Mutational Analysis of a Plant Defensin from Radish (Raphanus sativus L.) Reveals Two Adjacent Sites Important for Antifungal Activity*

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Genoveva W. De Samblanx‡, Inge J. Goderis‡, Karin Thevissen‡, Romaan Raemaekers‡, Franky Fant§, Frans Borremans§, David P. Acland¶, Rupert W. Osborn¶, Sunil Patel¶, and Willem F. Broekaert‡‡*‡‡

From the ‡F. A. Janssens Laboratory of Genetics, Katholieke Universiteit Leuven, Willem De Croylaan 42, B-3001 Heverlee, Belgium, the §Department of Organic Chemistry, Biomolecular NMR Unit, Universiteit Gent, Krijgslaan 248, S4bis, B-9000 Gent, Belgium, ¶Zeneca Agrochemicals, Jealott’s Hill Research Station, Bracknell, Berkshire, RG42 6ET, United Kingdom, and the ¶Department of Biochemistry and Molecular Biology, University College London, Gowerstreet, London WC1 E6BT, United Kingdom

Mutational analysis of Rs-AFP2, a radish antifungal peptide belonging to a family of peptides referred to as plant defensins, was performed using polymerase chain reaction-based site-directed mutagenesis and yeast as a system for heterologous expression. The strategy followed to select candidate amino acid residues for substitution was based on sequence comparison of Rs-AFP2 with other plant defensins exhibiting differential antifungal properties. Several mutations giving rise to peptide variants with reduced antifungal activity against Fusarium culmorum were identified. In parallel, an attempt was made to construct variants with enhanced antifungal activity by substituting single amino acids by arginine. Two arginine substitution variants were found to be more active than wild-type Rs-AFP2 in media with high ionic strength. Our data suggest that Rs-AFP2 possesses two adjacent sites that appear to be important for antifungal activity, namely the region around the type VI β-turn connecting β-strands 2 and 3, on the one hand, and the region formed by residues on the loop connecting β-strand 1 and the α-helix and contiguous residues on the α-helix and β-strand 3, on the other hand. When added to F. culmorum in a high ionic strength medium, Rs-AFP2 stimulated Ca²⁺ uptake by up to 20-fold. An arginine substitution variant with enhanced antifungal activity caused increased Ca²⁺ uptake by up to 50-fold, whereas a variant that was virtually devoid of antifungal activity did not stimulate Ca²⁺ uptake.

During the last decades, it has been recognized that many living organisms produce small antimicrobial peptides to protect their tissues from infectious microbial agents. Well known examples of peptides with antimicrobial properties are thionins (4), antimicrobial peptides (AMPs) (6), and cecropins (1) and magainins (2) of invertebrates (reviewed in Ref. 1) and mammalian defensins (3–5) of mammals and insects and are therefore termed plant defensins (8). Plant defensins are small cysteine-rich peptides consisting of 45–54 amino acids with a cysteine-stabilized α-helix motif (9), which is present in plant defensin A (10). Their three-dimensional structure consists of three antiparallel β-strands and an α-helix (11) and is similar to that of insect defensins (5) and some scorpion toxins (e.g. charybdotoxin; Ref. 12). Most plant defensins hitherto isolated exhibit antifungal activity. Some of them, for example SIα2 (Sorghum bicolor inhibitor 2 of α-amylase), are inhibitors of α-amylases but do not inhibit fungal growth (13, 14). The plant defensins with antifungal activity can be divided in two groups. The first group causes morphological distortions of the fungal hyphae resulting in swollen and hyperbranched fungal structures (9, 14). The second group merely inhibits fungal growth without inducing morphological changes. Mode of action studies performed on a representative of each class (Rs-AFP2 from radish and Dm-AMP1 from dahlia) has shown that plant defensins cause rapid ion fluxes upon addition to fungal hyphae, resulting in Ca²⁺ uptake, K⁺ efflux, and medium alkalinization (15).

In this study we have performed a structure-function analysis of Rs-AFP2, a plant defensin isolated from radish seed and member of the plant defensin group causing hyperbranching of fungal hyphae. It is the most potent among a number of plant defensin isoforms occurring in radish, including Rs-AFP1 isolated from seed and Rs-AFP3 and Rs-AFP4 isolated from invertebrates (4).

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** To whom correspondence should be addressed. Tel.: 32-16-322403; Fax: 32-16-322990; E-mail: willem.broekaert@agr.kuleuven.ac.be.
Mutational Analysis of a Plant Defensin

Table I

| Name       | Mutation         | Sequence                  |
|------------|------------------|---------------------------|
| OWB35      |                  | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB36      |                  | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB61      |                  | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB41      | Q6M              | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB43      | T10G             | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB45      | G16M             | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB46      | A31W             | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB47      | F40M             | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB48      | P41L             | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB49      | K44Q             | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB50      | Y48I             | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB51      |                  | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB52      | G9R              | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB53      | S12R             | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB54      | I46R             | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB55      | L28R             | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB56      | N37R             | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB57      | V39R             | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB58      | A42R             | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB59      | I26R             | 5′-GGCTAATTGGGTGCTGGAGG-3′ |
| OWB60      | F49R             | 5′-GGCTAATTGGGTGCTGGAGG-3′ |

