generates transformants of an NAD-ME type C_4 plant and the most closely related C_4 species to *A. thaliana*.

Materials and methods

Plant material

Cleome gynandra was grown in a greenhouse set at 22 °C with supplementary lighting from metal halide lamps for a 16 h photoperiod to produce seed for use in transformation experiments. Surface-sterilization of seeds involved rinsing in 70% ethanol followed by a 10 min soak in 5% sodium hypochlorite and three rinses in sterile water or by microwaving (Franco, 1993). Seed was also sown directly from freshly harvested intact pods, and placed in sterile germination plates of filter paper moistened with water, which were wrapped in Parafilm and incubated at 30 °C for 24 h. Healthy seed produced roots 1–5 mm in length after approximately 24 h, and these plants were transferred to sterile plastic pots containing MS medium (Murashige and Skoog, 1962) with B5 vitamins (Gamborg *et al.*, 1968) (Duchefa Biochimie BV), 10 g l⁻¹ sucrose, 8 g l⁻¹ agar (Melford), pH 6.0). These pots were kept in a tissue culture room at 24 °C with a 16 h photoperiod and a light intensity of 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$. After 6–8 d the expanded cotyledons and hypocotyls were used for transformation experiments.

Generation of uidA constructs for transformation

The binary plasmid SLJ1006 contains the *uidA* reporter gene under the *CaMV 35S* promoter and *Nos* terminator (Jones *et al.*, 1992). The *CgRbcS* construct consisted of the *CgRbcS* promoter (2.8 kb) and an unspliced coding region translationally fused to *GFP* and *uidA*, with 1 kb of the *CgRbcS* 3' region downstream of *uidA*. The

promoter, together with the coding region, was amplified from genomic DNA by PCR with primers (Table 1) including restriction enzyme sites, to allow the translational fusion to be generated. The 3' region was also amplified from genomic DNA with primers, including restriction sites (Table 1). The *uidA* gene was amplified, together with *gfp*, from pKGWFS7 (Plant Systems Biology, <http://www.psb.ugent.be/gateway/>). The reporter gene fragment was then ligated into pUC19 using T4 DNA ligase, amplified by PCR together with the pUC19 MCS using M13 primers and inserted into pENTR/D-TOPO (Invitrogen, <http://www.invitrogen.com/>). The *CgRbcS* promoter and unspliced coding region were ligated into the entry plasmid upstream of the reporter genes using T4 DNA ligase; the *CgRbcS* 3' region was ligated downstream. The assembled construct was recombined into the Gateway destination vector pGWB1 (Nakagawa *et al.*, 2007) using LR clonase (Invitrogen).

Bacterial stocks

A fresh plate of YEP medium (An *et al.*, 1988) (10 g l⁻¹ yeast extract, 10 g l⁻¹ Bacto-peptone, 5 g l⁻¹ NaCl, 15 g l⁻¹ Bacto-agar, pH 7.0) with streptomycin sulphate (100 µg ml⁻¹) and tetracycline (1 µg ml⁻¹) was streaked with LBA4404 containing SLJ1006 (Jones *et al.*, 1992) and grown at 30 °C overnight. A loop was used to inoculate 50 ml of liquid YEP medium (as above but without Bacto-agar) plus antibiotics in a sterile 250 ml flask and the suspension was grown for 18 h at 30 °C with shaking. The bacteria were pelleted and resuspended in 6 ml of 1-glycerol (50% glycerol, 10 g l⁻¹ Bacto-tryptone, 5 g l⁻¹ yeast extract, 5 g l⁻¹ NaCl, pH 7.0); 0.5 ml amounts were aliquoted into 1.5 ml microfuge tubes and stored at -80 °C for future use. A stock of LBA4404 with the *CgRbcS* construct was made in the same way but with the antibiotics kanamycin sulphate (50 µg ml⁻¹) and streptomycin sulphate (100 µg ml⁻¹) added to the growth medium.

