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Syringicin, a new α-elicitin from an isolate of *Phytophthora syringae*, pathogenic to citrus fruit

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Abstract

The primary structure of syringicin (syr), a new acidic α-elicitin, isolated from culture filtrates of *Phytophthora syringae*, causal agent of citrus fruit rot, has been determined using a combined approch based on Edman degradation and MALDI-MS (TTCTT TQQTA AYVAL VSILS DSSFN QCATD SGYSM LTATA LPTTA QYKLM CASTA CKTMI TKIVS LNAPD CELTV PTSGL VLNVY SYANG FSSTC ASL). Syr has 98 amino acids with a M_r of 10194.6±0.2, which was determined by electrospray ionisation-mass spectrometry (ES-MS) and in agreement with three disulphide bridges, located between Cys3-Cys71, Cys27-Cys56 and Cys51-Cys95. Syr induces a hypersensitive response and electrolyte leakage in tobacco. These are characteristic elicitor properties of the group and in agreement with the molecular mechanism recently proposed for this kind of protein. Finally, its possible applications in biological agriculture and biomedicine are briefly discussed. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Phytophthora syringae*; Citrus fruits; Elicitins; Amino acid sequence; Syringicin; Biological activity

1. Introduction

An approach to controlling plant diseases is to induce natural plant defence mechanisms without relying on synthetic pesticides.

In this view, proteins with elicitor activity named elicitins have been isolated from the culture filtrates of several *Phytophthora* species. Elicitins, 98 amino acid proteins with a M_r of about 10 kDa, are divided into α-elicitins and β-elicitins, of acid and basic nature, respectively (Ponchet et al., 1999; Churngchow and Rattarasarn, 2000).

Their characteristic biological activities are the induction of a hypersensitive response (HR) and systemic acquired resistance (SAR) in tobacco, with the formation of phytoalexins, PR proteins and salycilic acid (Ricci, 1997). Both types induce electrolyte leakage from the same plant cell (Zimmermann et al., 1998).

Moreover, they induce protection against phytopathogenic micro-organisms in tobacco and in some species of *Brassicaceae* (Kamoun et al., 1993; Bonnet et al., 1996; Blancard et al., 1998; Keizer et al., 1998; Tepfer et al., 1998; Keller et al., 1999). Churngchow and Rattarasarn (2000) have recently reported that palmivorein, a new α-elicitin purified from *Phytophthora palmivora*, isolated from rubber tree leaf, is also active on *Hevea* and a susceptible rubber clone.

The secondary and tertiary structure of some α-elicitins was also assessed by ^{1}H NMR (Bovaziz et al., 1994; Fefeu et al., 1997; Gooley et al., 1998) and X-ray diffraction studies (Boissy et al., 1996).

The gene (Inf1) encoding the α-elicitin infestin in *Phytophthora infestans* was identified and cloned (Kamoun et al., 1998). In addition, a mutant of a phytopathogenic *P. infestans* lacking Inf1 proved to be virulent to a tobacco species (*Nicotiana benthamiana*) (Kamoun et al., 1997), indicating an in vivo defence

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mechanism of the protein and, in all likelihood, of all elicitins.

It has recently been found that the bioactivity of the elicitins is correlated to the interaction of an elicitin-sterol complex with its plant plasmalemma receptor, which is proposed to be an allosteric calcium channel (Ponchet et al., 1999). The property of these proteins to load sterols has been exploited for the preparation of anticholesteremic agents, and their medical use has recently been patented (Mikes et al., 1998).

In a previous paper we reported the isolation and biochemical characterisation of elicitin 172, produced by an isolate pathogenic to tomato (Capasso et al., 1999). Elicitin 172 proved to be identical to parasiticein (Ricci et al., 1992) and elicitin 310 (Mouton-Perronet et al., 1995).

Here we describe the primary structure of syr, a new α-elicitin purified from the culture filtrates of *P. syringae,* which is the causal agent of rot of citrus fruit, and compare it to the other known α-elicitins (Huet et al., 1994; Churngchow and Rattarasarn, 2000).

