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ORIGINAL PAPER

Transgenic mimicry of pathogen attack stimulates growth and secondary metabolite accumulation

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Abstract Plant secondary metabolites, including pharmaceuticals, flavorings and aromas, are often produced in response to stress. We used chemical inducers of the pathogen defense response (jasmonic acid, salicylate, killed fungi, oligosaccharides and the fungal elicitor protein, cryptogein) to increase metabolite and biomass production in transformed root cultures of the medicinal plant, *Withania somnifera*, and the weed, *Convolvulus sepium*. In an effort to genetically mimic the observed effects of cryptogein, we employed *Agrobacterium rhizogenes* to insert a synthetic gene encoding cryptogein into the roots of *C. sepium*, *W. somnifera* and *Tylophora tanakae*. This genetic transformation was associated with stimulation in both secondary metabolite production and growth in the first two species, and in growth in the third. In whole plants of *Convolvulus arvensis* and *Arabidopsis thaliana*, transformation with the cryptogein gene led, respectively, to increases in the calystegines and certain flavonoids. A similar transgenic mimicry of pathogen attack was previously employed to stimulate resistance to the pathogen and abiotic stress. In the present study of biochemical phenotype, we show that transgenic mimicry is correlated with increased secondary metabolite production in transformed root cultures and whole plants. We propose that natural transformation with genes encoding the production of microbial elicitors could influence interactions between plants and other organisms.

Keywords Cryptogein · Flavonoids · *Withania* · *Tylophora* · *Convolvulus* · *Calystegia*

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Introduction

Plant secondary metabolites are the origin of many, if not most, of our pharmaceuticals, flavorings and aromas. Plants have a privilaged access to energy through photosynthesis; they can thus afford to synthesize a wide spectrum of exotic chemicals to aid their survival. Plant secondary metabolites are essential to functions ranging from allelopathy to resistance to UV light and biological aggression. Given the opportunistic nature of evolution, it is not

surprising that a single substance serves in several functions. For instance, the calystegines are nutritional mediators of plant/bacteria interactions (Tepfer et al. 1988), and they are potent glycosidase inhibitors (Molyneux et al. 1993), implicated in allelopathy directed at other plants and probably at animals, as well. Flavonoids are antioxidants, phytoalexins and UV protectors (Li et al. 1993; Harborne and Williams 2000; Pietta 2000). Thanks to the chemical and functional diversity of plant secondary metabolites, a given medicinal plant can be used for treating a variety of animal ailments, and plants form the basis of our pharmacopia.

Many medicinal plants are poorly adapted to mass cultivation, and the active ingredients they produce are too complex to synthesize in large quantities. Therefore, natural populations are under pressure from human harvesting (Shinwari and Gilani 2003; Olsen 2005). Genetic methods can be applied to increase the production of a given metabolite or a group of related compounds, e.g. via traditional breeding and screening (Buter et al. 1998; Long et al. 2006), through the expression of genes encoding key enzymes (Holmberg 1997; Dueckershoff et al. 2005; Tian and Dixon 2006; Inui et al. 2007; Liu et al. 2007) or through functional genomics (Goossens et al. 2003), but despite these successes, metabolic engineering of the relevant biosynthetic pathways is often hampered by a lack of knowledge of the enzymes and genes involved. Indirect approaches to improving metabolite production have thus been explored, including treating whole plants and in vitro cultures of organs or cells with inducers of plant defense responses, since the reaction to both biotic and abiotic stress can include increased accumulation of secondary metabolites (Lin and Wu 2002; Ye et al. 2004; Matthews et al. 2005; Wolucka et al. 2005; Zhao et al. 2005; Zobayed et al. 2005; Shi et al. 2007; Zhang and Fevereiro 2007).

Another indirect genetic approach has yielded increases in secondary metabolite production in a variety of species. Genetic transformation by the Ri TL-DNA of *Agrobacterium rhizogenes* produces roots that can be cultured in vitro (Tepfer and Tempé 1981; Tepfer 1984), which often show high accumulation of secondary metabolites (Flores et al. 1987; Jung and Tepfer 1987; Guillon et al. 2006). Furthermore, plants regenerated from transformed roots have profuse root systems, and they are easy to root by cutting (Tepfer 1983a, b, 1984). They could be used to improve secondary metabolite (or protein) production via plants grown in hydroponics or aeroponics. The morphological and physiological changes caused by the Ri T-DNA are similar in many dicot species (Tepfer 1984, 1989); thus, Ri T-DNA might be used to improve secondary metabolite production in multiple species, without knowledge of the underlying enzymology and genetics. In the following paper, we employ this transformation by *A. rhizogenes* in combination with another indirect genetic approach, which relies on transformation by a gene encoding a fungal elicitor.

β-cryptogein (Crypt) is a sterol scavenging protein secreted by the oomycite, *Phytophthora cryptogea* (Ricci et al. 1989; Boissy et al. 1996; Mikes et al. 1997). It elicits a defense response in tobacco through interaction with a receptor, followed by signal transduction, which leads to the expression of a transcription factor (Lecourieux et al. 2002; Guo et al. 2004; Kasparovsky et al. 2004; Lochman et al. 2005; Ren et al. 2006), which in turn is thought to be responsible for a generalized stress response. Knowledge of the amino acid sequence of the Crypt protein (Ricci et al. 1989) allowed the synthesis of a *crypt* gene, whose expression in *Escherichia coli* led to the production of Crypt protein that induced (in tobacco) the necrosis associated with the defense response (O'Donohue et al. 1995). Genetic transformation of tobacco with this synthetic gene, under the control of the 35S CaMV promoter, rendered two independent lines of tobacco plants resistant to *P. parasitica* var. *nicotianae* (Tepfer et al. 1998). Resistance was transmitted to progeny via meiosis, and it was higher in hemizygotes than in homozygotes. A lysine to valine mutation (K13V) at amino acid position 13 was also associated with resistance in hemizygotes in two lines. Presence of the Crypt protein was shown by immunoblot.