Regeneration

Seedlings were removed individually from pots and placed on a filter paper wetted with sterile water; with a sharp scalpel blade the hypocotyl was cut into 1–2 pieces 5–8 mm long and the cotyledons were excised at least 1 mm away from the apical meristem. Shoot regeneration from hypocotyl and cotyledon explants was assessed initially on medium Z (Yan You, 1995) (MS medium, 30 g l⁻¹ sucrose, 2 mg l⁻¹ BAP, 0.1 mg l⁻¹ NAA, pH 5.8, and 8 g l⁻¹ agar) with 0 or 20 mg l⁻¹ AgNO₃ and A5 (De Block *et al.*, 1989) solidified with 8 g l⁻¹ agar containing 0, 10, or 20 mg l⁻¹ AgNO₃, added after the medium had been autoclaved.

Transformation

One day before the transformation, 200 µl of bacterial stock stored at -80 °C were transferred to 50 ml of YEP liquid medium plus antibiotics in a 250 ml flask and grown overnight at 30 °C with shaking (*c.* 18–20 h). Bacteria were pelleted in a 50 ml centrifuge

tube for 10 min at 3500 rpm and resuspended in 40 ml liquid inoculation medium. Three liquid inoculation media were tested for their transformation efficiency: MSO+AS (MS medium with MS vitamins (Duchefa Biochimie BV), 30 g l⁻¹ sucrose, 0.2 mM acetosyringone, pH 5.5); A3 (De Block *et al.*, 1989) with 0.2 mM acetosyringone, pH 5.5; SIM (Alt-Mörbe *et al.*, 1989), consisting of 20 g l⁻¹ sucrose, 5.9 g l⁻¹ Tri-sodium citrate, and 0.2 mM acetosyringone at pH 5.5. All the media tested were set at pH 5.5 to improve transformation by *A. tumefaciens* (Alt-Mörbe *et al.*, 1989). Hypocotyl and cotyledon explants were cut as described above and transferred to dishes containing 10 ml of bacterial inoculum, placing cotyledons abaxial surface down into the inoculum. Twenty seedlings were inoculated in each 10 ml batch. After immersion for a total of 30 min the explants were transferred to co-culture plates [MS medium at 1/10 normal concentration, 30 g l⁻¹ sucrose, 1 mg l⁻¹ benzylaminopurine (BAP), 0.1 mg l⁻¹ naphthalene acetic acid (NAA), 8 g l⁻¹ agar, pH 5.5, covered with 1 ml MSO liquid medium without acetosyringone and a sterile filter paper], again placing cotyledons abaxial surface down. Co-culture plates were incubated for 2 d, after which the explants were transferred to regeneration medium. Two regeneration media, as well as several levels of kanamycin sulphate or paromomycin sulphate for the selection of transformed tissue, were tested for their effects on regeneration and transformation. Explants were transferred to medium A5 (Alt-Mörbe *et al.*, 1989) solidified with 8 g l⁻¹ agar or CR (modified from medium C of Shepard and Totten, 1977) as follows: MS major salts but with NH₄NO₃ replaced with NH₄Cl (107 mg l⁻¹), MS minor salts, Nitsch and Nitsch vitamins (Nitsch and Nitsch, 1969), all other addenda as in A5 (De Block *et al.*, 1989) including 20 g l⁻¹ sucrose, 1.0 mg l⁻¹ BAP, 0.1 mg l⁻¹ NAA, 0.5 g l⁻¹ MES, 40 mg l⁻¹ adenine sulphate, 0.5 g l⁻¹ PVP, 8 g l⁻¹ agar, pH 5.7; 10 mg l⁻¹ AgNO₃ was added to the medium after autoclaving. Media also contained the antibiotics carbenicillin (500 mg l⁻¹) for unselected tissue or carbenicillin (500 mg l⁻¹) and kanamycin sulphate (20, 40, 60, 80 mg l⁻¹) or paromomycin sulphate (20, 30, 40 mg l⁻¹) for the selection of transformed tissue. In one experiment, cotyledons were placed on A5 medium containing a matrix of BAP (0.5, 1.0, 2.0 mg l⁻¹) and NAA (0.1, 0.5, 1.0, 1.5 mg l⁻¹) concentrations to determine the optimum levels of these growth regulators.

