Biosynthesis of the proteasome inhibitor syringolin A: the ureido group joining two amino acids originates from bicarbonate

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Abstract

Background: Syringolin A, an important virulence factor in the interaction of the phytopathogenic bacterium Pseudomonas syringae pv. syringae B728a with its host plant Phaseolus vulgaris (bean), was recently shown to irreversibly inhibit eukaryotic proteasomes by a novel mechanism. Syringolin A is synthesized by a mixed non-ribosomal peptide synthetase/polyketide synthetase and consists of a tripeptide part including a twelve-membered ring with an N-terminal valine that is joined to a second valine via a very unusual ureido group. Analysis of sequence and architecture of the syringolin A synthetase gene cluster with the five open reading frames sylA-sylE allowed to formulate a biosynthesis model that explained all structural features of the tripeptide part of syringolin A but left the biosynthesis of the unusual ureido group unaccounted for.

Results: We have cloned a 22 kb genomic fragment containing the sylA-sylE gene cluster but no other complete gene into the broad host range cosmid pLAFR3. Transfer of the recombinant cosmid into Pseudomonas putida and P. syringae pv. syringae SM was sufficient to direct the biosynthesis of bona fide syringolin A in these heterologous organisms whose genomes do not contain homologous genes. NMR analysis of syringolin A isolated from cultures grown in the presence of NaH¹³CO₃ revealed preferential ¹³C-labeling at the ureido carbonyl position.

Conclusion: The results show that no additional syringolin A-specific genes were needed for the biosynthesis of the enigmatic ureido group joining two amino acids. They reveal the source of the ureido carbonyl group to be bicarbonate/carbon dioxide, which we hypothesize is incorporated by carbamylation of valine mediated by the sylC gene product(s). A similar mechanism may also play a role in the biosynthesis of other ureido-group-containing NRPS products known largely from cyanobacteria.

Background

Syringolins are a family of closely related cyclic peptide derivatives that are secreted by many strains of the phytopathogenic bacterium Pseudomonas syringae pv. syringae (Pss) in planta and under certain culture conditions [1,2]. Syringolin A, the major variant, was shown not only to
induce acquired resistance in rice and wheat after spray application, but also to trigger hypersensitive cell death at infection sites of wheat and Arabidopsis plants infected by compatible powdery mildew fungi [3,4]. Recently, syringolin A was shown to be an important virulence factor in the interaction of Pss B728a with its host plant Phaseolus vulgaris (bean), and its cellular target has been identified. Syringolin A irreversibly inhibits the eukaryotic proteasome by a novel mechanism, representing a new structural class of proteasome inhibitors [5,6].

Structure elucidation revealed that syringolin A is a tripeptide derivative consisting of an N-terminal valine followed by the two non-proteinogenic amino acids 3,4-dehydrolysine and 5-methyl-4-amino-2-hexenoic acid, the latter two forming a twelve-membered macrolactam ring. The N-terminal valine is in turn linked to a second valine via an unusual ureido group (Figure 1A; [1]). The minor variants syringolin B to syringolin F differ from syringolin A by the substitution of one or both valines with isoleucine residues, by the substitution of 3,4-dehydrolysine with lysine, and by combinations thereof [2]. The structure of syringolin A suggested that it was synthesized by a non-ribosomal peptide synthetase (NRPS), large modular enzymes that activate and condense amino acids according to the thiotemplate mechanism (for reviews see e.g. [7-9]). We previously cloned and delimited by mutational analysis a genomic region from Pss B301D-R containing five open reading frames (sylA-sylE) necessary for syringolin biosynthesis (Figure 1B; [10]). Whereas sylA and sylE encode a putative transcription activator and an exporter, respectively, sylC encodes a typical NRPS module predicted to activate valine, whereas sylD codes for two additional NRPS modules (of which the first is predicted to activate lysine and the second is predicted to activate valine [10]) and a type I polyketide synthetase (PKS) module. Type I PKSs are also modular enzymes that, similar to fatty acid synthesis, extend a starter molecule by condensation/decarboxylation of malonate extender units (for reviews see e.g. [11,12]). The analysis of the structure and architecture of the syl gene cluster led to the postulation of a model that completely accounts for the biosynthesis of the tripeptide part of syringolin A, including its ring structure with the 5-methyl-4-amino-2-hexenoic acid and the 3,4-dehydrolysine (Figure 1C, [10]). However, although the addition of the ureido group and its attached second valine could not be explained by the model, the syl gene cluster did not contain additional open reading frames, which, if present, could potentially have been involved in the biosynthesis of this unexplained part.

