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Agrobacterium tumefaciens can Obtain Sulfur from an Opine that is Synthesized by Octopine Synthase Using S-methylmethionine as a Substrate

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Summary

Agrobacterium tumefaciens incites plant tumors that produce nutrients called opines, which are utilized by the bacteria during host colonization. Various opines provide sources of carbon, nitrogen, and phosphorous, but virtually nothing was previously known about how *A. tumefaciens* acquires sulfur during colonization. Some strains encode an operon required for the catabolism of the opine octopine. This operon contains a gene, *msh*, that is predicted to direct the conversion of S-methylmethionine (SMM) and homocysteine (HCys) to two equivalents of methionine. Purified Msh carried out this reaction, suggesting that SMM could be an intermediate in opine catabolism. Purified octopine synthase (Ocs, normally expressed in plant tumors) utilized SMM and pyruvate to produce a novel opine, designated sulfonopine, whose catabolism by the bacteria would regenerate SMM. Sulfonopine was produced by tobacco and *Arabidopsis* when colonized by *A. tumefaciens* and was utilized as sole source of sulfur by *A. tumefaciens.* Purified Ocs also used thirteen other proteogenic and nonproteogenic amino acids as substrates, including three that contain sulfur. Sulfonopine and eleven other opines were tested for induction of octopine catabolic operon and all were able to do so. This is the first study of the acquisition of sulfur, an essential element, by this pathogen.

Keywords

S-methylmethionine; Sulfonopine; Agrobacterium; Opines

Introduction

Agrobacterium tumefaciens provides fascinating examples of the chemical ecology that underlies the interactions between plant-associated bacteria and their hosts (Brencic & Winans, 2005, Zhu *et al.*, 2000, Ranocha *et al.*, 2001). This bacterium uses a conjugation-like machinery to transfer discrete fragments of oncogenic DNA from the tumor-inducing (Ti) plasmid to the nuclei of host plants (Alvarez-Martinez & Christie, 2009, Gelvin, 2009, Tzfira & Citovsky, 2006). These DNA fragments are integrated into the host genome, and genes encoded within them are expressed in the plant nuclei. Several transferred genes direct the production of phytohormones such as auxin and cytokinin, which can lead to uncontrolled cell proliferation, producing a crown gall tumor (Kamada-Nobusada & Sakakibara, 2009, Tzfira & Citovsky, 2006). Other transferred genes direct the production of novel compounds called opines, which serve as nutrients for the colonizing bacteria (Savka *et al.*, 2002, White & Winans, 2007).

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Different strains of *Agrobacterium* collectively can direct plant tumors to synthesize a bewildering array of opines that can serve as sources of nitrogen, carbon or phosphorous (Dessaux *et al.*, 1998). First, different strains transfer diverse types of opine biosynthetic genes. Second, each strain generally transfers more than one opine biosynthetic gene. Third, particular opine biosynthetic enzymes can sometimes utilize a variety of substrates. For example, *A. tumefaciens* strains that carry a so-called octopine type Ti plasmid, such as pTiA6, pTiB6, pTi15955, pTiR10, or pTiAch5, transfer the opine biosynthetic genes *ocs, ags, mas1,* and *mas2*. Ocs can reductively conjugate pyruvate with several different amino acids, including arginine, lysine, ornithine, histidine, methionine, and glutamine, synthesizing the corresponding opines (Menage & Morel, 1964, Menage & Morel, 1965, Biemann *et al.*, 1960, Hack & Kemp, 1980, Otten *et al.*, 1977). Five of these are utilized as sources of carbon and nitrogen, while the compound containing methionine was described as a pseudo-opine, as it was not utilized by the bacterium (Firmin *et al.*, 1985). Mas1 and Mas2 conjugate mannose with glutamine, forming mannopine, which can be lactonized by Ags to form agropine or can spontaneously lactonize to form agropinic acid (Hong *et al.*, 1997, Dessaux *et al.*, 1988, Dessaux *et al.*, 1986).

Opine catabolic operons are generally located on non-transferred portions of the Ti plasmids, and are transcriptionally induced by the opines whose catabolism they direct (Dessaux et al., 1998). These operons invariably include genes that encode ABC-type uptake systems and one or more genes that direct opine catabolism. For example, the octopine catabolism *(occ)* operon of octopine-type Ti plasmids contains 15 genes (Fig. 1A), and is activated by the product of the divergent *occR* gene in response to octopine and similar opines (Wang *et al.*, 1992, Habeeb *et al.*, 1991, von Lintig *et al.*, 1991). This operon contains one confirmed (Valdivia *et al.*, 1991) and one putative ABC-type permease (Fig. 1A) and six genes known or suspected to be involved in opine catabolism. One such catabolic gene, *msh*, is described below. The last gene in the operon is *traR*, which encodes a LuxR-type transcription factor that directs the transcription of Ti plasmid vegetative replication and conjugative transfer genes (Fuqua & Winans, 1996a, Piper *et al.*, 1999). TraR functions only in the presence of 3-oxooctanoylhomoserine lactone (OOHL), whose synthesis is directed by TraI, also encoded on Ti plasmids (Fuqua & Winans, 1994, Piper *et al.*, 1993). TraR expression requires expression of the *occ* operon (Fuqua & Winans, 1996b), and quorum sensing therefore occurs only within or near colonized plants, as they are the only natural source of these opines in the rhizosphere.

The *msh* gene encodes a protein having a limited similarity to proteins involved in methionine biosynthesis, hence the mnemonic methionine synthase homolog (Fuqua & Winans, 1996b). However, this homology appeared to be limited to the amino and carboxyl termini. In this study, we describe an end-to-end homolog of Msh, and confirm that Msh, like its homolog, is a methyltransferase that converts S-methylmethionine (SMM) and homocysteine (HCys) to methionine. We also show that a T-DNA encoded protein, octopine synthase (Ocs), can provide a substrate for Msh by synthesizing a novel opine containing SMM, and can also utilize a broad variety of other amino acids, including three that contain sulfur. The ability of Ocs to derivatize one or more sulfur-containing amino acids provides a new perspective on nutrient acquisition during plant colonization.

Results

Msh converts SMM and HCys to methionine

As described above, the predicted Msh protein was previously found to have a limited sequence similarity to a family of proteins involved in methionine biosynthesis. Simply by decreasing the gap penalty of the BLAST algorithm (Altschul *et al.*, 1990), we detected an end-to-end sequence similarity between Msh and the MmuM protein of *E. coli* (Fig. S1).