After 3 weeks, explants were transferred to fresh selective medium; cotyledon pieces were placed on medium of the same composition, while hypocotyl explants that had already developed small shoots were transferred to medium A6 (De Block *et al.*, 1989) solidified with 8 g l⁻¹ agar+carbenicillin (250 mg l⁻¹)+kanamycin sulphate (60 mg l⁻¹). After a further 3 weeks cotyledon explants that had developed small shoots were transferred to A6 medium+agar+carbenicillin+kanamycin, and hypocotyl explants were transferred to A7 (De Block *et al.*, 1989) solidified with 8 g l⁻¹ agar+carbenicillin (250 mg l⁻¹)+kanamycin sulphate. Three weeks later the cotyledon explants were transferred to A7+agar+carbenicillin+kanamycin. Groups of shoots from cotyledons or hypocotyls could be maintained on this medium in the presence of the selective agent; once individual shoots had grown enough to be cut off for rooting they were placed on A8 medium (De Block *et al.*, 1989) solidified with 8 g l⁻¹ agar+carbenicillin (100 mg l⁻¹).

Table 1. Primers used in polymerase chain reactions to generate the translational fusion between the *RbcS* gene from *C. gynandra* and *uidA*

Restriction enzymes sites are shown in bold.

Primer	Nucleotide sequence
<i>CgRbcS</i> promoter F	5'- TCTGCAG CAAACACGCATTTATGGCTG
<i>CgRbcS</i> coding R	5'- TACTACT CATCTCTTTCTTCTTTGCTC
<i>gfp uidA</i> F	5'-CG TCTAG AGTGAGCAAGGG
<i>gfp uidA</i> R	5'-GCC GAGCTC ATTGTTGGCC
<i>CgRbcS</i> 3' F	5'- TGAGCTC TTAAGCCATCCCTCTTTGC
<i>CgRbcS</i> 3' R	5'- TGAATTC AAATATCCCTCACTTCACT

Seed production

Small shoots from tissue culture were used as scions for grafting onto 8–10-week-old *Cleome gynandra* stock plants grown in a growth chamber at 23 °C, with a 16 h photoperiod and light intensity of 350 µmol m⁻² s⁻¹. Grafting was carried out in the growth chamber and the stock plant was placed in a plastic bag to produce high humidity around the graft. Once the graft had established and the scion was producing new growth, the bag was removed slowly and the plant grown on for seed production.

Histochemical assay for β -glucuronidase

Tissue was assayed histochemically for β -glucuronidase activity (Jefferson *et al.*, 1987) at various stages in the regeneration process. Pieces of callus, leaves or whole seedlings were immersed in 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc), vacuum infiltrated, and incubated at 37 °C for 4–16 h, then destained in 70% ethanol. Leaf tissues destined for detailed microscopic examination of bundle sheath expression were treated further with 5% (w/v) NaOH solution at 37 °C for 4–20 h, washed several times in 70% ethanol, and clarified by immersing in a 75% solution of chloral hydrate for 20–24 h prior to examining microscopically. To make the chloral hydrate solution, 80 g chloral hydrate was stirred into 30 ml H₂O to give a saturated solution; to this was added 6 ml H₂O and 4 ml 10% glycerol.

Results and discussion

Sterilization of C. gynandra and incubation with A. tumefaciens

It was found that collecting mature unopened seed pods of *C. gynandra*, and sprinkling seeds from these onto moistened sterile filter paper prior to the transfer of germinated seedlings to agar plates led to very low levels of contamination and maximized the regeneration potential. Rates of seed germination of 80–90% were regularly obtained from fully-formed mature pods. Other more common methods of seed sterilization were problematic. For example, seed sterilization with ethanol and sodium hypochlorite reduced germination to 0–5% of controls. Microwaving of seed has reportedly been successful for tobacco and *Arabidopsis* (Franco, 1993) and so this was tested with seed of *C. gynandra*. Whilst microwaving for up to 10 min did not reduce the percentage of germination, it did not prevent low-level fungal contamination. Furthermore, explants derived from seeds that had been microwaved for longer than 5 min showed little or no callus development and regeneration during the following stages of tissue culture.