A similar transformation of tobacco was made using a natural *crypt* gene cloned from *Phytophthora cryptogea*, controlled by the promoter from the plant gene *hsr203J*, which is induced by pathogen attack (Keller et al. 1999). A resistance response was reported, although in a single transformed line, and transmission of the transformed phenotype through meiosis was not shown. However, resistance was detected against three other fungal pathogens. In a third rendition of this experiment (Jiang et al. 2002,

2004), a mutated *crypt* gene (K13V) was controlled by a rice phenylalanine ammonia-lyase promoter. Resistance was reported in transgenic tobacco to *Phytophthora parasitica*, *Alternaria alternata* and *Pseudomonas syringae*. In addition, transgenic plants were tolerant to salt.

In all, three different promoters were used, and the coding sequence was introduced in synthetic, natural and mutated forms. Resistance was observed in tobacco, using five pathogens and one abiotic stress. Thus, the principle of transgenetic mimicry of pathogen attack, leading to a generalized stress response, is established for the *crypt* gene in tobacco; however, the biological basis for the resistance is not known. Physiological correlates have been examined, including production of reactive oxygen species and the role of nitric oxide (Foissner et al. 2000; Lamotte et al. 2004; Planchet et al. 2006; Ashtamker et al. 2007). Crypt (as well as other elicitors) induced a transcription factor (OPBP1), probably responsible for the activation of multiple genes involved in stress responses, including genes involved in resistance to fungal pathogens (Guo et al. 2004). There was thus ample reason to expect the expression of *crypt* to lead to increased accumulation of some secondary metabolites.

In an effort to predict the effects of the *crypt* gene, before using it in transformation, we treated transformed root cultures (carrying just Ri T-DNA) with the cryptogein protein (Crypt), as well as with known chemical elicitors and a killed strain of *P. parasitica* var. *nicotianae*, which makes no known protein elicitor for tobacco. Effects on root growth and accumulation of withanoferin A in *W. somnifera* and calystegines in *C. sepium* were recorded. Withaferin A is steroidal lactone that resembles the ginsenosides in structure and medicinal use. Calystegines are potent glucosidase inhibitors and nutritional mediators in plant-bacteria interactions (Tepfer et al. 1988a; Goldmann et al. 1993; Molyneux et al. 1993).

In a test of the hypothesis that the gene encoding cryptogein (*crypt*) could also increase metabolite production via production of the Crypt protein *in planta*, the synthetic gene was introduced into the roots of *W. somnifera*, *C. sepium* and *Tylophora tanakae*, also transformed by *A. rhizogenes*. The roots in these experiments were transformed by *A. rhizogenes* to facilitate growth in vitro and to measure possible gain in metabolite production from elicitation by genetic mimicry in combination with transformation by Ri T-DNA.

The *crypt* gene was also transformed into whole plants. Morning glory (*Convolvulus arvensis*), was co-transformed with *crpt* and Ri T-DNA, and *A. thaliana* was transformed by *crypt* without Ri T-DNA. Calystegins were measured in *C. arvensis*, and *A. thaliana* served as a model for the production of flavonoids. In all cases, increases in growth and secondary metabolite accumulation were associated with the presence of the *crypt* gene.

Materials and methods

Plant materials

C. sepium and *C. arvensis* and were collected near the INRA, Versailles, campus. *W. somnifera* was from the tissue culture collection at the University of Calcutta, India. Seeds of *T. tanakae* were obtained from F. Abe.

Tissue culture and transformation

Methods for obtaining and culturing transformed roots are described elsewhere (Tepfer 1984; Chaudhuri et al. 2005; Bandyopadhyay et al. 2007). Briefly, surface-sterilized shoots (internodes and petioles) and leaves were infected with *A. rhizogenes* strains LBA9402 or A4, with or without the synthetic *crypt* gene (O'Donohue et al. 1995), under the control of the CaMV promoter (Tepfer et al. 1998). Roots induced at the site of inoculation were decontaminated by growth through Murashige and Skoog medium (Murashige and Skoog 1962) with 3% (w/v) sucrose (Sigma, USA) and one-fifth of the standard N-salts, solidified with 0.8% (w/v) agar (Sigma, USA). Transformed root cultures were subcultured every 4 weeks. For growth and metabolite extraction and analysis (see below), about 0.2 g fresh weight (FW) roots were grown in 100 ml liquid medium in 250 ml culture flasks on a rotary shaker at 100 rpm in the dark. Roots were cultured up to 13 days (*W. somnifera* and *C. sepium*) or 21 days (*T. tanakae*). Each root line was cultured in triplicate and the experiments were repeated at least once. The transgenic plants that spontaneously regenerated from transformed *C. arvensis* roots were cultured on agar

medium in culture tubes for 4–7 days, in a growth chamber (20°C, 16-h photoperiod, TFL 110 fluorescent Philips tubes, 65 µmol m^{-2} s^{-1}). Arabidopsis plants were transformed by the floral dip method (Desfeux et al. 2000) with *A. tumefaciens* strains carrying only the *nptII* gene (*nptII* control) or with both the *nptII* and *crypt* genes. One set of plants was not inoculated to serve as a negative (wild type) control. Plants were transferred to the greenhouse for seed production (see below). Seeds were collected, sterilized and germinated in vitro (Desfeux et al. 2000) on Arabidopsis-agar medium (Bechtold et al. 1993) in the presence of 50 mg l^{-1} kanamycin (Sigma, USA); kanamycin-resistant plants were transferred to the greenhouse.

Plant cultivation

Plants were grown in the greenhouse in the spring and summer in a mixture of peat, sand and vermiculite and watered three times per week with a nutrient solution (Coïc and Lesaint 1973). When necessary, day length was extended to 16 h using artificial light. Transgenic lines of *C. arvensis* were grown from stem cuttings of primary regenerants for 3 months before metabolite extraction and analysis (see below). Kanamycin-resistant Arabidopsis plants were checked for the presence of transgenes (see below), and their seeds were collected for flavonoid analysis.