MmuM transfers a methyl group from SMM to HCys to yield two molecules of methionine (Met) (Thanbichler *et al.*, 1999, Neuhierl *et al.*, 1999). If Msh were to carry out a similar reaction, it would suggest that SMM and/or HCys might be an intermediate in the catabolism of a previously undescribed opine. We hypothesized that one or both of these hypothetical new opines could be synthesized in crown gall tumors by Ocs (Fig. 1B), and could be converted back to the corresponding amino acids by octopine oxidase (Fig. 1B). These amino acids could then be converted by Msh to Met.

To test these ideas, we wanted first to determine whether Msh could convert SMM and HCys to two equivalents of Met. We purified recombinant His_6-Msh and quantitated reactants and products by electrospray ta ndem mass spectrometry (ESI-MS/MS). As predicted, Msh used SMM and HCys as substrates to produce methionine (Fig. 1C). Msh also carried out similar reactions with lower efficiency with three other methyl donors in place of SMM (Table 1). These properties are similar to those of MmuM (Thanbichler et al., 1999, Neuhierl et al., 1999), suggesting that these two enzymes are functionally similar.

Octopine synthase utilizes SMM and HCys as substrates

The model proposed above predicts that SMM and/or HCys could be derived from hypothetical opines, possibly synthesized by the Ocs protein that is expressed within crown gall tumors. In order to purify Ocs, we constructed a gene encoding an MBP-His_6-Ocs fusion protein that contains a cleavage site for TVMV protease between MBP and the His_6 tag. This fusion was co-expressed with TVMV protease to remove the MBP portion of the fusion (Donnelly *et al.*, 2006). We added an additional plasmid that overexpresses the chaperone proteins GroESL, as this caused a significant increase in His_6-Ocs solubility (data not shown). His_6-Ocs protein was purified to apparent homogeneity by sequential immobilized nickel affinity and gel filtration chromatography.

Enzymatic reactions were prepared containing Ocs, pyruvate, NADPH, and either SMM or arginine (Arg), and the reaction was monitored by using UV spectroscopy to detect the conversion of NADPH to $NADP^+$. Both amino acids stimulated NADPH oxidation (Table 2). We confirmed using ESI-MS/MS and NMR that reactions containing arginine produced octopine (N-(*R*-1-carboxyethyl)-arginine), while reactions containing SMM produced a novel compound, N-(*R*-1-carboxyethyl)-S-methyl-*S*-methionine (Fig. S2, S3B, S4) which we designate sulfonopine. The enzymatic efficiency (k_{cat}/k_m) for SMM was similar to that for arginine, indicating that Ocs utilized SMM with high efficiency. Similar reactions containing homocysteine produced yet another novel compound, (N-(*R*-1-carboxyethyl)-*S*-homocysteine, which in principle could be converted by octopine oxidase to homocysteine and pyruvate. The homocysteine thus produced could provide the second substrate for Msh.

To better understand Ocs substrate specificity, we compared all 20 proteinogenic amino acids as well as several nonproteinogenic ones. We also tested several α-keto acids for the ability to replace pyruvate in this reaction, and compared the utilization of NADPH and NADH. We confirmed that the enzyme can utilize lysine, ornithine, histidine, methionine, and glutamine. It also efficiently utilized canavanine, homoserine, cysteine, glycine, and homoarginine. Of these, histidine and canavanine were utilized even more efficiently than arginine. Ocs can also utilize other amino acids at reduced efficiencies (Table 2). The enzyme did not detectably utilize aromatic or acidic amino acids, nor did it utilize proline, carnosine, or any of three tested D-amino acids. The enzyme utilized α-ketobutyrate, oxaloacetate, and glyoxylate in place of pyruvate, although significantly less efficiently (Table 2). It did not detectably utilize α-ketoglutarate as a substrate. We confirmed an earlier report (Otten et al., 1977) that Ocs utilizes NADPH slightly more efficiently than NADH (data not shown).

Homogenates and exudates of plants colonized by *A. tumefaciens* contain sulfonopine

We set out to determine whether tobacco seedlings can synthesize and exude sulfur-containing opines when colonized by *A. tumefaciens*. To address this, tobacco seedlings were cultured hydroponically and inoculated using four different strains: R10 (wild type), KYC55 (which lacks a Ti plasmid), R10 *ΔvirD4* (which cannot transfer T-DNA to plant cells), and R10 *Δocs* (which fails to produce octopine-type opines). After three days of cocultivation, the bacteria were killed by addition of carbenicillin and the seedlings were suspended in water. Two weeks later the water and seedling tissues were assayed by ESI-MS/MS for sulfur-containing amino acids and sulfur-containing opines.

The amino acid SMM and the corresponding opine, sulfonopine, were readily detected in tobacco exudates and homogenates (Table 3, Fig. S3D–E). We also looked for the presence of three other sulfur-containing amino acids, homocysteine, methionine, and cysteine, and their corresponding opines, but did not detect them (data not shown). Sulfonopine was not detected in mock-infected seedlings, nor in seedlings infected with a strain lacking a Ti plasmid, nor in seedlings infected with a strain lacking *virD4* or *ocs* (Table 3). These results show that Ocs, when expressed in plant cells, can direct sulfonopine production, and that this opine is released to the rhizosphere, where it could be consumed by Agrobacteria (Savka *et al.*, 1996). It is plausible that transformed plants can also synthesize some or all of the opines that we detected with the purified enzyme, but that the substrates and products were not sufficiently abundant to be detected using our methods.

We wanted to know whether the production of sulfonopine by plants is dependent on SMM. We therefore used *Arabidopsis thaliana* wild type (Col-0), and two mutant lines, one of which (*hmt2*-2) overexpresses SMM, while the other (*mmt*) does not synthesize SMM (Lee *et al.*, 2008). Sulfonopine was produced by infected Col-0 and was produced at higher levels by infected *hmt2*-2 plants, but was not produced by infected *mmt* plants (Table 4, Fig. S3C). The concentration of SMM in the host therefore influences the production and accumulation of sulfonopine, consistent with SMM being a direct substrate for Ocs.

Sulfonopine induces transcription of the octopine catabolism operon

At least some octopine-type opines induce the transcription of the *occ* operon via the LysR-type regulator OccR (Habeeb et al., 1991). To investigate whether the octopine-type opines found in exudates and extracts from infected-tobacco seedlings were able to induce transcription of the *occ* operon, we used the strain KYC16, which harbors an octopine-inducible *ooxA-uidA* fusion made by transposition of Tn5*gus*A7 (Cho *et al.*, 1996). KYC16 was cultured in the presence of exudates or extracts from the infected-tobacco seedlings. Gus activity was elevated by exudates and extracts of tobacco seedlings infected with *A. tumefaciens* R10 (Fig. S5A–B).