Moreover, we report and discuss its elicitor activity identified by the usual HR and the electrolyte leakage test on tobacco (*Nicotiana tabacum* L., cv. Rustica).

We also briefly describe its potential applications in biological agriculture and biomedicine.

2. Results and discussion

The purification of syr was performed by a procedure slightly more simple than the one used for elicitin 172 (Capasso et al., 1999). The last step was low pressure chromatography through a reverse phase column (Lichroprep RP-18), giving rise to a very sharp and intense peak (Fig. 1). The protein (20 mg per litre of culture filtrate of *Phytophthora syringae*) was obtained in high purity grade as shown by native or SDS–PAGE.

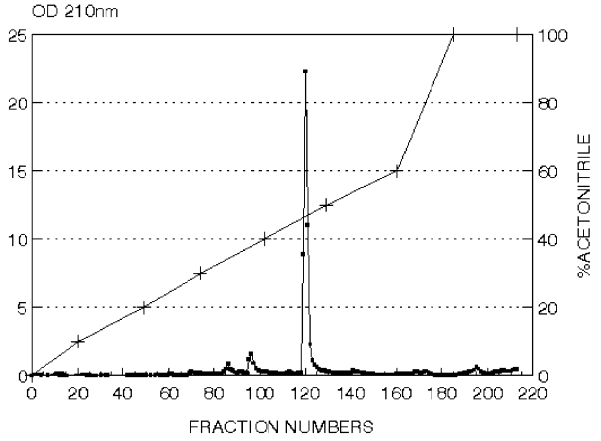

Fig. 1. Chromatogram of α-syringicin obtained by step-wise elution from an RP-HPLC C-18 column (31×2.5 cm), using H_2O/CH_3CN as eluant, under low pressure (3 bar). Flow rate: 15 ml/min; fractions of 5 ml.

After staining the tricine–SDS–PAGE electrophoregram with silver nitrate or with Coomassie blue, a single band appeared. Its apparent M_r was 10 kDa. The protein appeared as a single band in correspondence of 10 kDa on SDS-PAGE, even after heating in presence of 2-mercaptoethanol.

The protein, analysed by ES–MS, exhibited the characteristic bell-shaped distribution of multiple charged ions from which a M_r of 10194.6 ± 0.2 was calculated.

The experimental amino acid composition (Ala12, Asx7, Cys6, Glx5, Gly3, Ile3, Leu10, Lys3, Met3, Phe2, Pro3, Ser13, Thr17, Tyr5, Val6) of the purified syringicin account for a total of 98 amino acid residues and a M_r of 10195, considering the presence of three disulphide bridges.

Therefore, here we consider that threonine, serine and alanine are the most abundant amino acid residues; that the acidic nature, indicated by the theoretical pI value 4.31 and confirmed by the native alkaline PAGE, is consistent with the presence of seven Asx and five Glx and of only three lysyl residues and the absence of hystidinyl and arginyl residues.

The UV absorbance with a maximum at 277 nm (not shown) is in accordance with the presence of phenylalanyl and tyrosinyl residues and the absence of tryptophan.

Automated Edman degradation both on the native and *S*-pyridylethylated protein, allowed the N-terminal sequence of syr to be identificated up to residue 30. The rest of the syr sequence came from the CB-peptides (Fig. 2).

Native syr was treated with CNBr, *S*-pyridylethylated and then subjected to RP–HPLC chromatography, which furnished all CB-peptides.

CB-1 (positions 1-35), CB-2 (36-50) and CB-3 (51-59) were completely sequenced by Edman degradation, while CB-4 (60-98) was sequenced up to residue 94. The last four residues of syr were obtained by combining CPaseA digestion with MALDI–MS analysis as already reported for elicitin 172 (Capasso et al., 1999). In particular,

```
1         10        20        30        40        50
*         *         *         *         *         *
TTCTTTQQTAAYVALVSILSDSSFNQCATDSGYSMLTATALPTTAQYKLM
<=============================>
<---------------CB-1------------><-----CB-2----->

51        60        70        80        90      98
*         *         *         *         *       *
CASTACKTMITKIVSLNAPDCELTVPTSGLVLNVYSYANGFSSTCASL
<-CB-3-><-------------CB-4------------------>
                                            <<<<
```