DNA isolation and PCR analysis

Total DNA was extracted from transformed roots and transgenic plants (leaf tissue) according published methods (Dellaporta et al. 1983). The different transformed genotypes were verified by polymerase chain reaction (PCR) analysis for *rolC* (for the TL-DNA), *nptII* and *crypt*. Plasmid DNA from appropriate *Agrobacterium* strains (see above) was used as a positive control, and DNA isolated from the leaves of wild-type plants served as a negative control. Oligonucleotide primers for the *rolC* gene were 5′-TAACATGGCTGAAGACGACC and 5′-AAACTTG CACTCGCCATGCC (Slightom et al. 1986). The amplified product had the expected size of 534 bp. Samples were denatured at 95°C for 5 min, followed by 30 amplification cycles of 1 min at 94°C (denaturization), 1 min at 53°C (annealing) and 1.5 min at 72°C (extension), with a final extension step at 72°C for 5 min. The primers for *nptII* were 5′-GAACAA GATGGATTGCACGC and 5′-GAAGGCGATAGA AGGCGATGC (de Vries and Wackernagel 1998). The conditions were: 1 cycle at 94°C for 5 min; 40 cycles at 94°C for 1 min, 54°C for 2 min and 72°C for 4 min; 1 cycle of 72°C 12 min. Amplification resulted in a 412 bp amplified product. The primers for *crypt* were 5′-CCATGTCGACCTACAAGGAA-GAGCACTTGT and 5′-TCCGGTCGACATGGCT TGCACTGCTAC (O'Donohue et al. 1995). An initial 1 min step at 95°C was followed by 35 cycles (1 min at 95°C, 1 min at 55°C, and 1 min at 72°C) and a final 5 min step at 72°C. The amplified product had the expected size of 329 bp. In each case, the PCR mixture consisted of 200 ng DNA, 12.5 pmol of each primer, 200 mM of dNTPs, 1.5 U Taq polymerase (Invitrogen, USA) and buffer supplied by the manufacturer in a total volume of 25 µl. The PCR products were analyzed by electrophoresis on a 2% (w/v) agarose gel with a 1 kbp DNA ladder (Promega, USA).

Elicitor treatment

Purified β-cryptogein protein, a gift from M. O'Donohue and J.-C. Pernollet, was produced using *Pichia pastoris* (O'Donohue et al. 1996). Jasmonic acid (Sigma, USA) and salicylic acid (Sigma, USA) were dissolved in ethanol as filter-sterilized, 0.1 M stock solutions. α-1,4-oligogalacturonides from partial digestion of polygalacturonic acid with endopolygalacturonase from *Aspergillus niger* (Spiro et al. 1993) were provided by A. Aziz. They were dissolved in liquid medium to a concentration of 0.5 mg ml^{-1} and filtered-sterilized. *P. parasitica* var. *nicotianae* (strain INRA A183) was cultured on malt-agar medium. Agar plugs were cut out after 5–7 days and dissolved in liquid medium. The fungus was heat-killed by autoclaving, and the medium, with the cell-debris, was filter sterilized. Cryptogein protein (O'Donohue et al. 1996) was dissolved in water (0.1 mg ml^{-1}) and filter sterilized. Elicitors were added to 6-day-old (log phase) liquid cultures of *W. somnifera* and *C. sepium*, transformed with *A. rhizogenes* LBA9402. Jasmonate, salicylate and oligogalacturonides were added to the culture medium at a final concentration of 0.3 mM. One volume of fungal extract and 0.05 volumes of cryptogein protein stock were added to culture medium. When

necessary, controls received ethanol. Roots were harvested for growth studies and metabolite extraction and analysis (see below) after 48 h (5 days), 96 h (10 days) and 168 h (13 days) of elicitor treatment. Samples were in triplicates, and each experiment was repeated at least once.

Study of root growth

The initial FW was recorded at inoculation time, and roots were harvested over time. They were removed from the medium, washed with deionised water, blotted dry and weighed to determine the final FW. Growth was calculated as (Final FW − Initial FW)/Initial FW. Growth was also measured on a dry weight basis (DW), after desiccation for 16 h at 55°C.

Metabolite extraction and analysis

For the analysis of tylophorine and withanolides, roots were oven-dried at 55°C, powdered and extracted for HPLC as described previously (Chaudhuri et al. 2005; Bandyopadhyay et al. 2007). Calystegines in roots and root-regenerated shoots were extracted from 100 mg (FW) with 0.02 N HCl. The extracted samples were subjected to high voltage paper electrophoresis at 2,500 V for 20 min on 3 M chromatography paper (Whatman, USA) and visualized with silver staining (Tepfer et al. 1988a). Spots were analyzed by a Fluorchem 8000 Imaging system (Alpha Innotech Corporation, USA), coupled with a Multimage Light Cabinet II with automatic enhancer and detector. Calystegines were quantified using a standard curve, based on pure calystegine B2, which was linear between 1 and 10 µg. Flavonoids were extracted from 100 mg (FW) T1-seeds of Arabidopsis with 75% (v/v) methanol (in water), defattened with cyclohexane, eluted with methanol through a C-18 cartridge, dried under a stream of liquid nitrogen and redissolved in 15% (v/v) acetonitrile (in water). HPLC was performed on a Waters 990 system (Milford, USA), equipped with a Waters photodiode array detector and a Waters 600 multisolvent delivery system. Separation was with a Varian C-18 90A (reverse phase) column (150 × 4.6 mm, 5 µm particle size), and gradient elution was in 10–100% acetonitrile over 20 min at a flow rate of 1.0 ml min^{-1}. Quercitrine was detected at a retention time of 19.4 min, determined by spiking with a standard compound (Sigma, USA). Comparisons were made on the basis of peak areas at 230 nm absorption. The flavonoid content of the HPLC peaks was determined by mass spectroscopy (Routaboul et al. 2006).

Statistical analysis

Experiments were set up in a randomized design; sampling was in triplicate. Data were subjected to analysis of variance (ANOVA).

Results

Chemical elicitation in transformed roots

Transformed roots were induced on sterilized leaves of *W. somnifera* and on stem segments of *C. sepium*, using the LBA9402 strain of *A. rhizogenes* (pRi 1855), carrying only Ri T-DNA. Sterile root lines (representing independent transformation events) were derived and used to test the effects of known chemical inducers (including the cryptogein protein) on growth and secondary metabolite accumulation.