We tested sulfonopine and ten other enzymatically synthesized opines (Table 2), including two that contain sulfur (derived from methionine or cysteine) for induction of the *ooxA-uidA* fusion. We used a commercial source of octopine as a positive control and the corresponding amino acids as negative controls. Each of these opines activated the fusion (Fig. 2A), while the corresponding amino acids did not (Fig. S5C).

To examine whether OccR is required for induction, we cultured two congenic strains in the presence or absence of sulfonopine, and four other opines. Strains KYC1203(pKY148)(pRJM101) and KYC1203(pKY148) have a plasmid-borne *occQ-lacZ* fusion, and the former strain expresses OccR, while the latter strain does not. The former strain was induced by all five opines (Fig. 2B), while the latter strain was uninduced (Fig. S6A), indicating that OccR is required for detection of these opines.

A. tumefaciens can utilize sulfonopine as sole source of sulfur

We next determined whether *A. tumefaciens* is able to utilize sulfonopine as a source of sulfur. For this experiment, *A. tumefaciens* strains R10 and KYC55 (Ti-plasmid less) were cultured in the presence or absence of sulfonopine, SMM, or methionine as sole sulfur sources. All other essential nutrients were provided. Strain R10 utilized all three compounds (Fig. 3A and 3B). Unexpectedly, strain KYC55 also grew at the expense of any of these three compounds. This indicates that the *occ* operon is dispensable for the use of sulfonopine as a sulfur source.

Discussion

In *E. coli*, there are three known pathways for methionine biosynthesis; all involve methylation of homocysteine, but differ in the source of the methyl group. The MetE protein transfers the methyl group directly from N5-methyl-tetrahydrofolate (Gonzalez *et al.*, 1996). MetH transfers the methyl group from N5-methyl-tetrahydrofolate to a cobalamine coenzyme and from there to homocysteine (Frasca *et al.*, 1988). The third methionine synthase, MmuM, transfers the methyl group from SMM, converting it to a second molecule of methionine (Thanbichler et al., 1999). The Msh protein of *A. tumefaciens* has limited sequence and structural homology with MetE and MetH, restricted to their homocysteine binding domains (Evans *et al.*, 2004). In contrast, Msh has end-to-end sequence similarity with MmuM of *E. coli*. Msh showed a strong preference for SMM over three other methyl donors (Table 1), much like five other SMM-homocysteine methyltransferases found in *E. coli*, *Arabidopsis*, *S. cerevisiae*, and humans (Thanbichler et al., 1999, Ranocha *et al.*, 2000, Thomas *et al.*, 2000, Szegedi *et al.*, 2008).

SMM is a ubiquitous metabolite in many or possibly all plants, although it is not essential in *Arabidopsis* (Lee et al., 2008). The methylation of methionine creates a positive charge on the sulfur atom, causing a net positive charge and enhancing its solubility. Perhaps for this reason, SMM is the transported form of methionine, and is concentrated in floral tissues (Ranocha et al., 2001). It should perhaps not be surprising that SMM could be a substrate for Ocs, as Ocs can utilize a rather broad variety of amino acids, especially the positively charged amino acids arginine, lysine, ornithine and histidine, as well as the non-charged amino acids glutamine and methionine (Chilton *et al.*, 2001, Bates *et al.*, 1984, Menage & Morel, 1964, Hack & Kemp, 1977, Biemann et al., 1960).

All previous studies of Ocs were done using enzyme that was purified from crown gall tumors (Hack & Kemp, 1980, Otten et al., 1977). In the present study, we produced a highly purified recombinant Ocs, which was stable and provided highly reproducible kinetic data for different substrates. This enzyme utilizes a surprising variety of proteogenic and nonproteogenic substrates, and several different alpha-keto acids, as well as NADH or NADPH. Many of the nonproteinogenic substrates are found in plants, including canavanine, homocysteine, homoserine, homoarginine, and selenomethionine (Bell, 2003). From an evolutionary perspective, it would seem advantageous for *A. tumefaciens* to utilize such a wide array of compounds as substrates, using either NADH or NADPH, and to derivatize them in such a way that they are unavailable to most other bacteria or presumably to plants.

Among the new sulfur-containing compounds synthesized by Ocs, only sulfonopine was detected in plant exudates and homogenates, probably because its precursor, SMM, was far more abundant than homocysteine, methionine, and cysteine. However, Ocs efficiently utilized all four amino acids *in vitro*, suggesting that all four could be potential sources of sulfur. Of these, the methionine-containing compound was previously described as a pseudo-opine, in that it was found to be produced by crown gall tumors but not imported by

A. tumefaciens (Firmin et al., 1985). However, we showed that this compound induces expression of the *occ* operon via OccR. OccR is a cytoplasmic opine-responsive transcription factor of the LysR family. This indicates that the methionine-containing molecule can be internalized. Of the eleven compounds tested for induction of the *occ* operon, all were able to do so, suggesting that all can be internalized.

Production of sulfonopine by tobacco required transfer of *ocs* from the bacterium into plant cells, as a bacterial *vir* mutant or *ocs* mutant blocked sulfonopine production. Sulfonopine was exuded from tobacco, however, we do not know how sulfonopine or other opines are released from plant cells. An older report suggested that the *ons* gene facilitated opine export (Messens *et al.*, 1985), but Ons is now generally thought to be a member of the RolB-RolC family, whose members regulate the abundance or activity of phytohormones (Bulgakov, 2008). Therefore, any effect of Ons on opine export would probably be indirect. The export of opines from transformed plant cells is therefore an unanswered question. However, the roots of many nontransformed plants exude surprisingly high levels of amino acids and carbohydrates (Phillips *et al.*, 2004), suggesting that opine secretion may not require T-DNA encoded proteins.

Given the abundance of SMM in plants and the fact that it contains sulfur, an essential nutrient, it should not be surprising that at least some strains of *A. tumefaciens* can utilize it to form a sulfur-containing opine. We have not tested whether nopaline-type Ti plasmids can direct plant tumors to make a similar opine, but it seems quite plausible that nopaline synthase could conjugate SMM with α-ketoglutarate (rather than pyruvate) to create a compound similar to sulfonopine.