The composition of media used to suspend *A. tumefaciens* prior to, and during, incubation with plant tissue can alter transformation efficiency (Alt-Mörbe *et al.*, 1989; Godwin *et al.*, 1991; Humara *et al.*, 1999). The influence of three separate liquid suspension media (MSO, A3, and SIM) on the transfer of the *uidA* gene encoding β -glucuronidase (GUS) into *C. gynandra* was therefore assessed. Both *A.*

tumefaciens LBA4404 and EHA105 containing the binary plasmids SLJ1006 and pVDH (Jones *et al.*, 1992) with *uidA* under the control of the *CaMV 35S* promoter was used for this purpose. β -glucuronidase assays carried out on inoculated explants 8 d or 14 d after transfer to selective or non-selective regeneration medium showed more transformed tissue in those explants treated with LBA4404 compared with EHA105, therefore further experiments were conducted with LBA4404 as it appeared to give the best chance for the regeneration of transformed shoots. LBA4404 continues to be used for *Brassica* transformation (Chen *et al.*, 2008). A greater number of areas with GUS activity were found when explants were incubated with LBA4404 in SIM compared to either MSO or A3 (Table 2), and transformed areas were larger when SIM was used (Fig. 1). Future experiments were therefore conducted with SIM medium. It was discovered that subsequent explant growth was poor if tissue was left submerged in *A. tumefaciens* SIM inoculum for too long and so incubation was limited to 30 min.

Regeneration and selection of transformed C. gynandra

Regeneration of callus and then shoots from explants can vary between species, and is strongly influenced by factors such as the tissue used (van Wordragen and Dons, 1992; Akasaka-Kennedy *et al.*, 2005). The production of callus from both hypocotyls and cotyledons after incubation with *A. tumefaciens* was therefore assessed. After transfer to regeneration medium, hypocotyl explants became swollen at both ends, and tiny calli or shoot initials appeared at one or both ends as early as 10 d. These appeared to be produced from the cut ends of the vascular tissue as has been reported for *Brassica napus* (Akasaka-Kennedy *et al.*, 2005). By 4 weeks, well-formed shoots were visible (Fig. 2A) and these developed to produce clumps of shoots (Fig. 2B). Cotyledon explants developed more slowly and callus formation usually started after approximately 3 weeks following transfer to regeneration medium, with a few small shoots appearing after 4 weeks (Fig. 2C). Well-developed clumps were evident 8 weeks after incubation with *A. tumefaciens* (Fig. 2D).

To determine which medium gave the best regeneration efficiencies, the frequency of regeneration of cotyledon and

Table 2. Influence of *Agrobacterium* suspension media on *pCaMV35S::uidA::Tnos* transfer from LBA4404 containing SLJ1006 (Jones *et al.*, 1992) into hypocotyls and cotyledons of *C. gynandra*

Two independent experiments (A and B) are reported in which three media were tested: MSO, A3, and SIM. The regeneration and selection medium was A5 and contained either 500 mg l⁻¹ carbenicillin (A5+C) or 500 mg l⁻¹ carbenicillin and 20 mg l⁻¹ kanamycin (A5+CK). Details of media are reported in Materials and methods. Data are reported as the percentage of β -glucuronidase positive explants 8 d after incubation, with the total number of explants assessed in parentheses.

Inoculum medium		MSO		A3		SIM	
		A5+C	A5+CK	A5+C	A5+CK	A5+C	A5+CK
Hypocotyl	A	20% (20)	24% (46)	15% (20)	27% (44)	90% (20)	86% (42)
	B	10% (22)	2% (46)	5% (20)	2% (46)	40% (20)	50% (46)
Cotyledon	A	43% (21)	81% (31)	48% (21)	53% (38)	100% (17)	100% (26)
	B	47% (15)	100% (16)	60% (15)	100% (16)	100% (15)	100% (6)