In *C. sepium* roots, all elicitors stimulated growth, except the killed *Phytophtora* cells (Fig. 1a). In *Withania* roots, both the killed *Phytophthora* cells and salcylate had no effect on growth (Fig. 1b). Among the elicitors tested in both species, the Crypt protein was the most growth stimulating, particularly in *Withania*, where it was associated with a 211% increase in growth.

The Crypt protein also stimulated calystegine content in *C. sepium* (336%), but it did not increase withanolide content in *W. somnifera* (Fig. 1c, d). Nevertheless, oligogalacturonides and jasmonate did stimulate withanolide accumulation. Despite the limited effects of the Crypt protein on secondary metabolite production, the promotion of growth in the two species and calystegine content in *C. sepium* were sufficiently encouraging to warrant transformation with the *crypt* gene, since the stimulation of biomass accumulation in vitro can, by itself, increase overall metabolite production.

In order to test the effects of the gene (*crypt*) that encodes the Crypt protein, we made use of the synthetic *crypt* gene in the pBin19 transformation vector (Bevan 1984), under the control of the 35S

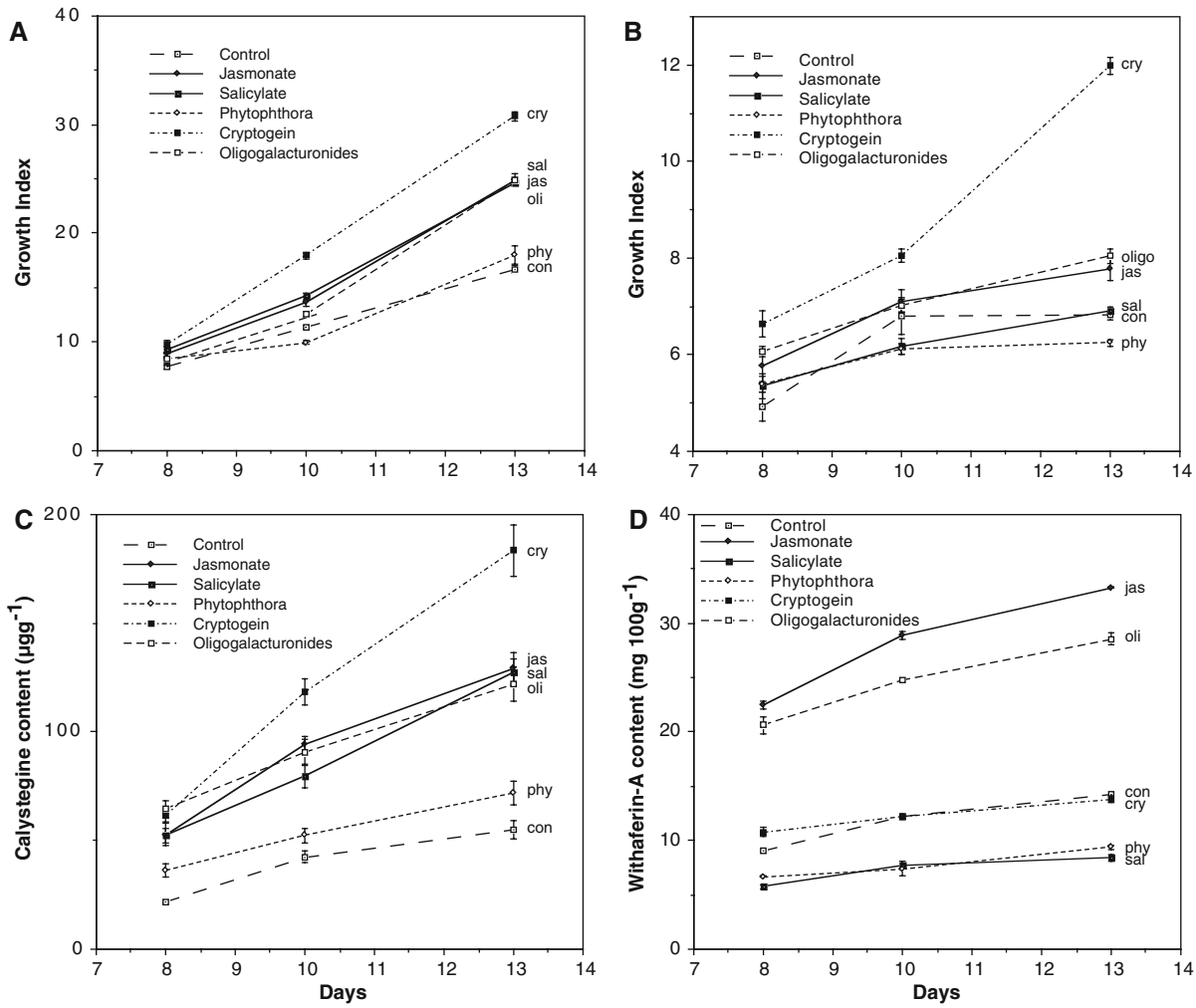

Fig. 1 Effects of chemical elicitors on transformed root growth and secondary metabolite production. Transformed root cultures, carrying just Ri T-DNA of *C. sepium* (**a**, **c**) and *W. somnifera* (**b**, **d**) were treated with known elicitors of defense responses: purified *β*-cryptogein protein (cry), jasmonic acid (jas), salicylic acid (sal) and α-1,4-oligogalacturonides (oli). An autoclaved and filtered culture of *P. parasitica* var. *nicotianae* (phy) was also tested, as well as no treatment (con). The effects were measured on growth (**a**, **b**) and secondary metabolite production (**c**, **d**). Calystegines were assayed in *C. sepium* and withaferin A was quantified in *W. somnifera*. Determinations were in triplicate; error bars represent standard deviations

CaMV promoter, which was previously shown to be associated with resistance to *Phytophthora* in tobacco (Tepfer et al. 1998). Transformed root lines were produced from single roots on different explants to insure that each line represented a distinct transformation event. The accumulation of secondary metabolites was measured in each root line over time. Controls consisted of root lines transformed by wild type *A. rhizogenes*. Transformation by *crypt* was demonstrated by PCR. In *W. somnifera*, three lines did not contain *crypt*, although they were produced using *A. rhizogenes* carrying the *crypt* construct. These lines, in which the binary vector T-DNA was not transferred, served as supplementary controls, since they carried only the Ri T-DNA.