Opines have long been known to induce genes required for their uptake and catabolism (von Lintig et al., 1991, Klapwijk *et al.*, 1978, Montoya *et al.*, 1977). Octopine has been reported to activate the transcription of the *occ* operon by binding to OccR. (Habeeb et al., 1991). In the present study, we found that all tested opines can activate the transcription of the *occ* operon and that for all five opines tested, OccR was required for induction. These findings suggest that OccR detects primarily the amino acid backbone and pyruvate moieties of these opines, and has little or no specificity for their amino acid side chains.

Our finding that the utilization of sulfonopine did not require the Ti plasmid was somewhat counterintuitive. Apparently the uptake and utilization of these compounds can be directed by other genes of the rather large (5.6 megabase) genome of this organism. Previous reports have shown that the first four genes of the *occ* operon direct octopine uptake into *E. coli* (Valdivia et al., 1991), but are not essential for its uptake into *A. tumefaciens* (Cho et al., 1996). Evidently, octopine uptake is encoded redundantly. The present study indicates that this is also true of sulfonopine uptake. However, utilization of octopine as a carbon or nitrogen source requires at least some genes of the *occ* operon (Cho et al., 1996). Our observations that sulfonopine can provide sulfur in the absence of *occ* indicates that its catabolism is encoded redundantly.

A. tumefaciens spp. are well known to have evolved an efficient mechanism to obtain nutrients from the plants they infect. Previously, opines have been described that can be used as a source of carbon, nitrogen or phosphorous. Here we show that also sulfur can be obtained by opine catabolism. This new opine, sulfonopine, represents a novel way to derivatize a source of sulfur, SMM, from the infected plant and make it available for colonizing Agrobacteria.

Experimental Procedures

Bacterial strains, plasmids, and oligonucleotides

The bacterial strains, plasmids and oligonucleotides used in this study are listed in Table S1 and S2, respectively. Luria Broth (LB) and Terrific Broth (TB) were used as complex media, while AT and AB media were used as defined media (Tartoff & Hobbs, 1987, Cangelosi *et al.*, 1991). AB medium. modified to lack sulfur, was used to test the ability of various opines and amino acids to provide sulfur. *E. coli* strains were grown at 37°C and *Agrobacterium tumefaciens* strains at 27°C. Octopine, amino acids, NADPH, NADH, and α-keto acids were purchased from Sigma-Aldrich. Sulfonopine was produced either by chemical synthesis or enzymatic synthesis and other opines were enzymatically synthesized (described below).

Chemical synthesis of SMM

L-SMM and D-SMM were synthesized using 1.5 g of L-methionine or D -methionine dissolved in 16 ml of 89% formic acid and 5 ml of acetic acid and combined with 2.5 ml of methyl iodide (Tuennies & Kolb, 1945). The reaction was incubated for 3 days at room temperature in the dark, and then evaporated to a syrup. Methanol (10 ml) was added to obtain granular particles, which were filtered and washed with methanol and acetone, and dissolved in 8 ml of warm 50% ethanol, and 25 ml of 100% ethanol was then added to allow crystallization in the dark. Crystals were filtered, washed and dried with acetone. Purity of L-SMM and D-SMM was determined using ESI-MS/MS and NMR.

Chemical synthesis of sulfonopine

The chemical synthesis of sulfonopine was done in two steps: 1) reductive condensation of methionine with pyruvate to yield N-(*R*-1-carboxyethyl)-*S*-methionine (methiopine), and 2) methylation of methiopine to yield sulfonopine. In the first step, 2 g of methionine methyl ester-HCl (Sigma-Aldrich) was combined with 10 ml of water and 1 M NaOH was added to reach pH 10.0 (~10 ml). The free base was extracted using chloroform and dried using Na_2SO_4. A solution of 35 ml of chloroform containing 1.5 g of methyl pyruvate and 4 g of sodium triacetoxyborohydride was added and incubated overnight under slight N_2 pressure at room temperature. Ten ml of $NaHCO_3$ solution (pH ~7.9), was then added and mixed until bubble formation ceased. The mixture was transferred to a separatory funnel, 20 ml of 1M NaOH was added, and extracted three times using 50 ml of CH_2Cl_2. The organic phases were combined and dried using MgSO4. The dried extract was dissolved in 3 ml of MeCN and then treated with 2 ml of water. After centrifugation, the supernatant was subjected to preparative HPLC using a 1×25 cm C-18 column (Phenomenex) and eluted with an acetonitrile-water gradient. Testing with ESI-MS/MS showed two major peaks to correspond to the two expected diastereomers of methiopine dimethyl ester. The pooled fractions of each diastereomer were evaporated to dryness, redissolved in 10 ml of 1M HCl, hydrolyzed by heating for 15 hours using an autoclave (120°C), and dried *in vacuo* to remove the HCl. The purity of each diastereomer was checked by ESI-MS/MS.

Conversion of the methiopine diastereomers to those of sulfonopine was carried out as for the methylation of methiopine to yield SMM (above). The purity of the sulfonopine diastereomers was checked by ESI-MS/MS (Fig. S3A) and NMR (Fig. S4). Only one of the diastereomers was found to have biological activity (Fig. S6B).

Enzymatic synthesis of opines

Octopine-type opines were produced using purified Ocs and glucose dehydrogenase, which regenerates NADPH from $NADP^+$ at the expense of glucose (Weckbecker & Hummel, 2005). Enzymatic assays were performed using 150 mM sodium pyruvate, 90 mM of the amino acid substrate, 10 mM NADPH, 150 mM glucose, 150 mM sodium phosphate (pH

6.6), 0.08 μM of His_6-Ocs and 2 units of glucose dehydrogenase. Reactions were incubated at 28°C for 24 hours. Enzymes were removed by methanol precipitation and filtration (Amicon Ultra, Millipore; molecular mass cut-off 3,000). The concentrations of reactants and products were determined using ESI-MS/MS.

DNA manipulation

Recombinant DNA techniques were performed using established procedures (Sambrook *et al.*, 1989). PCR amplification of genes was done using Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen). Plasmid DNA was isolated using QIAprep spin miniprep kits (Qiagen). DNA fragments generated by PCR or restriction digestion were gel purified using QIAquick Gel Extraction Kit (Qiagen). Restriction enzymes and other DNA modification reagents were purchased from New England Biolabs and used according to the methods described by the manufacturers. Plasmid DNA was introduced into *E. coli* and *A. tumefaciens* by electroporation (Cangelosi et al., 1991). Plasmids and bacterial strains are listed in Table S1, and primers are described in Table S2.