Here we show that the genes sylA-sylE are sufficient to direct the biosynthesis of bona fide syringolin A when heterologously expressed in Pseudomonas putida and Pss SM, two organisms which do not produce syringolin A and have no syl gene cluster homolog in their genomes. Thus, biosynthesis of the ureido group with its attached terminal valine is achieved without additional syringolin A-specific genes (i.e., genes with no other function than in syringolin A biosynthesis). We hypothesized that biosynthesis of the ureido group would most likely be accomplished by the product of the sylC gene, which would, in addition to the extracyclic peptidyl valine, also activate the terminal valine and join the two residues by incorporation of a carbonyl group derived from hydrogen carbonate/carbon dioxide, thus forming the ureido moiety. We demonstrate by NMR spectroscopic analysis of syringolin A isolated from Pss cultures grown in the presence of NaH13CO3 that the 13C isotope is preferentially found at the position of the ureido carbonyl atom. These results support our hypothesis, which may be of relevance for the hitherto unknown biosynthesis of other ureido-group-containing NRPS products largely known to be produced by cyanobacteria [13-20].

Results

Biosynthesis of syringolin A in heterologous organisms

In order to test whether the sylA-sylE gene cluster was sufficient to direct syringolin A biosynthesis, we constructed a cosmid containing the sylA-sylE genes but no other complete gene by taking advantage of AscI and NotI restriction sites flanking the syl gene cluster (Figure 1B). Southern blot analysis of AscI/NotI-digested genomic DNA of Pss B301D-R probed with a sylA gene fragment labeled the expected 22 kb fragment and thus confirmed the uniqueness of the restriction sites in the relevant genome region (data not shown). Thus, Pss B301D-R genomic DNA digested with AscI and NotI was separated by agarose gel electrophoresis. Fragments in the 20-23 kb size range were eluted and cloned into the wide host range cosmid pLAFR3 [21]. After packaging into lambda phages and transfection into E. coli XL-1Blue, the library was screened with a radiolabeled sylA gene probe. Positive clones were isolated and confirmed to contain the complete syl gene cluster by PCR amplification and sequencing of the expected insert ends. One of the confirmed clones was designated pPL3syl and chosen for further work.

To test the functionality of pPL3syl, the markerless Pss B301D-R mutant Δsyl was constructed in which the complete syl gene cluster was deleted. The pPL3syl cosmid was then mobilized into the Δsyl deletion mutant by triparental mating. We previously showed that infiltration of syringolin A-producing Pss strains or isolated syringolin A into rice leaves leads to the accumulation of transcripts corresponding to the defense-related Pfr7b gene (encoding an esterase; [22]), whereas strains or mutants unable to synthesize syringolin A do not activate this gene [1,23]. Syringolin A was originally identified and isolated based
on its action on the Pir7b gene in rice [1]. We thus infiltrated the B301D-R wild-type strain, the syringolin-negative mutants Δsyl and sylA_KO (contains a plasmid insertion interrupting the sylA transcription activator gene [10]), as well as Δsyl (pPL3syl), the deletion mutant complemented with pPL3syl, into rice leaves. RNA was extracted and subjected to gel blot analysis with regard to Pir7b transcript accumulation. As expected and in contrast to the wild type, the syringolin A-negative mutants did not induce Pir7b transcript accumulation, whereas the dele-

Figure 1
Structure and biosynthesis model of syringolin A. A. Structure of syringolin A. Amino acid constituents are delimited by bars. Val, valine. B. Genomic region of Pss B301D-R containing the sylA-sylE genes. Boxes above and below the line denote ORFs on the top and the bottom strand, respectively. Arrows indicate restriction sites used for cloning of the gene cluster into the cosmid pPL3syl. The sylA, sylB, and sylE genes encode a LuxR-type transcription activator, a rhizobitoxin desaturase-like protein thought to desaturate the lysine residue, and an efflux transporter, respectively [10]. The sylC gene encodes an NRPS module, while sylD codes for two NRPS modules and one PKS module [10] C. Biosynthesis model of the tripeptide part of syringolin A. The open boxes represent domains in modules of the syringolin A synthetase labeled with C, condensation domain; A, adenylation domain; PC, peptide carrier protein; KS, ketoacyl synthase; AT, acyl transferase; DH, dehydratase; KR, ketoreductase; AC, acyl carrier protein; TE, thioesterase. The A domains of the NRPS modules are thought to activate valine (NRPS mod1), lysine (NRPS mod2), and valine (NRPS mod3) [10]. The question mark indicates the unexplained synthesis and attachment of this group. The figures are adapted from [10].
tion mutant complemented with the pPL3syl cosmid led to a much stronger induction of the Pir7b gene (Figure 2A). This strongly suggested that pPL3syl contained a functional syl gene cluster able to direct syringolin A synthesis in the Δsyl deletion mutant. This does not exclude the possibility that genes not present in the syl gene cluster are necessary for syringolin A production because such genes would also be present in the Δsyl mutant background.

