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Research Note

Translocation and Exudation of Tumor Metabolites in Crown Galled Plants

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Crown gall tumors are induced on susceptible plants by pathogenic strains of *Agrobacterium*. These neoplastic plant cells produce metabolites, called opines, which provide a source of nutrients to colonizing agrobacteria. Opine production previously has been shown to influence microbial communities in the immediate vicinity of the tumor. We have obtained evidence for opine translocation to and exudation from distal uninfected regions of the plant host. Grafted plants made from an opine-producing transgenic scion with a wild-type stock, or with a wild-type scion and an opine-producing stock accumulate opines in the wild-type portions of the plant. Moreover, opines were detected in root, stem, and leaf tissues of non-transgenic plants on which stem crown galls had been induced by pathogenic strains of *Agrobacterium*. These plants exuded opines from their roots as a component of their root exudates. Translocation of opines from the tumor to other parts of the plant, and their exudation from roots, indicates that these biologically active compounds are available to opine-catabolizing microbes that have not induced the tumors but are present in the rhizosphere or on portions of the plant distant from the site of the gall.

Additional keywords: agrobacteria, opine degradation, opine synthesis, rhizosphere.

Agrobacterium tumefaciens is a member of the family Rhizobiaceae and the causal agent of crown gall (Smith and Townsend 1907). This disease state is characterized by uncontrolled plant cell proliferation at the infection site that results in formation of a neoplastic tissue, i. e., the crown gall tumor. After the induction of a tumor by the pathogenic *Agrobacterium* the tumor cells continue to proliferate in the absence of bacteria (Braun 1947, 1958). A region of the *A. tumefaciens* tumor-inducing (Ti) plasmid called the transfer-DNA (T-DNA) is transferred into plant cells were it becomes integrated into the nuclear genome during infection (Ream 1989; Hooykaas and Beijersbergen 1994). Some T-DNA genes encode enzymes that, in the plant cell, catalyze the synthesis of plant hormones responsible for the tumorous phenotype (Ream 1989). Other T-DNA genes encode enzymes for synthesis of novel compounds called opines (Petit et al. 1978; Dessaux et al. 1993).

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More than 20 different opines have been described, comprising several structural groups (Dessaux et al. 1993). Most opines are condensation products of amino acids with hexose sugars or with carbonyl compounds (Dessaux et al. 1993). These compounds, which are rich in carbon and nitrogen, accumulate in the tumors but cannot be utilized by the plant and therefore represent a diversion of photosynthate carbon and fixed nitrogen to a pool that is of no apparent value to the host (Petit et al. 1978; Dessaux et al. 1993). However, the inducing agrobacteria can catabolize the opines exuded by the neoplastic tissues. Ti plasmids encode the enzymes required for the uptake and degradation by the bacterium of specific sets of opines, the production of which is encoded by their T-DNA segments (Dessaux et al. 1993; Petit et al. 1983). Thus, the virulence plasmids of pathogenic *Agrobacterium* can be characterized by the opines present in transformed cells, and the opines degraded by the tumor-inducing bacterium (Dessaux et al. 1993). These observations led to the formulation of the "opine concept" which states that "opines are substances synthesized by the cells of the host-plant in response to a stimulus of the pathogen. Their presence creates favorable environmental conditions for the pathogen and contributes to its dissemination" (Tempé and Petit 1983). The opine concept, initially developed based on the Ti plasmid, was extended to include root-inducing (Ri) plasmids resident in *A. rhizogenes* (Petit et al. 1983).

Over the last decade several workers have shown that utilization of opines is not limited to *Agrobacterium*. Interestingly, certain soil isolates of pseudomonads and Gram-positive coryneforms can utilize opines as a sole source of carbon and energy (Beaulieu et al. 1983, Tremblay et al. 1987). This poses the question whether opines produced at the site of infection have the potential to affect bacterial growth at other locations in or around the plants. We took two approaches to determine whether these compounds could move through the plant and into the soil.

