Major biocontrol of plant tumors targets tRNA synthetase

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Materials and Methods

Strains and growth conditions

_A. tumefaciens_ strains used in this study were the following; NT1, a Ti plasmid-cured derivative of the wild-type tumorigenic C58 strain (S1), NTL4 (pTiC58)ΔaccR, an
Agrocin 84 supersensitive strain (construct has a deletion of the \textit{accR} gene responsible for regulation of agrocinopine permease operon) and NT1 (pAgK84::Tn5A1-B5), an Agrocin 84 overproducing strain. All \textit{A. tumefaciens} strains were grown in either LB or minimal media with D-glucose as a carbon source and the appropriate antibiotic at 28 °C to 30 °C.

\textit{E. coli} strains used for initial cloning and plasmid manipulations were DH5α or TA One shot cells (Invitrogen, Carlsbad, CA).

**Purification of Agrocin 84**

5 L of \textit{A. tumefaciens} NT1 (pAgK84::Tn5A1-B5) culture grown in minimal media containing kanamycin (50 μg/ml) for 48 h was centrifuged at 6,840 g at 4°C and the supernatant decanted off and filtered through 0.2 μm filters. The filtrate was then lyophilized in small batches. The crude supernatant was then resuspended and initially purified by HPLC using multiple injections on a preparative scale (22 x 250 mm, 300 Å, 10 micron) Vydac C18 protein and peptide reverse phase column (Flow rate 15 ml / min with 20 mM TEAA (pH 7.5) buffer and a 100 – 50 % acetonitrile isocratic gradient. Subsequent purification of bioassay active fractions was performed on a semi-preparative scale (10 x 250 mm, 300 Å, 5 μm) Vydac C18 column. Agrocin 84 was further purified using an Affi-Gel 601 Boronate affinity gel (Biorad, Hercules, CA) column (15 mm diameter x 80 mm length) using 0.1 M sodium phosphate buffer (pH 7.6) to load and wash and eluted with 0.1 M D-ribose in 0.1 M sodium phosphate buffer (pH 7.6). The toxic moiety can be produced by short incubation of Agrocin 84 in sodium phosphate buffer (pH 7.0, 100 °C, 15 min) and purified by reverse phase HPLC. Purified Agrocin
84 and the toxic moiety were desalted before analysis by ESI-MS. Agrocin 84 and its toxic moiety were stored at –80°C until use.

**Cloning of the C58 LeuRS and agnB2 genes**

Oligonucleotide primers were designed to PCR-amplify the open reading frame of the *A. tumefaciens* LeuRS gene introducing *Nde*I and *Xho*I sites. The gene was cloned into the pCR4 using the Topo-TA ligation system (Invitrogen, Carlsbad, CA). A natural *Nde*I restriction site in the gene was removed by site-directed mutagenesis using the QuikChange system (Stratagene, La Jolla, CA) and the mutation was confirmed by DNA sequencing analysis. The gene was then subcloned into the expression vector pET-21b, which adds an N-terminal His6-tag, using the *Nde*I and *Xho*I restriction sites. The *agnB2* gene encoded on the pAgK84 plasmid was PCR-amplified using primers designed to introduce a 5′ *Nco*I restriction site (producing a conservative N→E mutation in the gene product) and a 3′ *Xba*I restriction site. The PCR product was then cloned into a pET-28a expression plasmid (Novagen, Madison, WI).

**Purification of His6-tagged LeuRS enzymes**

The cloned *agnB2*-coded LeuRS and *A. tumefaciens* genomic LeuRS cloned in the pET vectors were overexpressed in *E. coli* BL21(DE3) (Novagen, Madison, WI). 3 x 2 L cultures that were grown to a cell density of *A*₆₀₀ = 0.4 (mid-log phase), before induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and grown for an additional 3 h before harvesting. Cells were harvested by centrifugation at 6,330 g, lysed by French press, centrifuged at 111, 000 g to clear insoluble cellular debris, and batch-
purified by Ni-NTA chromatography. The proteins were then concentrated and buffer exchanged into 50 mM Tris-HCl pH 8.3, 20 mM NaCl and 10 mM β-mercaptoethanol and purified by anion-exchange column chromatography. Protein concentration was determined by Bradford protein assay and active site titration.