Next we wanted to mobilize pPL3syl into Pseudomonas strains not carrying syl gene homologs and lacking syringolin A production as evidenced by PCR, DNA gel blot analysis of genomic DNA, high performance liquid chromatography (HPLC) analysis of culture supernatants with regard to syringolin A content, infiltration into rice leaves followed by monitoring of Pir7b transcript accumulation, and whole genome sequence comparisons where possible (data not shown). After repeated unsuccessful attempts to transfer pPL3syl into the P. syringae pv. tomato DC3000 strain (all tetracycline-resistant putative transformants analyzed contained deletion variants of pPL3syl), the cosmid was successfully transferred into the non-pathogenic bacterium P. putida P3 [24] and Pss SM, a strain originally isolated from wheat [23,25]. Gel blot analysis of RNA extracted from rice leaves infiltrated with parental and transformed strains showed that, as expected, P. putida P3 and Pss SM did not induce Pir7b transcript accumulation. In contrast, both strains lead to Pir7b gene induction when carrying the pPL3syl cosmid (Figure 2B), suggesting that pPL3syl conferred the ability for syringolin A biosynthesis to these strains.

To confirm this, the transformed strains were grown in shaken cultures in SRM_{AF} medium and conditioned media were analyzed by HPLC. As shown in Figure 3, both strains produced a compound eluting at 15.5 min, the elution time of the syringolin A standard. Peaks were collected from multiple HPLC runs and subjected to mass

![Figure 2](http://www.biomedcentral.com/1471-2091/10/26)

**Figure 2**

**Gel blot analysis of Pir7b transcript accumulation.** A. RNA was extracted from rice leaves infiltrated with water (C), Pss B301D-R (WT), a sylA plasmid insertion mutant (sylA_KO), a syl cluster deletion mutant (Δsyl), and Δsyl (pPL3syl), the deletion mutant complemented with the wild-type syl gene cluster. Top panel, autoradiogram (exposed for 5 h); bottom panel, ethidium bromide (EtBr)-stained agarose gel. B. Lanes were loaded with RNA extracted from rice leaves infiltrated as indicated. C, water control; B301D-R, Pss wild-type strain; P.p, P. putida P3; P.p. (pPL3syl), P. putida P3 transformed with the syl gene cluster; SM, Pss SM; SM (pPL3syl); Pss SM transformed with pPL3syl, and syringolin A solutions of the indicated concentrations. Top panel, autoradiogram (exposure times indicated on top), bottom panel, EtBr-stained gel.

![Figure 3](http://www.biomedcentral.com/1471-2091/10/26)

**Figure 3**

**HPLC analysis of syringolin A content in conditioned SRM_{AF} media.** Conditioned media were sterile-filtrated and 20-μl aliquots were loaded on the column. Absorption was monitored at 206 nm. Labels of HPLC traces are the same as in the legend to Figure 2B.
The ureido carbonyl group of syringolin A is incorporated from bicarbonate/carbon dioxide