Plasmid construction

The *ocs* gene was PCR amplified by using primers ALFM28 and ALFM29, and inserted into pMCSG19 using ligation-independent cloning (LIC) (Donnelly et al., 2006), resulting in pAFM04, which encodes MBP-His_6-Ocs fusion. Between MBP and His_6- tag, there is a recognition site for TVMV protease. pAFM04 and pT7-*groESL* were electroporated into *E. coli* BL21/DE3(pRK1037) (pRK1037 encodes TVMV protease), so the MBP portion of the tripartite fusion was removed immediately after protein synthesis. Plasmid pT7-*groESL* was provided to enhance accumulation of soluble protein (Choi & Greenberg, 1991). The *msh* gene was subcloned by PCR amplification using primers ALFM21 and ALFM27 (Table S2) and pMCSGG19, resulting in pAFM11, which encodes a MBP-His_6-Msh fusion. This plasmid was electroporated into BL21/DE3(pRK1037)(pT7-*groESL*).

Mutagenesis of *ocs* and *virD4* by Campbell-type integration

Plasmids pAFM110 and pAFM111 are suicide plasmids containing 0.5 kb internal fragments of *ocs* and *virD4*, respectively. These fragments were PCR amplified using ALFM218 and ALFM219 (for *ocs*) and ALFM220 and ALFM221 (for *virD4*), and inserted between the *Kpn*I and *Bam*HI sites of the suicide plasmid pVIK107 (Kalogeraki & Winans, 1997). The resulting plasmids, pAFM110 and pAFM111, respectively, were transferred into strain R10 by conjugation (Kalogeraki & Winans, 1997), and transconjugants were selected using AB minimal agar plates containing 200 μg/ml of kanamycin. Since these plasmids cannot replicate in *A. tumefaciens*, they conferred antibiotic resistance by Campbell-type integration into *ocs* and *vir*D4, respectively, creating null mutations in each gene. Campbell-type integration was confirmed by PCR amplification.

Overproduction and purification of Ocs and Msh

To overproduce *Ocs, E. coli* strain BL21/DE3(pAFM04)(pRK1037)(pT7-*groESL*) was cultured at 37°C in 1 L of TB containing 400 μg/ml of ampicillin, 400 μg/ml of kanamycin, and 35 μg/ml of chloramphenicol until OD_{600} 0.6 was reached. The culture was cooled on ice to 28°C and overexpression of Ocs was induced by adding 0.3 mM IPTG. Incubation was continued at 28°C for 5 additional hours. Cells were concentrated by centrifugation for 10 min at 4°C. The pellet was suspended in lysis buffer (20 mM sodium phosphate buffer (pH 7.4), 200 mM NaCl, 20% glycerol, and 10 mM imidazole) and disrupted by passage through a French pressure cell (20,000 psi). The lysate was cleared by ultracentrifugation (25,000 × g at 4°C for 30 min). The supernatant was applied to Ni Sepharose™ 6 Fast Flow (GE Lifescience) chromatography resin. The column was washed extensively using lysis

buffer, and Ocs was eluted using lysis buffer supplemented with 250 mM imidazole. Fractions containing Ocs were combined and concentrated by using an Amicon Ultra cell with YM-30 filter membrane (30,000 MWCO; Millipore, Eschborn, Germany). During concentration the buffer was changed to 20 mM sodium phosphate buffer (pH 7.4), 200 mM NaCl, 20% glycerol and 1 mM DTT. Ocs was further purified by gel filtration chromatography using a Superdex 200 column (GE Lifescience). Peak fractions were pooled and concentrated as described above and dialyzed using a buffer containing 150 mM sodium phosphate buffer (pH 6.6), 50% glycerol, and 1 mM DTT. Protein purity was analyzed using 12% SDS-PAGE gels and visualized by Coomassie staining. Msh was purified according to the same techniques using strain BL21/DE3(pAFM11)(pRK1037)(pT7-groESL). After purification, Msh was dialyzed using a buffer containing 20 mM Tris (pH 7.9), 50% glycerol and 1 mM DTT.

Msh Activity Assays

Msh enzymatic reactions were carried out by using 0.61 μM His_6-Msh in 20 mM Tris buffer (pH 7.9), 10 mM homocysteine, and different concentrations of a variety of methyl donors such as SMM, SAM, methylcobalamine, betaine and, dimethylglycine. The reactions were stopped by adding an equal volume of 75% methanol and 0.25% formic acid solution and centrifuged for 30 min to precipitate Msh. The supernatants were analyzed for methionine production by ESI-MS/MS. Enzyme kinetics were calculated using the initial velocities for various concentrations of methyl donor.

Ocs Activity Assays

Enzymatic assays were performed by using 10 mM sodium pyruvate, 0.3 mM NADPH and different concentrations of amino acids in 150 mM PIPES (pH 6.6) and 0.08 μM of His_6-Ocs. The reaction was started by the addition of the amino acid to be tested and followed by measuring the oxidation of NADPH spectrophotometrically at 340 nm at room temperature by using a Synergy™ HT multi-detection microplate reader (Biotek Intruments). The concentrations of NADPH in solution were calculated on the basis of the molar absorption coefficient 6200 M^{-1} cm^{-1} at 340 nm. Production of opines in the enzymatic mixture was confirmed by ESI-MS/MS. Enzyme kinetics were calculated using the initial velocities. Initial velocities obtained from Msh and Ocs enzymatic reactions were used to calculate the kinetics constants by using nonlinear least square analysis of the data fitted to the Michaelis-Menten and Lineweaver-Burk rate equation using SIGMA PLOT 9.0 (Systat Software, Ekrath, Germany) and the enzyme kinetic module 2.0.

Plant cultivation and inoculation

Arabidopsis thaliana wild-type Col-O, and mutants *hmt2-2* and *mmt-2* (Lee et al., 2008) were obtained as seeds from G. Jander (Boyce Thompson Institute). *Nicotiana tabacum* seeds were obtained from A. Colmer (Cornell University). Seeds were surface-sterilized by soaking in 50% bleach and 0.1% SDS for 10 min, followed by extensive washing in sterile water. Seeds were transferred to sterile water in Petri dishes and incubated for 48 h at 4°C in the dark. *A. thaliana* sterilized seeds were transferred to trays containing sterilized soil. Plants were maintained at room temperature with exposure to natural and artificial lighting.

Prior to plant infection, *A. tumefaciens* strains were cultured overnight in AT medium. Cells were then washed with sterile water and suspended to OD_{600} of 0.5 in a solution containing 5% sucrose and 0.005% of Triton-X100. *A. thaliana* plants cultivated in soil were infected by the floral dip method (Zhang *et al.*, 2006). Plants were then cultivated for 2 weeks. Floral bolts were collected and homogenized using a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 5 min at 10,000× g at 4°C and the supernatant was evaporated to dryness under a vacuum. The resulting solid was resuspended in 3:1 (v/v)

methanol: 1% formic acid in water and centrifuged. The clear supernatant was analyzed by ESI-MS/MS.