Fig. 2. Complete amino acid sequence of syringicin. Automated Edman degradation (< = = = >, < — — — >) and CPase A digestion (< < < < <), and MALDI–MS were employed for sequence determination. CB-peptides are numbered according to their position in the polypeptide chain. Alignment of CB peptides 2 and 3 was obtained by homology with other elicitins. CB, Cyanogen bromide.

aliquots of CB-4 peptide, incubated with the enzyme, were analysed at five minute intervals and the amino acid residues removed in sequence from the C-terminus were identified by the mass change relatively to the mass (4259.14) of *S*-pyridylethylated CB-4 peptide, as shown in Table 1. Therefore, the C-terminal sequence was determined as Cys-Ala-Ser-Leu, which is characteristic for all α-elicitins, with the exception of cactorein (cact).

The alignment of CB-2 and CB-3 peptides was inferred from the sequence homology with elicitin. The complete sequence of syr is reported in Fig. 2.

Syr contains no Trp, His and Arg residues. Furthermore, as found for all other known α-elicitins, the six cysteine residues are located in positions 3, 27, 51, 56, 71 and 95 and are, very likely, engaged in disulphide bridges. This conclusion is based on the negative reaction of native syr with Nbs_2 [5,5′-(ditiobis(2-nitrobenzoic acid)] (Parente et al., 1985), showing no free sulphydryl groups and confirmed by the value for the M_r of 10194.6±0.2, found for the native syr by mass spectrometry (see above). The three disulphide bridges are located, in all likelihood, between Cys3-Cis71, Cys27-Cys56 and Cys51-Cys95, as demonstrated for capsicein (Bovaziz et al., 1994), from which syr differs only in four amino acid positions (i.e. positions 1, 57, 68 and 93; Fig. 3).

Table 1
Time course digestion of CB-4 peptide with CPase A

t (min)	M_r (u)	ΔM_r	Removed residue
0	4259.14	–	–
5	4146.14	113.0	Leu/Ile
10	4059.04	87.1	Ser
15	3987.64	71.4	Ala
20	3779.54	208.1	Cys (pyridylethyl)

The comparison of the primary structure of syr with all other known α-elicitins allowed us to assess the novelty of this protein, as shown in Fig 3. In particular, cact from *P. cactorum* shows the higher number of amino acid differences (thirteen) and caps, from *P. capsicum,* the lower one (four). The other α-elicitins with known amino acid sequence show intermediate amino acid differences: (i) twelve [α-drechselerin (dre-α) from *P. drechseleri*]; (ii) nine [parasiticein (Ricci et al., 1992); elicitin 310 (Mouton-Perronet et al., 1995) and elicitin 172 (Capasso et al., 1999)]; and seven [α-megaspermin (meg-α) from *P. megasperma* and infestin (inf.) from *P. infestans*].

Palmivorein, isolated from *Phytophthora palmivora* and sequenced up to residue 26, shows, with respect to syr, an amino acid replacement in position 22 (Ser replaced by Thr, see Fig. 3). When we compare the amino acid composition of syr with that of palmivorein we found six amino acid differences: Ala (+2), Val (+1), Pro (−2) and Asx (−1).

The identification of syr as α-elicitin was also confirmed by the hypersensitive response (HR) (Ricci, 1997) and the electrolytes leakage (Zimmermann et al., 1998) induced on tobacco.