The above results raised the question of how the ureidovaline is synthesized and incorporated into syringolin A. We hypothesized that this would most likely be accomplished by the product of the sylC gene, which would, in addition to the N-terminal valine of the tripeptide part of syringolin A, also activate the second valine and join the two residues via their amino groups formally by amidation of carbonic acid, thus forming the ureido moiety. If true, feeding syringolin A-producing cultures with 13C-labeled hydrogen carbonate should result in syringolin A that is preferentially labeled with 13C at the ureido carbonyl position. Thus, Pss B301D-R transformed with pOEAC, a plasmid carrying the sylA transcriptional activator gene under the control of the lacZ promoter, was grown in SRMAF medium. After 48 h, 13C-labeled syringolin A (Figure 4A, B). Inspection of the resolved 13C spectrum of labeled syringolin A (data not shown) suggests a 13C content close to natural abundance. Therefore, the 45-fold signal enhancement in labeled syringolin A directly corresponds to the absolute 13C enrichment at this site. The normalized signal strengths of all other C atoms were equal in labeled and unlabeled syringolin A, with the exception of the C4 position of 3,4-dehydrolysinone, whose signal was enhanced approximately 16-fold in 13C-labeled syringolin A (Figure 4A, B). Inspection of biosynthetic pathways using the KEGG database [26] revealed that this can be attributed to a carboxylation reaction in the biosynthesis of lysine. The C4 atom of lysine represents the C4 atom of L-aspartate-4-semialdehyde, a derivative of aspartate, which is condensed to pyruvate to yield the intermediary compound L-2,3-dihydribipicolinate in bacterial lysine biosynthesis. The C4 atom of aspartate in turn originates from the carboxylation of pyruvate to oxaloacetate, an intermediary compound in the tricarboxylic acid cycle, which is transaminated to aspartate. Thus, enhanced 13C-labeling of lysine with H13CO3- at the C4 position is to be expected. We note that malonate will also be labeled by H13CO3- as it is derived from acetate by carboxylation. However, the label will be removed by the condensation/carboxylation of malonate to the peptide chain during syringolin A biosynthesis. We conclude from this analysis that our hypothesis is correct, i.e. that the ureido carbonyl moiety in syringolin A originates from the incorporation of hydrogen carbonate/carbon dioxide.

Discussion

We have demonstrated that the syl gene cluster is sufficient to direct syringolin A synthesis in heterologous organisms. Although the biosynthesis model presented earlier [10] plausibly explained every structural feature of the syringolin A tripeptide part through the enzymatic actions of the sylB, sylC, and sylD gene products, the generation and condensation of the ureido valine remained enigmatic. As the other genes present in the syl cluster encode a transcriptional activator (sylA gene) and an exporter (sylE gene), a plausible hypothesis was that the sylC-encoded NRPS module not only activated the N-terminal peptidyl valine, but also the ureido valine, and that the ureido carbonyl moiety is incorporated from hydrogen carbonate/carbon dioxide. As shown above, in vivo labeling of syringolin A with 13C-hydrogen carbonate supports this hypothesis. Currently, we can only speculate how this is achieved. One possibility is that the quaternary syringolin A synthetase complex may contain two (not necessarily identical) molecules derived from the sylC gene per SyID polypeptide. Both sylC gene products would activate valine, or, to a certain degree, isoleucine in minor syringolin variants [2]. The first valine would then be carboxylated by HCO3-/CO2, perhaps without the action of another enzyme, as has been reported for the carboxyla
tion of a catalytic lysine residue in β-lactamases of class D [27,28]. The ureido moiety would then be formed by amide bond formation between the carboxylated valine and the second valine. In this scenario, it remains unclear how the first valine, which, like the second one, is envis
ed to be bound to the peptide carrier protein domain by a thioester bond, is released upon ureido bond formation. It is also conceivable that ureido bond formation is achieved by a single SyIC protein, which contains a condensation domain usually absent from starter modules.
that may be involved. To clarify these issues, more structural information about the large syringolin A synthetase and the SyIC module must be obtained. The reconstitution of the enzymatic activities of the module(s) derived from the sylC gene in vitro will be challenging.

In addition to the syringolin family of compounds, a number of other natural cyclic peptides mostly isolated from cyanobacteria have been described in the literature that contain extracyclic ureido groups linking two different amino acids. These include anabaenopeptins from Anabaena, Oscillatoria, and Planktothrix species [13-16], ferintoic acids from Myrcocystis aeruginosa [17], pompanopeptins from Lyngbya conforevoides [18], as well as moza-mides and brunsvicamides, compounds of presumably cyanobacterial origin isolated from sponges [19,20]. Bicarbonate/CO₂ may also be the source of the ureido carbonyl group joining two extracyclic amino acids in the biosynthesis of these compounds, which, to our knowledge, has not been elucidated so far.

**Conclusion**

Our results show that the syl biosynthesis gene cluster was sufficient to direct the biosynthesis of bona fide syringolin A, including the enigmatic ureido group joining two amino acids. They reveal the source of the ureido carbonyl group to be bicarbonate/carbon dioxide, which we hypothesize is incorporated by carbamylation of valine mediated by the sylC gene product(s). A similar mechanism may also play a role in the biosynthesis of other ureido-group-containing NRPS products known largely from cyanobacteria.