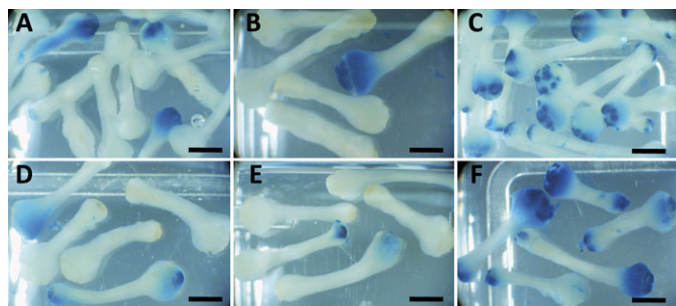


Fig. 1. Hypocotyl explants of *C. gynandra* assayed for β -glucuronidase activity 8 d after transfer to regeneration medium A5 with (A–C) or without (D–F) 20 mg l^{-1} kanamycin. Hypocotyls were initially inoculated with LBA4404 containing the construct SLJ1006 in MSO (A, D), A3 (B, E) or SIM (C, F). Scale bars=2 mm.

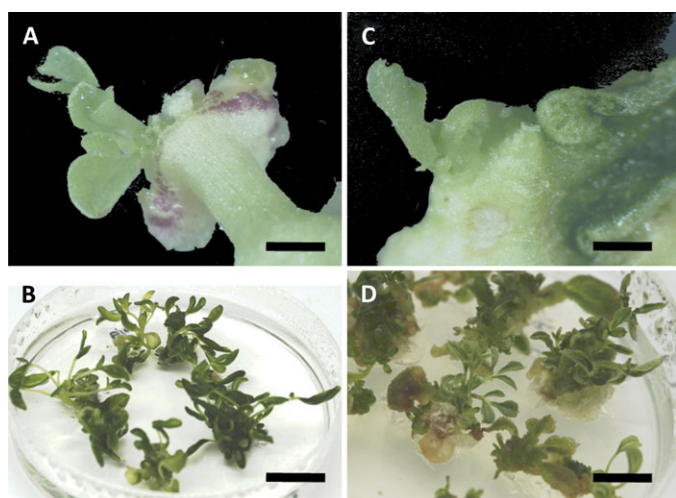


Fig. 2. Regeneration of hypocotyl and cotyledon explants of *C. gynandra*. Hypocotyl explants after 4 weeks (A) and 8 weeks (B) on regeneration medium CR. Cotyledon explants after 4 weeks (C) and 8 weeks (D) on regeneration medium CR. Scale bars: (A, C) 2 mm, (B) 14 mm, (D) 12 mm.

hypocotyl explants was assessed 4 weeks after incubation with *A. tumefaciens* (Table 3). Hypocotyl explants showed the highest level (79%) of regeneration on A5 medium containing 10 mg l^{-1} AgNO_3 and A5 without AgNO_3 the lowest. AgNO_3 has previously been used for *Brassica* transformation (De Block *et al.*, 1989; Akasaka-Kennedy *et al.*, 2005). After 4 weeks, cotyledon explants showed callusing and incipient shoot formation frequency of 52% on A5 plus 20 mg l^{-1} AgNO_3 and 44% on medium Z. Thus, hypocotyls gave the most efficient regeneration, and addition of AgNO_3 to the regeneration medium enhanced regeneration particularly in hypocotyl explants, but addition of more than 10 mg l^{-1} did not appear to be beneficial (Table 3). The choice of these media was informed by previous work. For example, Z has previously been used for tissue culture and propagation of *C. gynandra* (Yan You, 1995) and, because of the phylogenetic proximity of the Cleomaceae and Brassicaceae (Shepard and Totten, 1977;

Table 3. Regeneration efficiencies from hypocotyl and cotyledon explants of *C. gynandra* after 4 weeks on regeneration medium A5 (De Block *et al.*, 1989) or Z (Yan You, 1995)

Regeneration was determined on four media with varying amounts of AgNO_3 , and data are presented as the percentage of explants with callus or shoots and the total number of explants assessed in parentheses. For details of media constituents see Materials and methods.