Approximately 200 *N. tabacum* seeds were germinated in water supplemented with MS salts (Murashige, 1962). When the seedlings were 1–2 cm in length (approximately 2 weeks), they were submerged in the bacterial suspensions for 5 min, washed gently in 5% sucrose, and transferred to MS salts medium. Three days later, the infected seedlings were extensively washed with medium containing 300 μg per ml of carbenicillin, and incubated for 1 day in medium containing carbenicillin, then transferred to water and incubated for 2 weeks. Seedlings and water were collected for ESI-MS/MS analysis. Seedling extracts were obtained following the same protocol as for *A. thaliana* described above. Exudates were obtained by evaporating the water to dryness and resuspending in 3:1 (v/v) methanol: 1% formic acid in water for further ESI MS/MS analysis.

Electrospray mass-spectroscopy and nuclear magnetic resonance

Mass spectroscopy analysis was carried out using a Micromass Quattro II tandem MS operated in positive ion electrospray mode. Samples were injected directly using a syringe pump at a rate of 4 μl per min. Data acquisition and processing for the MS scans was controlled by the MassLynx software (Waters Corporation, Milford, MA). Twenty or more scans were averaged for each sample. When possible, spectra of all compounds were compared to those of commercial preparations of the same or similar compounds. NMR analysis was done by using a JEOL ECX-400 NMR spectrometer (Peabody, MA)

Utilization of sulfonopine and other sulfur source compounds

Sulfonopine, SMM, and methionine were tested as sources of sulfur by culturing strains in modified AB broth containing all essential nutrients except sulfur and supplemented with 1 mM of the tested compound as a sole sulfur source. Cultures were incubated at 28°C with vigorous aeration. Experiments were performed in triplicate using three different isolates for each strain.

Induction of the occ operon by opines

Opines were tested for induction of the *occ* operon using strain KYC16 (*ooxA-uidA*) cultured in AT medium containing 100 μg/ml of kanamycin at 27°C. β-glucuronidase specific activity was measured as described previously (Gallagher, 1992).

To determine whether OccR is responsible for induction, KYC1203(pKY148) and KYC1203(pKY148)(pRJM101) were cultured in AT medium supplemented with 1 mM of opines under vigorous aeration at 27°C to an OD_{600} of 0.3–0.4, and assayed for β-galactosidase specific activity (Miller, 1972).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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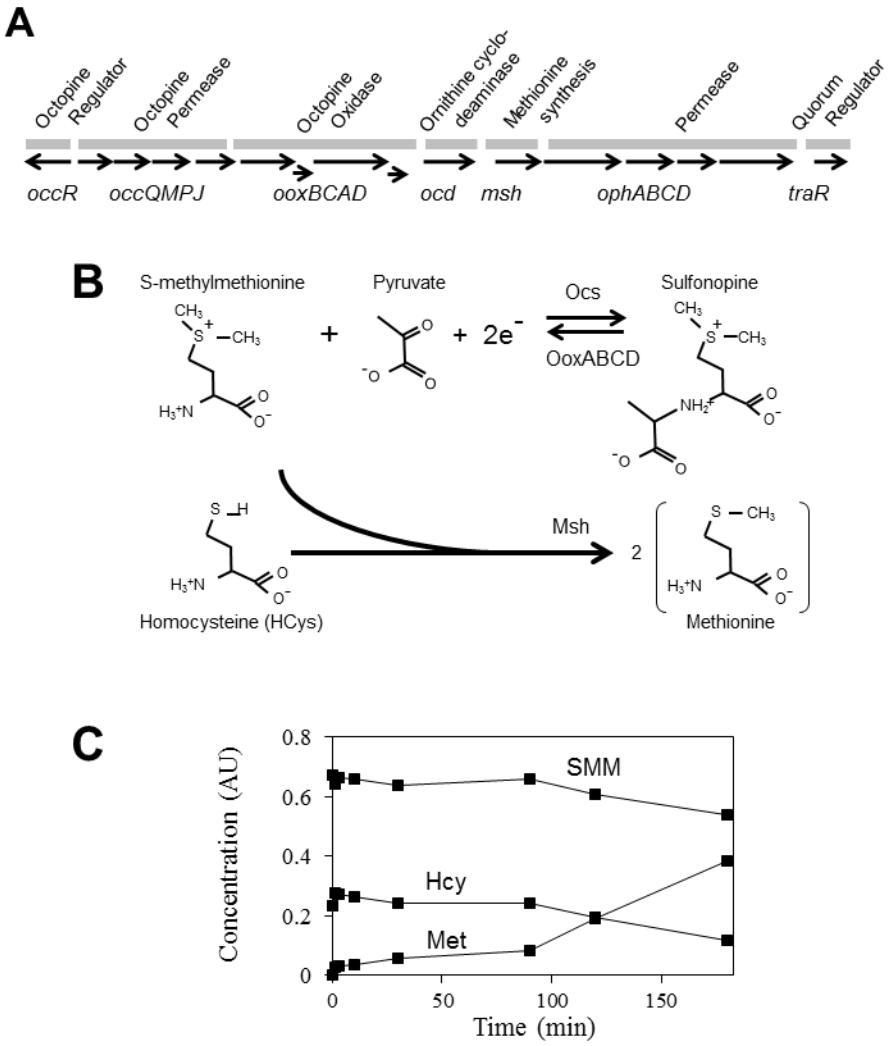

Fig. 1.

Proposed synthesis and catabolism of sulfonopine. (A) Genetic map of the octopine catabolic operon. The *occQMPJ* genes direct the uptake of octopine and probably related opines, while *ooxBCAD* direct the oxidative hydrolysis of octopine-type opines, yielding pyruvate and the corresponding amino acid. The *ooxC* and *ooxD* genes are provisional and based upon our DNA sequence analysis (data not shown). The *ocd* gene directs the conversion of ornithine to proline (Schindler *et al.*, 1989). The *msh* resembles *S*-methylmethionine-homocysteine methyltransferase of *E. coli*. The remaining five genes constitute an ABC-type permease for an unknown substrate, and the *traR* gene, which directs quorum-dependent Ti plasmid conjugation and vegetative replication. OccR positively regulates the operon in the presence of octopine-type opines. (B) Proposed synthesis of sulfonopine in plant cells by Ocs and its metabolism in bacterial cells by OoxABCD (top). SMM and HCys, undergo transmethylation by Msh, yielding Met (bottom). (C) Purified Msh can convert SMM and HCys to two equivalents of Met via a methyltransferase activity.