In fact, from 1 to 20 μM, syr induced necrosis in tobacco. When applied to the leaves at 1 μM, syr

```
                1       10          20          30          40
                *        *          *           *           *
Syr             TTCTTTQQTA AYVALVSILS DSSFNQCATD SGYSMLTATA
Elic-172        TTCTTTQQTA AYVALVSILS DTSFNQCSTD SGYSMLTATS
Cact            ATCTSSQQTA AYVALVSILS DTSFNQCSTD SGYSMLTATS
Caps            ATCTTTQQTA AYVALVSILS DSSFNQCATD SGYSMLTATA
Dre-α           TTCTSTQQTA AYVTLVSILS DSSFNQCATD SGYSMLTATS
Meg-α           TTCTSTQQTA AYVTLVSILS DSSFNQCATD SGYSMLTATA
Inf             TTCTTSQQTV AYVALVSILS DTSFNQCSTD SGYSMLTATS
Pal             TTCTTTQQTA AYVALVSILS DTSFNQ

                41      50          60          70          80
                *        *          *           *           *
Syr             LPTTAQYKLM CASTACKTMI TKIVSLNAPD CELTVPTSGL
Elic-172        LPTTEQYKLM CASTACKTMI NKIVTLNPPD CELTVPTSGL
Cact            LPTTAQYTLM CGSTACKTMI NKIVSLNPPN CELTVPTSGL
Caps            LPTTAQYKLM CASTACNTMI TKIVSLNPPD CELTVPTSGL
Dre-α           LPTDAQYKLM CSSTACNTMI KKIVSLNAPN CDLTVPTSGL
Meg-α           LPTTAQYKLM CASTACNTMI KKIVTLNPPD CELTVPTSGL
Inf             LPTTRQYKLM CASTACKTMI NKIVSLNAPD CELTVPTSGL

                81      90        98
                *        *        *
Syr             VLNVYSYANG FSSTCASL
Elic-172        VLNVFTYANG FSSTCASL
Cact            VLNVYSYANG FSTTCSSL
Caps            VLNVYSYANG FSATCASL
Dre-α           VLNVYEYANG FSTKCASL
Meg-α           VLNVYSYANG FSATCASL
Inf             VLNVYSYANG FSSTCASL
```

Fig. 3. Comparison of syr with the all known α-elicitin sequences.

induced necrosis estimated in amount of 1 in the relative evaluation scale (see experimental) and 4 when applied at 20 μM; when tobacco leaves were immersed in a solution of syr at 1 μM, little necrotic spots appeared on tobacco leaves (1); at 10 μM, necrosis was observed on entire leaves (4). These data indicate that syr induced in tobacco a weak HR at 1μM, whereas the highest HR was obtained at 10 μM.

As expected, syr significantly stimulates the electrolytes leakage through the cell membranes of tobacco plant tissues (Fig. 4) at 0.02 mM, which is the lowest concentration used in our experiments.

It is also noteworthy that, even if syr stimulates the electrolyte leakage from tobacco with increasing intensity, corresponding to the concentration values of 0.1 and 0.2 mM, as shown by the two respective curves in Fig. 4, however, these converge to the same value after 9 h, and remain constant for an extended period of time. This result is fully consistent with the molecular mechanism of elicitins, recently proposed by Ponchet et al. (1999). These authors report that four elicitin molecules, loaded with an ergosterol molecule, which is trapped from a plant cell, interact with the calcium channel of the plant cell plasmalemma, triggering the considered biological and physiological responses. At 0.1 mM, all tobacco cells have, in all likelihood, the site saturated in nine hours, while at 0.02 mM only a part, in our experimental conditions.

In conclusion, syr is a new α-elicitin (see Fig. 2) with the closest primary structure to caps and the farthest from cact (Fig. 3).

The structural specificity of the elicitins produced by the different species of *Phytophthora* suggests that syr also could be used as a taxonomic marker for *P. syringae*.

Furthermore, syr could be used to extend the studies on triggering the resistance of tobacco and other plants towards many pathogenic microrganisms, using directly the protein or by means of the preparation of transgenic plants, as already performed in the last decade for other well known elicitins (Kamoun et al., 1993; Bonnet et al., 1996; Blancard et al., 1998; Tepfer et al., 1998; Keller et al., 1999; Churngchow and Rattarasarn, 2000).

Finally, the property of elicitins as sterol carriers has recently been exploited, by patenting the use of some of them, including caps, as anticholesteremic agents (Mikes et al., 1998), suggesting a similar use for syr for their high analogy.

3. Experimental

3.1. General

Protein was estimated by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard. UV (Perkin Elmer): H_2O.