	A5	A5+10 mg l^{-1} AgNO_3	A5+ 20 mg l^{-1} 20 mg l^{-1}	Z
Hypocotyl	31% (167)	79% (67)	71% (56)	39% (137)
Cotyledon			52% (90)	44% (337)

De Block *et al.*, 1989), media previously used for *Brassica* transformation and regeneration were also tested.

Following inoculation with *A. tumefaciens* LBA4404 harbouring the binary plasmid SLJ1006, hypocotyl and cotyledon explants were placed on regeneration medium with the addition of carbenicillin and kanamycin sulphate. Hypocotyl and cotyledon explants were tested for regeneration on medium A5 (De Block *et al.*, 1989) and CR (modified from medium C; Shepard and Totten, 1977) and four levels of kanamycin sulphate. Regeneration from hypocotyls or cotyledons was assessed after 4 weeks (Table 4). The addition of antibiotics to plant regeneration media, which prevent the growth of *A. tumefaciens*, has been shown to affect the regeneration process (De Block *et al.*, 1989; Lin *et al.*, 1995; Nauerby *et al.*, 1997). Addition of carbenicillin alone to A5 or CR led to higher regeneration levels from *C. gynandra* explants (Table 4) compared with the same medium lacking carbenicillin (Table 3). The presence of kanamycin in the medium reduced the levels of regeneration from both explant types but was not sufficient to prevent regeneration altogether. Medium CR was superior in terms of explant appearance and quantity of regenerable tissue or shoots and was therefore adopted for future experiments. Kanamycin sulphate at 60 mg l^{-1} was chosen as the best concentration to use because, in addition to the selection of GUS positive explants, tissue was healthy. Both hypocotyl and cotyledon explants were also cultured on medium containing paromomycin sulphate as the selective agent at 20, 30 and 40 mg l^{-1} and although regeneration frequencies were similar to those on kanamycin sulphate (data not shown), the tissue appeared vitreous and this approach was discontinued.

The efficiency of shoot regeneration after transformation can be modified by the relative amounts of benzylaminopurine (BAP) and naphthalene acetic acid (NAA). To investigate the best concentrations of these compounds for *C. gynandra*, a matrix of cotyledon and hypocotyl explants was set up on BAP at 0.5, 1.0, and 2.0 mg l^{-1} and NAA at 0.1, 0.5, 1.0, and 1.5 mg l^{-1} (Table 5). Hypocotyl explants seemed less responsive to this matrix of BAP and NAA than cotyledons. Although there was a relatively broad range of BAP and NAA concentrations that have reasonable regeneration

Table 4. Regeneration from hypocotyl and cotyledon explants of *C. gynandra* after 4 weeks on regeneration medium containing carbenicillin and kanamycin sulphate after inoculation with LBA4404 containing SLJ1006

Material was selected on carbenicillin alone (C), or carbenicillin and kanamycin (CK) with kanamycin concentrations of 20, 40, 60, and 80 mg l⁻¹. Data are shown as the percentage of explants with shoots, and in parentheses the total number of explants assessed.

	C	CK-20	CK-40	CK-60	CK-80
Hypocotyls on A5	89% (160)	12% (107)	28% (167)	43% (156)	40% (116)
Hypocotyls on CR	100% (43)	–	42% (116)	59% (114)	60% (118)
Cotyledons on A5	100% (25)	42% (50)	43% (112)	43% (116)	45% (77)
Cotyledons on CR	100% (11)	–	54% (76)	56% (75)	46% (74)

efficiencies (Table 5), when the quality of the tissue was also assessed it was decided that combining 1 or 2 mg l⁻¹ BAP with 0.1 or 0.5 mg l⁻¹ NAA gave the best results. Therefore, a medium composition of 1.0 mg l⁻¹ BAP + 0.1 mg l⁻¹ NAA was adopted for future experiments.