Transgenic *Nicotiana tabacum* plants that express the three mannityl opine biosynthetic genes (*mas0*, *mas1*, and *mas2*) of the *A. tumefaciens* T-DNA accumulate the four members of the mannityl opine family, mannopinic acid (MOA), mannopine (MOP), agropinic acid (AGA), and agropine (AGR) in

all tissues (Savka and Farrand 1992). We used grafting techniques to determine if opines produced in one part of the plant are translocated to the rest of the plant. Mannityl opine-producing plants (*N. tabacum* L. cv. Xanthi nc) of homozygous line 2-26 (Savka and Farrand 1992) and wild-type *N. tabacum* L. cv. Havana 425 (H425) can be distinguished by leaf morphology and plant form. A complementary pair of grafts containing an opine-producing scion on wild-type rootstock (2-26/H425) and a wild type scion on opine-producing rootstock (H425/2-26) were constructed using 10-week-old plants (Fig. 1A). Shoot meristems of approximately 10 cm length and 1.5 cm diameter were cut to a wedge point at the basal end and these scions were grafted to stems of rootstock plants prepared in a similar manner. The graft junction was sealed with grafting tape, and the entire plant was covered with a plastic bag. The plants were grown in reduced light at room temperature for 7 days, the plastic bags were removed, and the plants were transferred to the greenhouse.

Plants of each graft type, along with the parents, were grown in potting soil, and various tissues were examined for the presence of opines. Chimeric grafts as well as transgenic and nontransgenic plants were harvested at 2, 3, 4, 5, and 8 weeks after grafting and tissue extracts were prepared as previously described (Savka et al. 1990). Opines in the extracts were separated by high voltage paper electrophoresis (HVPE) on sheets of Whatman 3MM paper in pH 1.7 buffer as previously described (Savka et al. 1990). The mannityl opines were detected according to established methods (Savka et al. 1990). Spots appearing on the electrophoretogram were identified as opines by comparing their electrophoretic mobilities and staining properties with those of authentic standards. Extracts prepared from tissues obtained from the wild-type and the transgenic parent plants were included on electrophoretograms as negative and positive controls. After 2 weeks, mannityl opines were detected in the nontransgenic leaves from H425/2-26 plants in amounts similar to those detected in the transgenic 2-26 plant line (Fig. 1B). Opine levels did not vary significantly through the 8 weeks of growth (data not shown). Opines also were detected in the nontransgenic roots of the 2-26/H425 plants after 2, 3, 4, 5, and 8 weeks of growth. The levels of opines that accumulated in the roots of this graft type were similar irrespective of the graft age and were much lower than the levels of opines detected in the roots of the 2-26 line (Fig. 1C). The reduced levels of opines present in the normal roots may be due to the carbon translocation stream being preferentially directed to the more extensively growing shoots.

The observation that opines can be translocated from the site of synthesis to other parts of the plant raised the question as to whether plants with crown galls contain these compounds in tissues distal to the site of the tumor. To investigate this, we induced tumors on stems of *Kalanchoe diagremontiana* using *A. tumefaciens* strain 15955, which produces tumors containing mannityl opines, and strain C58, which produces tumors containing nopaline (Dessaux et al. 1993). The stems of greenhouse-grown 6-week-old *K. diagremontiana* plants were inoculated at a position about 10 cm above the soil line with *A. tumefaciens* strains 15955 or C58 using sterile wooden dowels (0.2 × 14 cm) previously dipped into overnight L broth cultures of one or the other bacterium. Other plants were inoculated with the avirulent strain NT1 (Watson et al. 1975) or with an uncontaminated sterile dowel dipped in sterile L broth. The inoculated plants were grown in the greenhouse for 12 weeks. At this time the tumors were well developed (2 to 4 cm in diameter). Tissue samples were taken from six plants of each treatment, extracts were prepared (Savka et al. 1990), and the mannityl opines were separated by HVPE and detected by staining as described above. Nopaline was separated and detected according to established methods (Yang et al. 1987). The presence of spots on HVPEs corresponding to synthetic standards and extracts from tumors were considered positive indicators for the presence of the opine in tissue extract samples. At least three members of the mannityl opine family were detected in representative leaf, root, and stem tissue samples assayed from six plants bearing galls induced by 15955. Similarly, nopaline was detected in leaf, root, and stem tissue samples taken from plants with galls induced by C58. Plants inoculated with the avirulent *Agrobacterium* strain NT1, or plants mock-inoculated with sterile L broth failed to develop galls, and tissues from