**Methods**

**Construction and expression of pPL3syl**

Unless stated otherwise, standard protocols were used [29]. Genomic DNA from Ps B301D-R was isolated and
11 μg were digested with the restriction enzymes Ascl and NotI. Ascl and NotI sites are both unique in the syl gene region (GenBank: AJ548826) located at position 2052 and 24124, respectively, within the ORFs flanking the sylA-sylE ORFs (3507-23596). A DNA gel blot was prepared with 1 μg of the digested DNA and probed with a 32P-labeled sylA gene fragment PCR-amplified from genomic DNA with primers P1 (5' - cacatcagtagagtattggc) and P2 (5' - ggaattctcaaaaattccattcg). The rest of the digested DNA was separated on a 0.4% agarose gel and the DNA in the 20-23 kb size range was cut out, electrophoretically eluted into a dialysis bag (10 kDa cutoff), precipitated with ethanol and finally taken up in TE (10 mM Tris-HCl, pH 8; 1 mM EDTA). Fragments were ligated into the HindIII/BamHI-cleave host range cosmid vector pLAFR3 [21] using adaptors prepared by annealing the oligonucleotide 5'-cgcggcgtataacccgaaacga and 5'-gaatttggaagagcgttgg (Ascl/HindIII adaptor) and 5'-ggaattctcctgactagc (BamHI adaptor), respectively. Ligation products were packaged into lambda phage particles using the Gigapack III Gold Packaging Kit (Stratagene, La Jolla, California) and the library lambda phage particles using the Gigapack III Gold Packaging Kit (Stratagene, La Jolla, California) and the library was plated out on E. coli XL1-Blue (Stratagene) and screened according to the instructions of the manufacturer using the 32P-labeled sylA gene fragment described above as a probe. Positive clones were isolated and confirmed to contain the complete syl gene cluster by PCR amplification and sequencing of the insert end fragments using primers 5'-ccggcctacacgcattc (sylA end) and 5'-agcaacctgtcagctgg (sylE end) with the respective adaptor oligonucleotides (see above).

pPL3syl was transferred from XL1-Blue to Pseudomonas strains by triparental mating using the E. coli helper strain HB101 (pRK600) [30,31].

**Construction of the syl gene cluster deletion mutant syl**

Two fragments of 783 bp and 655 bp length flanking the syl gene cluster on the 5’ and 3’ side, respectively, were amplified by PCR from Psst301D-R genomic DNA using the primer pairs P3 (5’-ccggcctcagatgtaatactcg; base given in bold at position 2297 in GenBank:AJ548826) and P4 (5’-agtctctgtaattctgtagatatg; reverse primer; EcoRI site in italics, reverse stop codon in bold), digested with Clal and EcoRI, and cloned into the respective polylinker sites of the pME6001 (GmR) expression vector [34], thereby placing it under the control of the lacZ promoter. The resulting plasmid was named pOEA. As it turned out that pOEA did not confer gentamycin resistance in SRMAE medium, pOEA was used, a derivative of pME6014 (TetR) [35], which, in addition to the LacZ::sylA chimeric gene, contained a sylC::lacZ reporter fusion gene in opposite orientation (the reporter gene is of no relevance in the present context). To construct pOEAC, the LacZ::sylA fusion gene was amplified from pOEA with primers P8 (see above) and P9 (5’-acctgtcaacacagtacggc; upstream of lac promoter; bold base complementary to position 987 of pBluescript vector (GenBank:X52329)) and joined with a sylC promoter fragment (position 5409-5649 of GenBank:AJ548826) that was amplified with primers P10 (5’-ctgcctagctgacggccctgcctag; bold base at position 5409) and P11 (5’-aactcgtcagctgacggcctgag; Psst site in italics, bold base at position 5649) by overlap extension PCR using primers P8 and P11. The resulting fragment was digested
with EcoRI and PstI and cloned between the respective sites in the poly linker of pME6014.

**Bacterial infiltration of rice leaves and RNA gel blot analysis**

Bacterial strains were grown on a rotary shaker (220 rpm) over night at 28 °C in LB containing, where appropriate, 10 μg/ml tetracycline. Bacteria were pelleted by centrifugation, washed twice in distilled water, resuspended in distilled water at an optical density at 600 nm (OD_{600}) of 0.4 (approximately 10^8 cfu), and infiltrated into first leaves at 3 s. The samples were dissolved in MeOH and analyzed via continuous flow HPLC analysis on a Bruker maXis QTOF-MS instrument (Bruker Daltonics GmbH, Bremen, Germany). The samples were infiltrated leaves 16 h after infiltration and subjected to gel blot analysis using a 32P-labeled Pir7b cDNA probe (GenBank:Z34270[23]) according to standard procedures [29].