3.2. Production of culture filtrates

A pathogenic strain of *Phytophthora syringae* was isolated from Citrus lemon plants in Meta di Sorrento and deposited in the culture collection at the Dipartimento di Aboricoltura, Botanica and Patologia Vegetale, Università di Napoli "Federico II", 80055 Portici, and numbered Ph 254. The stock culture was maintained on V-8 agar Petri plates. Liquid cultures were obtained by growing the fungus in flasks containing ASYT medium composed of: L-asparagine, 1 g; sucrose, 10 g; yeast extract, 5 g; thiamine, 0.001 g; $KH_2PO_4 \cdot 7H_2O$, 0.5 g; distilled water, 1000 ml. The flasks were incubated and stirred for 12 days at 26°C. Then, the culture fluid was strained through cheese cloth and filter

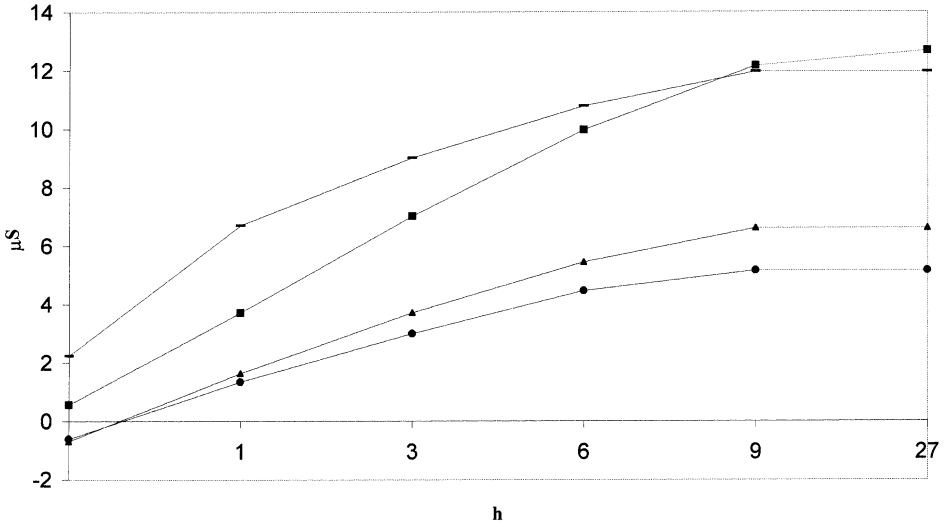

Fig. 4. Electrolytes leakage curves stimulated by syr on tobacco (*Nicotiana tabacum* L., cv. Rustica) leaf discs: the curves are corresponding to concn 0.2 mM (–), 0.1mM (■), 0.02 mM (▲) and control (0.0 mM) (●).

paper, sterilised by filtration through a 0.22 μm membrane and stored at −20°C.

3.3. SDS–PAGE

Polyacrylamide gel electrophoresis in the presence of SDS, using also tricine–SDS or tricine–SDS with urea 6 M and alkaline PAGE performed at pH 8.3 with glycine were performed as reported for elicitin 172 (Capasso et al., 1999).

3.4. Purification of syringicin

Syringicin was purified using a four-step procedure. This is slightly more simple than that used for the purification of elicitin 172 (Capasso et al., 1999), which consisted of five steps. The only difference regarding the purification of syringicin was the elimination of the rechromatography (fourth step) of the raw elicitin through a Sephadex G 50 column, which was directly substituted by the chromatography through a Lichroprep RP-18 column (Merck). In particular, the partially pure syingicin (19 mg each time of a raw sample of 38 mg coming out from the third step) was eluted stepwise with H_2O containing CH_3CN (0, 1, 5, 10, 20, 30, 40, 50 and 100% of acetonitrile in water, 150 ml every step) at low pressure (3 bar). Fractions of 5 ml were collected and monitored by UV absorbance at 210 nm. The purity of syr in every step was controlled by SDS–PAGE, whereas alkaline native PAGE was only used for the monitoring the purity after the final purification step.

3.5. Electrospray–mass spectrometry (ES–MS)

Syr was analysed by ES–MS using a Platform single quadrupole mass spectrometer (micromass). Aliquots (1 μg/10 μl) of protein solution were injected into the ion source at a flow rate of 2 μl/min. The mass spectrometer was scanned from m/z 1000 to 2500 at 10 s/scan, using a capillary voltage of 3.6 kV and a cone voltage of 40 V.