Genetic elicitation using transformed roots

The presence of the *crypt* gene in transformed root lines was associated with a stimulation of root growth and secondary metabolite accumulation in both *C. sepium* and *W. somnifera* (Fig. 2). After 13 days

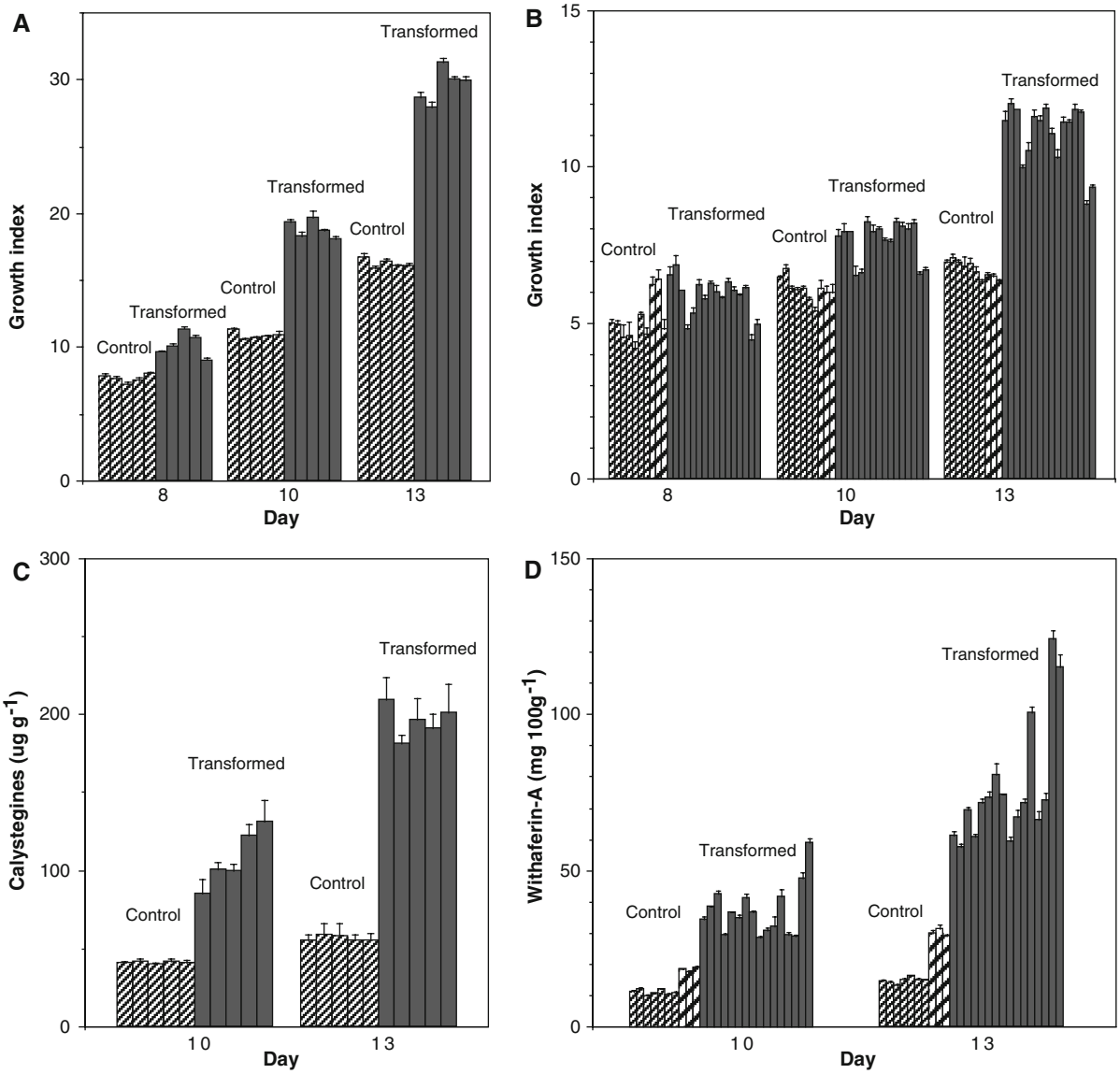

Fig. 2 Effects of genetic transformation with the *crypt* gene on growth and secondary metabolite production. The *crypt* gene was introduced into *C. sepium* (**a**, **c**) and *W. somnifera* (**b**, **d**) using *A. rhizogenes*. Growth (**a**, **b**) and secondary metabolite accumulation (**c**, **d**) were recorded over time. Bars represent individual root clones from independent transformation events: dark hatching, roots containing *crypt* and Ri T-DNA; medium density hatching, roots containing just Ri T-DNA produced by wild type *A. rhizogenes*; light hatching, roots produced by *A. rhizogenes* carrying the *crypt* gene construct, but which did not receive the *crypt* gene from the pBin19 binary vector

of culture, root growth increased by at least 50% and secondary metabolite production by as much as 8-fold in exceptional root clones (Fig. 2d). These results were taken as support for the hypothesis that genetic (endogenous) expression of the Cry protein can mimic the effects of exogenous treatment on growth and secondary metabolite accumulation. In order to validate and extend this finding, we introduced the *crypt* gene into three other species: *C. arvensis* (a morning glory that regenerates from roots), *Tylophora* (an Indian medicinal plant) and *Arabidopsis* (a model for flavonoid production).

Tylophora root clones were established in liquid culture, as for other species. Their growth was studied by incubating 200 mg of roots (in triplicates) in 100 ml N/5 liquid medium in 250 ml flasks, for

21 days in the dark at 100 rpm. Root lines were analyzed after 3 months in culture. One-way ANOVA analysis showed a significantly ($P \leq 0.05$) greater growth and biomass productivity in root clones carrying *crypt*, compared to control clones (Fig. 3). These *crypt* clones grew and accumulated biomass (FW and DW) about 1.5-fold faster than the corresponding control clones. The difference in growth was evident in both DW and FW measurements. Thus, the stimulation of growth observed in *Withania* and *Calystegia* was repeated in *Tylophora*.