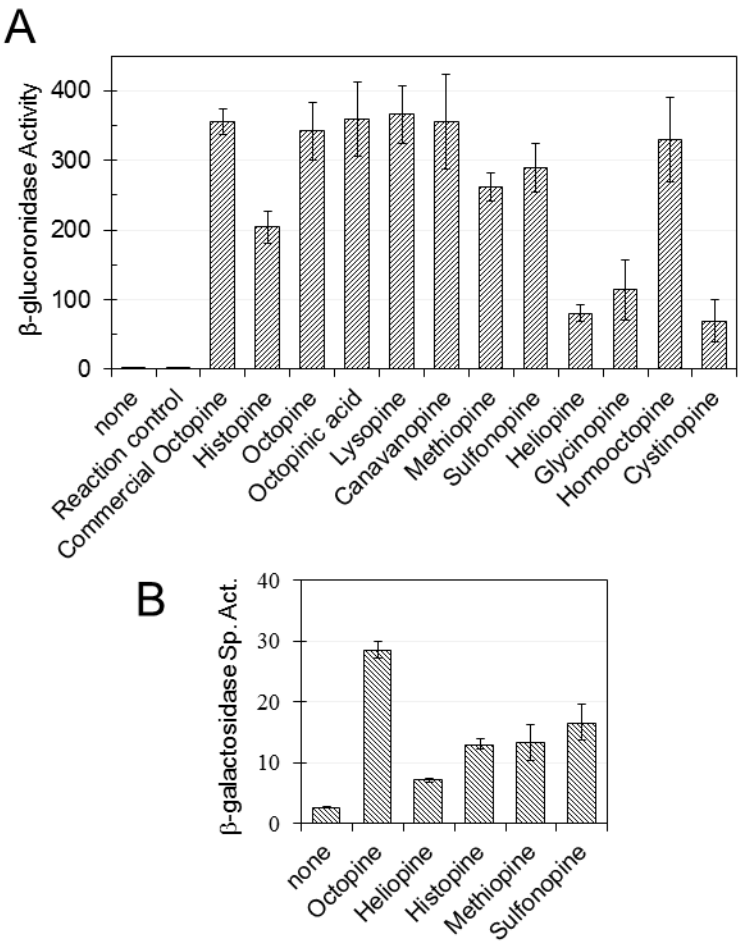

Fig. 2.

Induction of *occ* operon of strain KYC16 (R10 *ooxA-uidA*) by octopine-type opines. (A) Enzymatically produced opines induce expression of an *ooxA-uidA* fusion on the Ti plasmid. “Reaction control” refers to an enzymatic reaction mixture lacking any amino acid. “Commercial octopine” was purchased from Aldrich. (B) β-galactosidase activity of *A. tumefaciens* KYC1203(pKYC148)(pRM101) cultured in the presence of different opines. pKYC148 contains an *occQ-lacZ* translational fusion, while pRM101 contains a P*lac-occR* fusion. Histopine, N-(*R*-1-carboxyethyl)-Histidine; Octopinic acid, N-(*R*-1-carboxyethyl)-Ornithine; Lysopine, N-(*R*-1-carboxyethyl)-Lysine; Canavanopine, N-(*R*-1-carboxyethyl)-Canavanine; Methiopine, N-(*R*-1-carboxyethyl)-Methionine; Heliopine, N-(*R*-1-carboxyethyl)-Glutamine; Glycinopine, N-(*R*-1-carboxyethyl)-Glycine; Homooctopine, N-(*R*-1-carboxyethyl)-Homoarginine; Cystinopine, N-(*R*-1-carboxyethyl)-Cysteine. Mean ± SD of n=3.

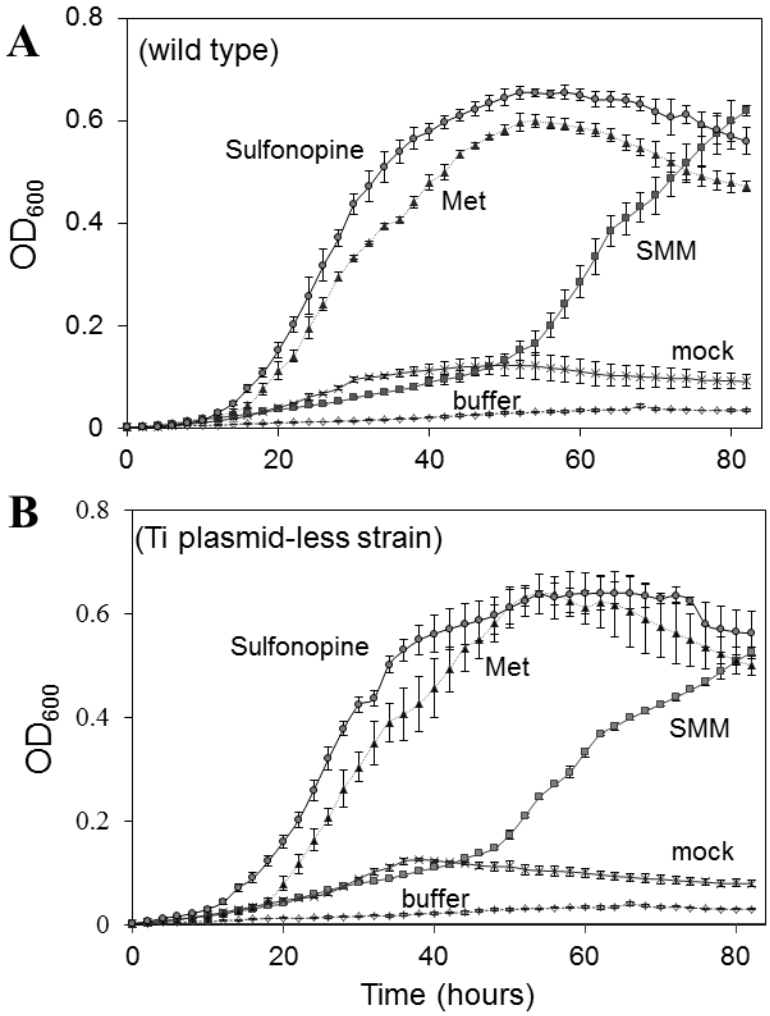

Fig. 3.