3.6. Cyanogen bromide cleavage

Cleavage of native syr with cyanogen bromide was carried out in 70% formic acid as already reported (Parente et al., 1993).

3.7. S-Pyridylethylation

Samples of native (10 μg) or CNBr-treated (25 μg) syr were *S*-pyridylethylated as previously reported (Scudiero et al., 1995). At the end of the incubation time the modified syr absorbed on ProSorb™ to remove reagents and solvent. The modified protein was then sequenced at the N-terminus. CNBr-treated syr, after *S*-pyridylethylation, was brought to 50% formic acid and chromatographed by RP-HPLC on a Beckman C_{18} column, using acetonitrile containing 0.1% TFA as eluant.

3.8. Amino acid composition

Samples of native syr were hydrolysed at 110°C for 20 h in the presence of 0.02% phenol. Amino acid analyses were obtained with an amino acid analyser (Beckman) equipped with the post-column, ninhydrin detection system. Chemicals and experimental were as suggested by the manufacturer.

3.9. pI Determination

Theorical pI was determined by CHARGPRO in PC-GENE software package.

3.10. Amino acid sequencing

Samples of native, *S*-pyridylethylated syr or CB-peptides were sequenced by automated Edman degradation on a pulsed phase sequencer (Model 473A; Applied Biosystems) as previously reported (Parente et al., 1993).

3.11. CPase A digestion and MALDI–mass spectrometry (MALDI–MS)

Cyanogen bromide peptide CB-4 was dissolved in 0.02 M ammonium bicarbonate and incubated with CPase A (Sigma; final enzyme: substrate ratio of 1:50, w:w), at 37°C. Aliquots of 10 μl were removed from the incubation mixture after 0, 5, 10, 15 and 20 min and analysed by MALDI-MS. The timed aliquots (1 μl, about 0.5 μg) of CPase A-treated CB-4 peptide were prepared in the following way, using α-cyano-4-hydroxycinnamic acid (Fluka) as matrix. The sample was loaded on the target and dried. Then 1 μl of a mixture composed by 0.1% TFA in water–ethanol–10 μg/μl of matrix in H_2O (l:1:1; v/v). The sample was analysed using a Voyager (Persective Biosystem) mass spectrometer in linear mode.

3.12. Hypersensitivity induction assay

Induction of hypersensitivity by pure syr was determined by infiltrating sterile distilled water solutions of protein into tobacco leaves (*Nicotiana tabacum* L., cv. Rustica). A hypersensitive response (HR) was scored when a brown necrosis occurred in the infiltrated area 48 h after inoculation. HR was scored on the basis of necrotic areas: 0 = necrosis of 0–5% infiltrated tissues; 1 = 6–25% infiltrated tissues; 2 = 26–50% infiltrated tissues; 3 = 51–75% infiltrated tissues; 4 = 76–100% infiltrated tissues. To test the induction of distal HR, petiole dip assays (Huet et al., 1992) were carried out on cut tobacco leaves. Leaf petioles were dipped into a 50 ml-solution of protein. The solution was taken up after

about 2 h. Then the leaves were transferred to sterile water. Necrotic lesions were visible after 24–48 h and ranged from minute necrotic spots to large, confluent necrotic areas.

3.13. Electrolytes leakage assay (LEA)

Thirty tobacco leaf discs (5 mm diameter) were incubated overnight in 0.0, 0.2, 1.0 and 2.0 mg/ml (corresponding to 0.0, 0.02, 0.1 and 0.2 mM, respectively) of syr, respectively. Than six leaf disks were transferred in sterile distilled water 20 ml and the corresponding conductance was measured in microsiemens using a conductivity meter CRISON 525 with 20 electrodes (range 20 μS–200 mS cm; $K=1$) connected to a computer with a specific program. Each value was the mean of at least five replicate samples and was calculated as the difference from the reading at the beginning of the assay, after 1, 3, 6, 9 and 27 h. These values where then corrected by subtracting those obtained for the control.

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