Tylophorine content in root lines carrying *crypt* was not significantly increased, compared to the controls. Thus, tylophorine production (a function of growth and metabolite content) in liquid culture by these *crypt* clones would be significantly improved. After 6 months in liquid culture, the differences in growth between the controls and the lines containing *crypt* were no longer detected (results not shown.) We conclude that the *crypt* gene has the potential to stimulate tylophorine production, but to a lesser extent than the other metabolites studied in other species, and that this stimulation is unstable.

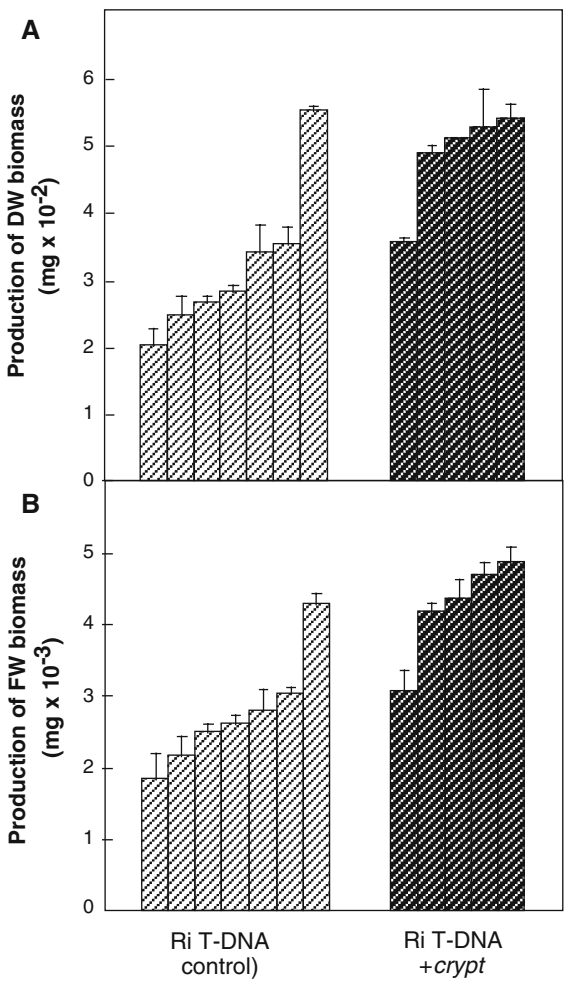

Fig. 3 Effects of *crypt* on growth of transformed *Tylophora* roots. Growth was significantly stimulated in root clones carrying both *crypt* and Ri T-DNA, independently of water content. (**a**) DW; (**b**) FW. Each bar represents an independently transformed line

Genetic elicitation using transformed plants

The effects of the *crypt* gene in whole plants was first studied in *Convolvulus arvensis*, a close relative of *C. sepium*, which regenerates directly from its roots. When the *crypt* gene was present in *C. arvensis* plants, calystegine content in the roots of *C. arvensis* plants carrying the *crypt* gene increased by 35% ($P \leq 0.05$) (Fig. 4). In the shoots, mean calystegine content increased by 42% ($P \leq 0.05$). Thus the stimulation in calystegine content, previously observed for root cultures of *C. sepium*, was repeated in whole transgenic plants of *C. arvensis*. (Changes in growth and development were not observed). We concluded that the initial metabolite accumulation results from *Withania* and *C. sepium* were validated in whole plants of *C. arvensis*.

The above experiments relied on *A. rhizogenes* for transformation, because we were interested in measuring a possible gain in secondary metabolites, due to co-transformation by Ri T-DNA plus the *crypt* gene, particularly in transformed root cultures. In Arabidopsis, we transformed without Ri T-DNA, using Agrobacterium *tumefaciens* and a standard floral dip transformation method, because transformation with the genetic marker alone (*nptII*) in Arabidopsis generates considerable genetic and phenotypic variability (Fig. 5), and we did not want to combine this with the previously described variation associated with Ri T-DNA (Tepfer 1984).

Since flavonoids accumulate in seeds and (particularly) in seed coats, whole seed flavonoids from controls and transformants were extracted and separated by HPLC, and the two major flavonoid peaks were examined by mass spectrometry to identify their components (Table 1). Peak I contained quercitrin almost exclusively, while peak II contained three flavonoid derivatives (Table 1) and traces of two

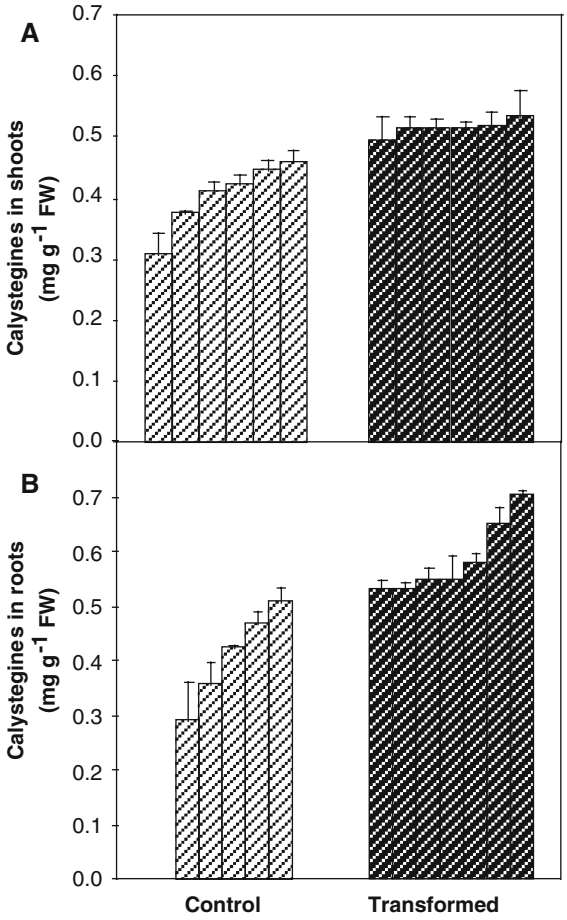

Fig. 4 Effects of the *crypt* gene on calystegine content in *C. arvensisi* shoots and roots. Transformation by *crypt* and Ri T-DNA resulted in increased calystegine accumulation in whole plants, both in shoots (**a**) and roots (**b**). Each bar represents an independently transformed line

others (not shown). HPLC was then routinely used to measure possible effects of the *crypt* construct on these seed flavonoids.