Utilization of sulfonopine as a sulfur source by strain R10 (wild type, A) and KYC55 (lacking a Ti plasmid, B). *S*trains were cultured in modified AB minimal lacking sulfur supplemented with 1 mM of the tested compounds. Sulfonopine was synthesized enzymatically, and "mock" refers to a control reaction conducted in the absence of any amino acid. "Buffer" refers to growth medium lacking any additions. Mean ± SD of n=3.

Table 1

Msh catalytic specificity for methyl donor substrates[a].

Methyl Donor	k_m (mM)	k_{cat} (min^{-1})	Enzymatic efficiency (k_{cat}/k_m)	Relative activity (%)
SMM	3.7 ± 2[b]	114	31	(100)
Methylcobalamin	17 ± 8	14	0.8	2.7
Dimethylglycine	21 ± 8	2.4	0.114	0.4
SAM	18 ± 4	0.7	0.04	0.12
Betaine	>1.3 ± 0.6[c]	<0.04 [c]	>0.03 [c]	>0.9 [c]

[a]Production of Met from homocysteine (10 mM) and the indicated methyl donors was carried out by using 0.61 μM His6-Msh in 20 mM Tris buffer (pH 7.9). Met production was analyzed by ESI-MS/MS.

[b]Mean ± SD of n=3 enzymatic reactions.

[c]Detection limit.

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Table 2

Substrate specificity of octopine synthase for amino acids and α-keto acids.

Amino Acid	α-keto acid	k_m (mM)	k_{cat} (min-1)	Enzymatic Efficiency (k_{cat}/k_m)	Relative activity (%)
L-Arginine	Pyruvate	3.5 ± 0.3	720	210	(100)
L-SMM	Pyruvate	0.31 ± 0.04	49	160	76
L-HCys	Pyruvate	8.9 ± 1.1	1600	180	86
L-Methionine	Pyruvate	27 ± 6	4700	170	81
L-Cysteine	Pyruvate	11 ± 3	670	61	29
L-Histidine	Pyruvate	2.0 ± 0.21	1200	600	286
L-Canavanine	Pyruvate	2.8 ± 0.5	1000	360	171
L-Ornithine	Pyruvate	3.0 ± 0.4	540	180	86
L-Lysine	Pyruvate	0.74 ± 0.08	130	178	85
L-Glutamine	Pyruvate	5.6 ± 0.6	780	140	67
L-Homoserine	Pyruvate	15 ± 3	1800	120	57
L-Glycine	Pyruvate	30 ± 6	1300	43	20
L-Homoarginine	Pyruvate	11 ± 2	410	37	18
L-Leucine	Pyruvate	11 ± 2	410	37	18
L-Serine	Pyruvate	12 ± 2	220	18	9
L-Alanine	Pyruvate	47 ± 14	340	7.2	3.4
L-Asparagine	Pyruvate	41 ± 13	220	5.4	2.6
L-Threonine	Pyruvate	976 ± 30	4600	4.7	2.2
L-Valine	Pyruvate	36 ± 5	170	4.7	2.2
L-Isoleucine	Pyruvate	70 ± 30	180	2.6	1.2
L-Glutamic acid	Pyruvate	$>1.5 \times 10^6$	$<6.9 \times 10^4$	<0.6	<0.09
L-Aspartic acid	Pyruvate	$>1.5 \times 10^6$	$<6.9 \times 10^4$	<0.6	<0.09
L-Tryptophan	Pyruvate	$>1.5 \times 10^6$	$<6.9 \times 10^4$	<0.6	<0.09
L-Tyrosine	Pyruvate	$>1.5 \times 10^6$	$<6.9 \times 10^4$	<0.6	<0.09
L-Phenylalanine	Pyruvate	$>1.5 \times 10^6$	$<6.9 \times 10^4$	<0.6	<0.09
L-Proline	Pyruvate	$>1.5 \times 10^6$	$<6.9 \times 10^4$	<0.6	<0.09

Amino Acid	α-keto acid	k_m (mM)	k_{cat} (min-1)	Enzymatic Efficiency (k_{cat}/k_m)	Relative activity (%)
L-Carnosine	Pyruvate	$>1.5 \times 10^6$	$<6.9 \times 10^4$	<0.6	<0.09
D-Arginine	Pyruvate	$>1.5 \times 10^6$	$<6.9 \times 10^4$	<0.6	<0.09
D-Methionine	Pyruvate	$>1.5 \times 10^6$	$<6.9 \times 10^4$	<0.6	<0.09
D-SMM	Pyruvate	$>1.5 \times 10^6$	$<6.9 \times 10^4$	<0.6	<0.09
Arginine	Pyruvate	0.44 ± 0.07	561	1275	(100)
Arginine	α-ketobutyrate	6 ± 1	573	96	7.5
Arginine	Oxaloacetate	28 ± 4.5	1073	38	3
Arginine	Glyoxylate	22 ± 5	354	16	1.3
Arginine	α-ketoglutarate	$>1.5\ e^{+6}$ [a]	$<6.9\ e^{+4}$ [a]	>0.6 [a]	>0.09 [a]

Mean ± SD of n=3 enzymatic reactions.

[a] Detection limit

Table 3

SMM and sulfonopine content in tobacco tissue extracts and exudate of infected seedlings with *A. tumefaciens* R10 and mutants strains.

Compound	Location	R10	KYC55	Δ*vir*D4	Δ*ocs*	Mock infected
SMM[a]	Homogenate	21±16[a,c]	18±14	31 ±25	26 ±19	40±25
Sulfonopine[a]	Homogenate	5±0.6[a,c]	n. d.[d]	n. d.	n. d.	n. d.
Sulfonopine[b]	Exudate	2 ±0.7[b,c]	n. d.	n. d.	n. d.	n. d.

[a]: $nmol\,mg^{-1}$ of plant tissue

[b]: $nmol\ ml^{-1}$

[c]: Mean ± SD of triplicate samples.

[d]: ND. Not detected

Table 4

Sulfonopine is detected only in plants that produce SMM.

	Sulfonopine Contect in Floral Tissue		
Arabidopsis Strain	**Wild type**[a]	***mmt***[b]	***hmt2-2***[c]
Infected	7 ± 1[e,f]	n. d.	140 ± 90
Noninfected	n. d.[d]	n. d.	n. d.

[a]: Wild type (Columbia ecotype).

[b]: does not produce SMM.

[c]: SMM hyperaccumulation.

[d]: Not detected

[e]: (nmol mg^{-1})

[f]: Mean ± SD of triplicate samples.