Transformed shoots could only be rooted in the presence of kanamycin at an overall frequency of 0.4%. Different combinations of media, growth regulators, omission of antibiotics, addition of charcoal, use of vermiculite (De Block *et al.*, 1989) all had little or no effect. For example, medium A8 (De Block *et al.*, 1989) was tested with reduced levels of organic additions, different levels of IAA or IBA, the addition of activated charcoal, and the removal of kanamycin from the medium. In addition, incorporating a rooting medium previously used for *Brassica* (Bidney *et al.*, 1983) with IBA or IAA, plus or minus activated charcoal, or kanamycin was also tested. Small shoots were also transferred to vermiculite with rooting media as in De Block *et al.* (1989). Overall, no consistent effects on rooting were seen. Consequently, shoots were grafted onto 8–10-week-old *C. gynandra* stock plants in order to produce seed of transgenic lines (Fig. 3A). Grafting was successful as long as the scion was kept in a humid atmosphere and held securely in place while the tissues made a connection. 80% of grafts were successful if healthy scions were used.

Histochemical assays for GUS

To confirm that putative transformants were expressing transgenes, analysis of GUS activity was carried out on shoots resistant to kanamycin as well as T₁ plants. Shoots from hypocotyl explants incubated with *A. tumefaciens* harbouring the *pCaMV 35S::uidA::Tnos* transgene developed quickly and some were visible as early as 10 d after the hypocotyl was placed on the regeneration medium. Assays of GUS activity after 4 weeks of culture, when quite large shoots were present, often showed areas of GUS at the base of shoots but not in the apical meristem (Fig. 3B). The same results were found for 4- and 8-week-old regenerating shoots from cotyledon explants (Fig. 3C, D). This was particularly frequent in those explants selected at a low level

Table 5. Callus production from hypocotyls and cotyledons on a matrix of benzylaminopurine (BAP) and naphthalene acetic acid (NAA)

Explants were scored for callus production after 5 weeks growth on regeneration medium A5 with 500 mg l⁻¹ carbenicillin and 60 mg l⁻¹ kanamycin sulphate. The effect of varying concentrations of BAP and NAA on callus production was assessed. Data are presented from two experiments as the percentage of explants that produced callus, and the total number of calli assessed in each experiment are in parentheses.

Hypocotyls	BAP 0.5 mg l ⁻¹	BAP 1.0 mg l ⁻¹	BAP 2.0 mg l ⁻¹
NAA 0.1 mg l ⁻¹	53% (40)	40% (40)	28% (40)
	45% (40)	40% (40)	73% (40)
NAA 0.5 mg l ⁻¹	28% (40)	30% (40)	40% (40)
	18% (40)	35% (40)	63% (40)
NAA 1.0 mg l ⁻¹	15% (40)	20% (40)	28% (40)
	10% (40)	25% (40)	43% (40)
NAA 1.5 mg l ⁻¹	5% (40)	25% (40)	15% (40)
	3% (40)	50% (40)	18% (40)
Cotyledons	BAP 0.5 mg l ⁻¹	BAP 1.0 mg l ⁻¹	BAP 2.0 mg l ⁻¹
NAA 0.1 mg l ⁻¹	8% (24)	15% (13)	28% (25)
	31% (19)	53% (19)	89% (19)
NAA 0.5 mg l ⁻¹	0% (13)	84% (19)	15% (13)
	74% (19)	20% (20)	20% (39)
NAA 1.0 mg l ⁻¹	47% (19)	68% (19)	31% (19)
NAA 1.5 mg l ⁻¹	12% (8)	20% (10)	16% (19)
	10% (20)	5% (20)	0% (6)