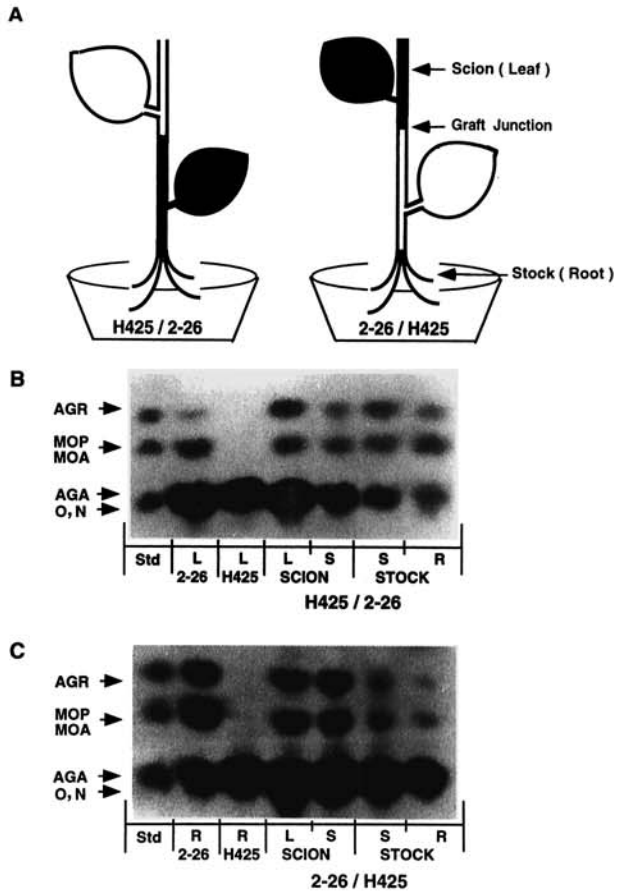

Fig. 1. Translocation of opines in chimeric grafted plants. **A,** Diagram of the chimeric grafts using H425 ❑ and 2-26 ■ plants. **B,** Presence of opines in tissues of H425/2-26 grafted plants. **C,** Presence of opines in tissues of 2-26/H425 grafted plants. Lanes in B and C contain extracts prepared from leaf (L), stem (S), or roots (R) of parent plants (2-26 or H425) or of the grafts. Standards (Std) represent 2 µl of a stock containing 2 mmoles/ml of each of the four mannityl opines, agropinic acid (AGA), mannopine (MOP), mannopinic acid (MOA), and agropine (AGR). At pH 1.7, MOP and MOA coelectrophorese and AGA remains at the origin (0). Thus, AGA is obscured by the electrophoretically neutral $AgNO_3$-positive compounds (N) present in normal and transgenic tissues.

these plants did not contain detectable levels of either opine type (Table 1). These results show that opines are translocated from the site of synthesis, the crown gall tumor, to other portions, the leaves, stems and roots, of the host plant.

When grown under autotrophic conditions the transgenic tobacco line 2-26 exudes large quantities of mannityl opines from its roots (Savka and Farrand 1992). Since opines are translocated from galls to nontransformed tissues of the plant, we asked whether these metabolites produced by stem tumors are exuded from the roots of galled, but otherwise normal plants. *N. tabacum* L. cv. Xanthi nc plants were grown from seed for 3 weeks on MS tissue culture agar medium and subsequently inoculated with a hypodermic needle dipped into an overnight culture of *A. tumefaciens* strain 15955, or the avirulent strain, NT1. After an additional 3 weeks of growth, the plants were transferred to separate test tubes (25 × 95 mm) containing MS liquid medium supplemented with Timentin (200 mg/L; Smithkline Beecham) to prevent the growth of *Agrobacterium* in the hydroponic medium. Timentin is a mixture of the β-lactam antibiotic ticarcillin and the β-lactamase inhibitor potassium clavulanate. Ungalled normal plants and the transgenic opine-producing line 2-26 were grown in a similar manner. The root systems of the plants were supported on sterile cheesecloth such that only the roots were submerged in the medium while the tumor remained well above the meniscus. Samples of the hydroponic fluids were collected after an additional 8 weeks of growth. The samples were concentrated 10-fold in a Savant Speed Vac and 10 µl volumes of the concentrated fluids were analyzed by HVPE as described above. Mannityl opines were detected in the hydroponic fluids in which the root systems of tobacco plants galled by strain 15955 had been immersed during the course of the experiment (Fig. 2). The levels of opines detected in this culture medium were similar to those observed in hydroponic root growth supernatants from the transgenic 2-26 plant line. The mannityl opines were detected in hydroponic fluids from all eight galled plants tested. Hydroponic fluids in which ungalled plants were grown did not contain detectable levels of the mannityl opines. These results indicate that opines are efficiently translocated from the crown gall tumors to the roots where they are released as a component of the root exudate.