**HPLC analysis and mass spectrometry of syringolin A**

To analyze conditioned media with regard to syringolin A content, *Pseudomonas* strains were grown in SRM_{AF} medium [36,37] at 28 °C for 60 h on a rotary shaker (220 rpm). Bacteria were pelleted by centrifugation and the supernatant was sterile filtered (0.22 μm pore size). Two-hundred-microliter aliquots were acidified with trifluoroacetic acid (TFA: 0.3% final concentration) and subjected to reverse-phase HPLC with a Reprosil 100-5 C 18 250/4.6 column (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) on a Dionex UltiMate 3000 system (Dionex Corporation, Sunnyvale, CA). Elution was performed isocratically with 20% acetonitrile and 0.06% TFA in water at a flow rate of 1 ml/min.

High-resolution electrospray mass spectra were recorded on a Bruker maXis QTOF-MS instrument (Bruker Daltonics GmbH, Bremen, Germany). The samples were dissolved in MeOH and analyzed via continuous flow injection at 3 μl/min. The mass spectrometer was operated in positive ion mode with a capillary voltage of 4 kV, an endplate offset of -500 V, nebulizer pressure of 5.8 psig, and a drying gas flow rate of 4 l/min at 180 °C. The instrument was calibrated with a Fluka electrospray calibration solution (Sigma-Aldrich, Buchs, Switzerland) that was 100 times diluted with acetonitrile. The resolution was optimized at 30'000 FWHM in the active focus mode. The accuracy was better than 2 ppm in a mass range between m/z 118 and 2721. All solvents used were purchased in best LC-MS qualities.

**13C-labeling and NMR Spectroscopy**

Pss B301D-R was transformed with pOEAC and grown in LB containing 10 μg/ml tetracycline on a shaker at 28 °C until an OD_{600} of approximately 0.5 was reached. Bacteria were collected by centrifugation, washed twice with SRM_{AF} medium, and taken up in SRM_{AF} medium at an OD_{600} of 0.3. Fifty-ml cultures were inoculated with 0.01 volume of the bacterial suspension and incubated at 28 °C on a shaker (220 rpm). After 48 h, NaH^{13}CO_3 (98%; Sigma-Aldrich, Buchs, Switzerland) was added to a final concentration of 70 mM and incubation was continued for 20 h. Bacteria were pelleted and syringolin A was isolated from sterile-filtrated conditioned media as described [4].

1H broadband decoupled 13C NMR spectra were recorded at 25 °C on a Bruker Avance III 600 MHz spectrometer equipped with a cryogenic 5 mm CPDCH probe head optimized for 13C detection. Two samples were prepared by dissolving 200 μg of labeled syringolin A in 130 μl DMSO-d6 and 5 μg of unlabeled syringolin A in 750 μl DMSO-d6, respectively. The labeled sample was transferred to a 3 mm Shigemi tube, the unleaded sample was transferred to a regular 5 mm NMR tube. The spectral width in both spectra was 248.5 ppm, the transmitter was set to 100 ppm. The excitation pulse angle was set to 45°. The acquisition time was 2.1 s with a waiting time of 0.3 s between two scans. Both spectra were 1H broadband decoupled using the waltz16 composite-pulse decoupling scheme. The resulting fid consisted of 157890 total data points. For the unlabeled syringolin A sample 4000 scans were accumulated. For the labeled syringolin A sample 29605 scans were accumulated. Both spectra were zero filled to 131072 complex data points and processed using an exponential line broadening of 2 Hz. The samples contained no internal chemical shift reference and the spectra were referenced to the solvent peak (39.5 ppm). By comparison with chemical shifts listed in [1] the signals at 157.8 ppm and 132.8 ppm were assigned to the ureido group and the olefinic C at position 4 in the 3,4-dehydrosyne moiety, respectively.

**Authors’ contributions**

CR carried out the majority of experiments. The pPL3syl cosmid and the Δsyl deletion mutant were constructed by MT and MM, respectively. Mass spectrometry and NMR spectroscopy were performed and analyzed by LB and MOE, respectively. BS performed RNA gel blot analyses in the rice infiltration experiments. RD, CR, and BS designed experiments and RD wrote a draft manuscript. All authors provided critical inputs to the manuscript.

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