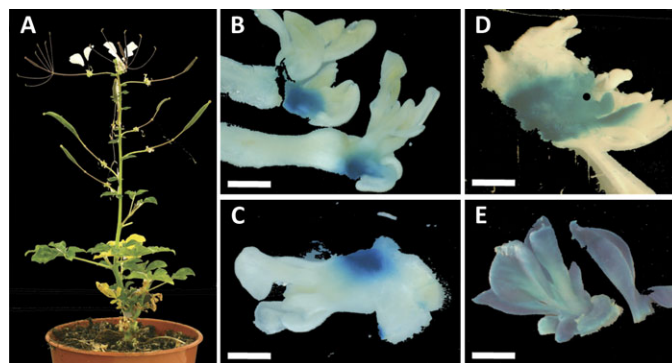


Fig. 3. Stable transformation of *C. gynandra*. (A) Graft of a transformed shoot onto a wild-type scion allows seed set. (B–E) Regenerating hypocotyl and cotyledon explants after 4 weeks (B, D) or cotyledon explants at 8 weeks (C, E) of culture on regeneration medium containing 500 mg l⁻¹ carbenicillin and 60 mg l⁻¹ kanamycin. (B–D) Regenerants that show partial β-glucuronidase (GUS) staining. (E) Regenerants stained for GUS throughout the explant. Scale bars=2 mm.

of kanamycin such as 20 mg l⁻¹. However, it was possible to recover plants that stained for GUS in all tissues (Fig. 3E). Thus in order to optimize the production of transgenic shoots it was necessary to maximize the areas of GUS at the ends of the hypocotyl segments, so that fast-growing calli would be more likely to incorporate transgenic cells. Use of SIM medium as the inoculation medium appeared to give

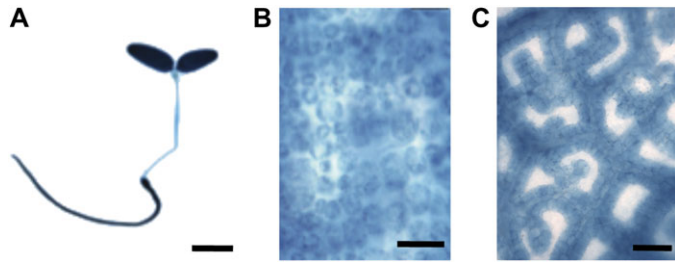


Fig. 4. Constitutive and bundle sheath specific accumulation of β -glucuronidase (GUS) in *C. gynandra* plants. (A) Seedling expressing *uidA* under the control of the CaMV 35S promoter. (B) Mature leaf expressing *uidA* under the control of the CaMV 35S promoter, GUS is visible in mesophyll cells. (C) Mature leaf expressing *uidA* translationally fused to a *C. gynandra RbcS* gene, GUS is restricted to bundle sheath cells. Scale bars: (A) 5 mm, (B) 50 μ m, (C) 100 μ m.

the most opportunity for the regeneration of transgenic shoots.

To confirm that the transformation procedure could be used to investigate mechanisms underlying the patterns of gene expression associated with C_4 photosynthesis, GUS accumulation in lines of *C. gynandra* containing the *pCaMV35S::uidA::Tnos* construct was compared to lines with *uidA* translationally fused to a *C. gynandra RbcS* gene. In the latter lines, we would expect GUS to be restricted to BS cells (Marshall *et al.*, 2007). Imaging of T_1 seedlings of plants expressing *pCaMV35S::uidA::Tnos* showed that GUS was present in leaves and roots (Fig. 4A). Bright-field microscopy of leaves showed that GUS was present in both M and BS cells (Fig. 4B), consistent with the constitutive expression associated with the *CaMV35S* promoter. By contrast, when *uidA* was translationally fused to a *CgRbcS* gene, it accumulated in a strictly BS specific manner (Fig. 4C).

Overall, suspending the inoculum in SIM medium and regenerating hypocotyls and cotyledons on CR medium, followed by media A5–A8 as detailed in De Block *et al.* (1989), led to a transformation efficiency (the number of independent transgenic shoots produced relative to the total number of explants that were cultured) of 14%. This compares reasonably with transformation efficiencies in the C_3 species oilseed rape (De Block *et al.*, 1989) as well as the NADP-ME type C_4 plants *F. bidentis* (Chitty *et al.*, 1999). Although the time taken to generate transgenic shoots of *C. gynandra*, and *F. bidentis* appear similar (this study; Chitty *et al.*, 1999), because the life cycle of *C. gynandra* is faster, analysis should be faster with *Cleome*. The approach described here should therefore facilitate analysis of NADP-ME-type C_4 photosynthesis, and through comparative analysis with *A. thaliana*, the identification of genes that determine the cell biology and development associated with leaves of C_4 dicotyledons.

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