The imino diacid opines, octopine and nopaline, are detectable at very low levels in wild-type cultured tissues of soybean, tobacco, and cotton grown on media containing 100 mM arginine (Christou et al. 1986). The mannityl opines, however, have not been detected in tissues from normal plants uninfected by *Agrobacterium* under any conditions. In our study, the chimeric grafts were not stressed any more than non-grafted wild-type H425 plants at the time tissues were harvested for opine analysis. Thus it is unlikely that the detection of opines in chimeric grafts and galled plants could be stress related.

Although agrobacteria generally remain in the region of the primary infection (Hill 1928; Tarbah and Goodman 1987), there are reports of systemic movement and of secondary tumors arising at sites distal from the primary galls (Braun 1941, de Cleene and de Ley 1976). While we never observed such secondary galls on our infected plants, it is possible that the opines detected in tissues distal from the tumor could have been produced by small foci of infections by agrobacteria that had migrated from the primary infection site. We find this unlikely considering that opines were present in all tissue types tested of the galled plants. Moreover, the opines in the nontransgenic tissues of our grafted plants could not have been produced at secondary transformation sites because the transgenic plants used to construct the grafts were grown from microbiologically sterile seed stock and are free of *Agrobacterium*.

The opine concept proposes the bacteriocentric notion that the inducing agrobacteria engineer the plant to produce opines, thereby gaining an advantage for growth in the opine-rich environment provided by the tumor (Petit et al. 1978; Dessaux et al. 1993). Recent work supports this hypothesis (Guyon et al. 1993; Farrand et al. 1994; Wilson et al. 1995). Opines exuded from the roots and leaves of transgenic plants provided a competitive advantage to opine-catabolizing bacteria colonizing these organs (Farrand et al. 1994, Wilson et al. 1995). The results presented here suggest that the sphere of influence exerted by the opines extends beyond the tumor. We propose that in nature, the opines produced by crown gall tumors are translocated to other portions of the galled plant and can provide a resource to opine-catabolizing microorganisms colonizing the rhizosphere and other sites of the plant distal from the tumor.

It also is possible that the opines themselves might be beneficial to the plant. Opines present in leaves of regenerated

Table 1. Presence of opines in tissues of galled plants[a]

Inoculation treatment	Tissue type				
	R	SB	G	SA	L
NT1	–	–	nt	–	–
15955	+	+	+	+	+
C58	+	+	+	+	+
L broth	–	–	nt	–	–

[a] Analysis of tissue extracts prepared from root (R), gall (G), stem above (SA) or below (SB) the inoculation site, and leaf (L) from plants inoculated with strains 15955 or C58 bearing a stem gall (G). For ungalled plants inoculated with NT1 or with L broth, SB and SA samples were obtained from the crown of the plant and from the third internode, respectively. Abbreviations are: nt, no tumor present; +, presence of opine; –, absence of opine.

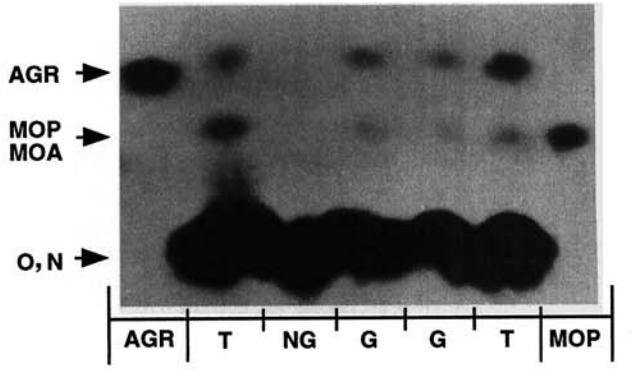

Fig. 2. Opines are exuded by roots of galled plants. Lanes contain hydroponic supernatants in which had been grown a normal plant (NG), two different normal plants galled on their stems with *Agrobacterium tumefaciens* 15955 (G), and the opine-producing transgenic plant line, 2-26 (T). Other abbreviations and concentrations of standards are as described in Figure 1.

hairy root plants are antagonistic to herbivorous lepidopteran insect larvae (Sauerwein and Wink 1993). Moreover, these opine-producing plants exhibited allelopathy against weed seeds (Sauerwein and Wink 1993). Given that our results demonstrate that opines produced by galls can translocate to other parts of the plant, we suggest that the opines can be of value to the galled plant. By virtue of the translocation and exudation of bioactive opines, the *Agrobacterium*-plant interaction could well be a form of mutualism rather than parasitism. In this scenario, the opines produced by crown gall tumors may be more important to both partners of the *Agrobacterium*-plant association than has been previously recognized. Given a mutual benefit from the synthesis, exudation and translocation of opines, we propose that the opine concept be extended to include the plant host.

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