Flavonoids varied among individual transformants in both the controls, carrying *nptII*, and in plants carrying the *crypt* gene. Similar variability was not seen in the wild type control. Thus, transformation alone with the *nptII* gene altered the content of seed flavonoids in individual transformants, and it complicated detection of differences due to *crypt*.

Flavonoid peak I, composed of quercitrin (quercetin-3-*O*-rhamnoside) did not statistically increase in the lines transformed by the crypt gene, but the chances of finding high-producing lines in this population were increased (see Discussion).

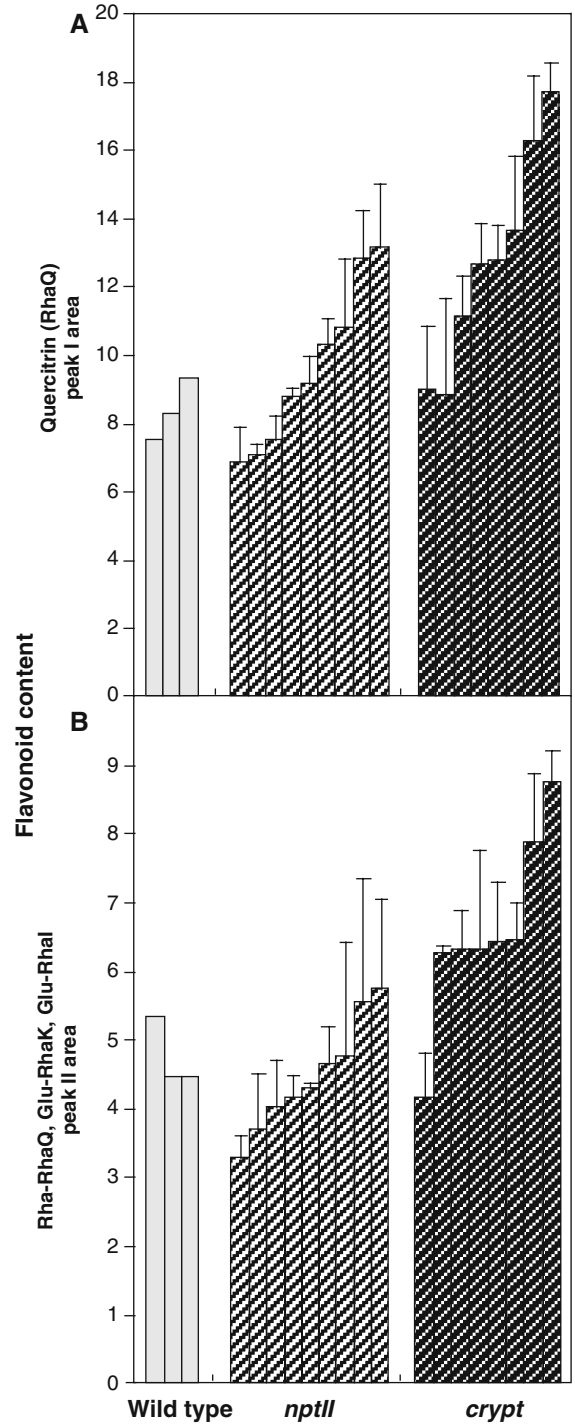

Fig. 5 Flavonoids in the seeds of T1 generation transgenic Arabidopsis plants. Arabidopsis was transformed with the *crypt* gene, using *A. tumefaciens*. Flavonoids were analyzed using HPLC. The results for two peaks are shown (**a**, peak I; **b**, peak II). The *crypt* gene was not associated with a significant increase in peak I, but it was for peak II (see Table 1 for peak compositions)

Table 1 Flavonoid content of HPLC peaks

Flavonoid peak (HPLC)	Common name	Chemical abbreviation	Chemical name	Molecular weight	Retention time
Peak I	quercitrin	RhaQ	Quercetin-3-*O*-rhamnoside	448	19.4
Peak II		Glu-RhaI	Isorhamnetin-hexoside-rhamnoside	624	15.6
		Rha-RhaQ	Quercetin-di-rhamnoside	594	15.3
		Glu-RhaK	Kaempferol-3-*O*-glucoside-7-*O*-rhanoside	594	15.2

Flavonoid peak II, containing three flavonoid derivatives, increased by about 40% ($P = 0.005$), relative to the *nptII* transformants, which were not significantly different ($P = 0.59$) from the wild type control. (Changes in growth and development were not observed). We conclude that transformation with the cryptogein gene can stimulate the accumulation of certain (but not all) flavonoids in Arabidopsis seeds.

Discussion

These findings validate the hypothesis that transgenetic mimicry of fungal attack can lead to increased secondary metabolite production. To better focus on testing transgenetic elicitation in multiple species, we limited the analysis to phenotypic changes, deferring verification of underlying biochemical and genetic changes to future studies. We assumed that, as for other elicitors (Zhao et al. 2005), the synthesis of the Crypt protein in plant tissues initiates a signal transduction cascade that results in the induction of one or more transcription factors, responsible for a generalized defense response. Inducible expression and secretion of *crypt* could be used to verify this assumption. The CaMV promoter used here is considered to be constitutive, but no signal peptide was attached to the *crypt* coding sequence, and we have no information about the fate of the Crypt protein after synthesis, although the protein encoded by this construct was detected in transgenic tobacco plants (Tepfer et al. 1998). Cryptogein has sterol scavenging activates, and it could be toxic for the plant cell. Thus, high-producing transformants were likely counterselected after transformation. How the protein, produced on the inside of the cell, can act with the receptor, presumed to be on the outside, is not known, although leakage, e.g. through cell injury, was proposed (Tepfer et al. 1998). Despite these uncertainties, the phenomen of secondary metabolite and root growth stimulation seems clear, and it is reproducible, albeit to varying degrees, in different plant species producing different secondary metabolites.