**Aminoacylation assays**

Aminoacylation assays were carried out as previously described (S2) in 50 mM HEPES (pH 7.0), 20 mM KCl, 20 mM β-mercaptoethanol, 10 mM MgCl₂, 4 μg / ml pyrophosphatase. For Fig 1B aminoacylation of bulk *E.coli* tRNA by *A.tumefaciens* LeuRS and purified AgnB2 enzyme from *A. radiobacter* K84 strain was carried out at 28 °C with enzyme (10 nM), ATP (100 μM), bulk *E.coli* tRNA (0.4 mM), leucine (100 μM), trace [3,4,5-³H]leucine and Agrocin 84 (250 nM) or the toxic moiety of Agrocin 84 (250 nM). Data points in Fig 1B represent the average of 3 separate experiments. Enzyme concentrations were determined by active site titration (S3).

For IC₅₀ calculations of the *A.tumefaciens* LeuRS and purified AgnB2 enzyme from strain K84 for the toxic moiety of agrocin 84 were determined using enzyme concentrations of 2 nM and inhibitor concentrations ranging from 10 nM–100 μM.

**Bioassays.**

Cultures of *A. tumefaciens* Agrocin 84 supersensitive strain NTL4(pTiC58ΔaccR) were grown overnight in LB and diluted 100-fold into fresh LB. 5 mL of diluted culture was washed over each 25 mL LB-agar plate, and then removed before the assay plates were dried. Central 6 mm diameter wells were then placed in each assay plate and the
solution (typically 50 µl) to be tested was added to the well. Plates were then incubated typically for 24 hours at 28 °C and zones of growth inhibition were measured.

**Uptake and inhibition of aminoacylation.**

Uptake of agrocin 84 was assessed by measuring the disappearance of the antibiotic from culture supernatants. Cultures of *A. tumefaciens* strains NT1 and NTL4(pTiC58 ΔaccR) strains were grown in 50 mL of LB to a cell density of $A_{600} = 0.4$. Fractions (10 ml) of the culture were centrifuged at 16,000 g and the supernatant decanted off. The cell pellets were then resuspended in LB (1 ml) containing purified Agrocin 84 at a final concentration of 700 nM and incubated for 17 h at 30 °C on an orbital shaker. The cells were pelleted and the supernatant decanted off and a 75 µl sample was examined for Agrocin 84 uptake by bioassay. The cell pellets were resuspended in 1.5 ml fresh LB and centrifuged again to remove any excess Agrocin 84 and the cell pellets were then stored at −20°C. The frozen cells were thawed, and suspended in 500 µl of buffer containing 50 mM HEPES (pH 7.5), 20 mM KCl, 10 mM β-mercaptoethanol and 2 ‘EDTA free’ protease inhibitor cocktail tablets (Roche, Indianapolis, IN) per 10 ml of buffer. The agrobacterial cells were lysed on ice by sonication and the insoluble cellular debris was removed by centrifugation at 16,000 g at 4 °C. Each of cell extract (12.5 µl) was assayed for Leu-tRNA$^{\text{Leu}}$ and Ile tRNA$^{\text{Ile}}$ production (by LeuRS and IleRS activity respectively) after 20 mins incubation in a final assay reaction volume of 50 µl. Assays were carried out at 28 °C using ATP (100 µM), bulk *E.coli* tRNA (0.2 mM) and leucine (50 µM). The production of Leu-tRNA$^{\text{Leu}}$ and Ile-tRNA$^{\text{Ile}}$ after 20 mins of incubation of cell extracts under identical reaction conditions
monitored LeuRS and IleRS activities. These results were converted to % aminoacylated-tRNA for each enzyme activity by comparison with cell extracts from control cells incubated in the absence of Agrocin 84. Data points represent the average of 3 separate experiments, with error bars corresponding to one standard deviation.

References and Notes


Figure S1 Structure of the plant tumor-derived substrate Agrocinopine A (S4)

Figure S2A,B. Bioassay detecting the sensitivity of *A. tumefaciens* NTL4(pTiC58 ΔaccR) strain to purified Agrocin 84 (A) or to the toxic moiety of Agrocin 84 (B)

Purified Agrocin 84 (3 $\mu$M) or the toxic moiety of Agrocin 84 (3 $\mu$M) in PBS buffer (pH 7.4) were added to the central well of each bioassay plate and incubated at 28 °C for 1-2 days.

Figure S2C,D. ESI-MS spectra of purified Agrocin 84 (C) and the toxic moiety of Agrocin 84 (D)

The samples were electrosprayed with MeOH as a solvent using negative ionization mode. The mass spectrum of the purified agrocin 84 spectrum (C) showed two peaks, m/z = 701 and m/z = 350, corresponding to the -1H$^+$ and -2H$^+$ deprotonated masses of Agrocin 84 respectively. The mass spectra (D) of the toxic moiety of Agrocin 84 showed one peak, m/z = 459, corresponding to the -1H$^+$ deprotonated mass of the toxic moiety.
Figure S1
Figure S2