In all species examined, root transformation by the *crypt* gene was correlated with increased biomass accumulation in vitro. FW and DW accumulations were similar (Fig. 3, other data not shown), thus increases in biomass were not due to increases in water content. Since metabolite production is a function of concentration and biomass accumulation, the effect of *crypt* on growth could significantly contribute to the production of metabolites in transformed root cultures, even if increases in metabolite content are modest. Other chemical inducers of plant defenses also stimulated root growth; thus, increased root growth might be a generalized reaction to stress. It is logical that pathogen attack leading to necrosis in one part of a root system would be compensated by increased growth in other parts of the root system—i.e. that a general defense response would stimulate the growth of the surviving root system.

Increases in metabolite content in transformed root cultures were recorded in all but one (*Tylophora*) of the five species tested. For a given species, levels of stimulation varied among clones, but results were reproducible within a given clone, indicating that the chemical measurements were accurate. Clonal variation was expected, due to diverse factors, including insertion site and transgene copy number. Clonal variation did not prevent finding that the root lines containing *crypt* contained statistically more secondary metabolites than the controls. Variation can, in itself, contribute to obtaining high-producing clones. The lack of a stable increase in *Tylophora* could be due to the high stimulation of tylophorines in roots transformed by the Ri T-DNA alone (Chaudhuri et al.

2005). We presume that maximum metabolite accumulation is limited by carbon availability and toxicity.

Statistically significant stimulation of calystegine production was observed in *C. arvensis* leaves and roots (increases of 30% and 40%, respectively). Calystegins are allelopathic glycosidase inhibitors that likely poison the plants that produce them, probably limiting the amount that these plants can produce. They are tropane derivatives (Goldmann et al. 1990) that served here as a convenient model to determine whether an exotic tropane alkaloid pathway can be stimulated by transformation with the *crypt* gene. In nature, their allopathic properties are likely used in defense against microbial pathogens and in competition among plant species (Goldmann et al. 1996). They also serve as nutritional mediators in interactions with *Rhizobium meliloti* (*Sinorhizobium meliloti*) Tepfer et al. (1988a, b). Changes in calystegine production might thus alter rhizosphere population dynamics.

A. rhizogenes was used for transformation (except in *Arabidopsis*); thus, Ri T-DNA was co-introduced with the *crypt* gene. The phenotypic expression of the Ri T-DNA was optimized by screening for rapid root growth in vitro. In contrast to the other species, *Arabidopsis* was transformed by *A. tumefaciens*. Transformation of *Arabidopsis* with Ri T-DNA plus *crypt* (via floral dip using *A. rhizogenes*) might further improve flavonoid production. It would be appropriate to test the effects of *crypt* on other substances known to accumulate in response to pathogen attack in *Arabidopsis*, including polyamines and other polyphenols, such as camalexin (Glawischnig 2007).

In the case of quercitrin (peak I), the population of clones transformed by *crypt* was not significantly different from the controls transformed by *nptII*. Nevertheless, transformation by *crypt* increased the chances of obtaining high-producing lines. If a 50% increase in quercitrin content is used to define a high-producing line, than the population of crypt lines included five out of eight (63%) high producers, whereas the population transformed by *nptII* alone included two out of nine (22%) high producers. The highest producing line transformed by *crypt* showed a stimulation of 111%, and the highest producing line transformed by *nptII* alone showed a stimulation of 57%. Thus, even in the absence of a statistically significant difference between the two populations, the chances of obtaining high quercitrin production were increased when the *crypt* gene was present.

Flavonoid peak II contained three flavonoid derivatives. The lines containing *crypt* showed a statistically significant, 39% stimulation in peak II, compared to the control lines carrying just *nptII*, which was not statistically different from the wild type controls. The best producing line contained 85% more flavonoids than the control. The ranking of the lines was the same for both peaks I and II. Increases in flavonoids were not observed for a third flavonoid peak (not shown). The effects of *crypt* on flavonoid accumulation in *Arabidopsis* seeds would seem to be limited to certain substances, possibly due to a redistribution of carbon in the phenylpropanoid pathway.

The metabolites stimulated by *crypt* represent a variety of natural products, produced by widely different species, perhaps reflecting the wide host specificity of *Phytophthora* species (Erwin and Ribeiro 1966). The metabolites included diverse structures: calystegines A and B (tropane derivatives), withaferin A (a steroidal lactone), and a group of flavonol glycosides. Although tylopohorine B (a phenanthroindolizidine alkaloid) was not increased, the ability of the *crypt* gene to stimulate the accumulation of substances of varying chemical nature suggests that the production of certain medicines, aromas and colours might be improved without understanding the underlying biochemistry and genetics. The increases recorded here varied from a few tens of a percent to several fold, but even small, significant increases suggest that improvement might be possible by using, for instance, inducible and tissue-specific promoters. A general method for improving the production of key metabolites could have economic applications in the pharmaceutical and food industries and at the same time provide a scientific tool for studying the biology and function of secondary metabolites.

We previously showed in tobacco that the *crypt* construct conferred resistance to *Phytophthora*, and we suggested that natural genetic transformation by a gene such as *crypt* could play a role in evolution (Tepfer et al. 1998). Here, we extend the analysis of the phenotypic changes associated with *crypt* to secondary metabolites and to five new species. Secondary metabolites serve multiple functions,

ranging from attractants to allelopathic poisons. Changes in chemical phenotype could therefore have wide-ranging effects on fitness. *A. rhizogenes* is capable of inducing transformed roots in a variety of plant species, and in many of these species, shoots regenerate spontaneously from roots, propagating the transformed genotype and phenotype through meiosis (Tepfer 1983a, b, 1984; Tepfer et al. 1989). In nature, enhanced secondary metabolite production could be acquired via genetic transformation with an *A. rhizogenes*, carrying a T-DNA encoding a defense response eliciter. Artificial transformation by the *crypt* gene thus illustrates how natural transfer to plants of a single gene from the soil metagenome could have important repercussions in relationships between plants and other organisms.

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