The mutational analysis of the Rs-AFP2 coding sequence was performed by two sequential polymerase chain reactions (PCR) as described in Ref. 18. Primers used in the PCR reaction are listed in Table I. In the first PCR, the Rs-AFP2 coding sequence was amplified using a sense mismatch primer containing the desired mutation and primer OWB35, a derivative of the M13 reverse primer elongated with a 5′ tag (28 cycles; 1 min at 94°C, 1 min at 55°C, 1 min at 72°C). For design of the mismatch primer, the yeast preferential codon usage was taken into account (17). Ten ng of Poul I-linearized plasmid pBlueScript/RsAFP* (20) was used as a template for the first PCR. The amplified product containing the mismatch served as a mega-primer to further elongate the Rs-AFP2 sequence (6 cycles; 1 min at 94°C, 1 min at 55°C, 1 min at 72°C). In a second PCR, this elongated fragment was amplified by primer OWB61, binding to the 5′ end of the Rs-AFP2 gene, and OWB36, an oligonucleotide identical to the 5′ tag of OWB35 (28 cycles; 1 min at 94°C, 1 min at 55°C, 1 min at 72°C). OWB61 contains a restriction site allowing in-frame cloning into the HindIII site in the MF41 pro-sequence region of pVD4 (20). Amplification products of the second PCR were digested in HindIII-BamHI and introduced in the corresponding sites of pVD4. After verification of the occurrence of the desired mutations by nucleotide sequence determination, the expression blocks containing the MF41 promoter and propeptide sequence followed by the mutated Rs-AFP2 gene were isolated by SalI-BamHI restriction digestion and subcloned into the SalI-BgII-digested yeast shuttle vector pTG3828 (21). After subcloning, the sequence of the mutated Rs-AFP2 domain was verified by nucleotide sequencing. Restriction enzymes were purchased from Boehringer Mannheim (Mannheim, Germany), T4 DNA ligase from Life Technologies, Inc. (Life Technologies, Merelbeke, Belgium), and Taq DNA polymerase from Appligene (Pleasanton, CA). DNA sequencing was performed on a Pharmacia A.L.F. DNA sequencer using the AutoRead Sequencing Kit (Pharmacia, Uppsala, Sweden) according to the manufacturer’s instructions.

Heterologous Expression and Purification of Rs-AFP2 Variants—Transformation of S. cerevisiae, growth of the yeast cultures, and purification of the Rs-AFP2 variants from the culture supernatants were essentially done as described previously for native Rs-AFP2 (20). Briefly, 250 ml of culture supernatant (minimal selective SD medium: 0.8 g/liter CSM-URA from BDP 101, La Jolla, CA; 6.5 g/liter yeast nitrogen base from Difco; 20 g/liter glucose (Merck); 5 g/liter casamino acids from Difco) was passed over an anion-exchange chromatography column (Q-Sepharose Fast Flow, Pharmacia) connected on-line with a disposable reversed phase C8 silica column (Bond Elut, 500 mg solid phase, Varian, Harbor City, CA). The C8 silica column was subsequently rinsed with 6 ml of 10% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. The Rs-AFP2 variants were eluted from the latter column with 4 ml of 30% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. After drying in a rotating vacuum concentrator, the eluted fractions were purified by reversed-phase chromatography on a C8/AQ silica column (Bond Elut, 500 mg solid phase, Varian, Harbor City, CA). The C8 silica column was subsequently rinsed with 6 ml of 10% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. The Rs-AFP2 variants were eluted from the latter column with 4 ml of 30% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. After drying in a rotating vacuum concentrator, the eluted fractions were purified by reversed-phase chromatography on a C8/AQ silica column (Bond Elut, 500 mg solid phase, Varian, Harbor City, CA). The C8 silica column was subsequently rinsed with 6 ml of 10% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. After drying in a rotating vacuum concentrator, the eluted fractions were purified by reversed-phase chromatography on a C8/AQ silica column (Bond Elut, 500 mg solid phase, Varian, Harbor City, CA). The C8 silica column was subsequently rinsed with 6 ml of 10% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. After drying in a rotating vacuum concentrator, the eluted fractions were purified by reversed-phase chromatography on a C8/AQ silica column (Bond Elut, 500 mg solid phase, Varian, Harbor City, CA). The C8 silica column was subsequently rinsed with 6 ml of 10% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. After drying in a rotating vacuum concentrator, the eluted fractions were purified by reversed-phase chromatography on a C8/AQ silica column (Bond Elut, 500 mg solid phase, Varian, Harbor City, CA). The C8 silica column was subsequently rinsed with 6 ml of 10% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. After drying in a rotating vacuum concentrator, the eluted fractions were purified by reversed-phase chromatography on a C8/AQ silica column (Bond Elut, 500 mg solid phase, Varian, Harbor City, CA).

Protein Analysis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to Ref. 22 using a 15% (w/v) acrylamide, 0.5% (w/v) bisacrylamide separating gel and a 5% (w/v) acrylamide stacking gel. Gels were either stained with Coomassie Brilliant Blue R250 or immunoblotted using anti-Rs-AFP1 antibodies as described previously (8). Protein concentrations were determined by the bicinchoninic acid method (23) using authentic Rs-AFP2 as a standard. Free cysteine thiold groups were determined by the Ellman assay on both reduced and unreduced protein samples as described previously (17). Circular dichroism spectra were recorded on a Jasco J-810 spectropolarimeter with a cell path of 0.02 cm. Proteins were dissolved at 0.5 mg/ml in distilled water. The spectra were acquired in a single scan mode (10 nm/min) in the ultraviolet region of 265–185 nm. Circular dichroism data were base line-corrected and are presented in units of Δε (M⁻¹ cm⁻¹) (24).

Large Scale Purification of Rs-AFP2 Variants—Recombinant yeast (S. cerevisiae) cells containing vectors for expression of Rs-AFP2 (Y38G) and Rs-AFP2 (V39R), respectively, were grown for 7 days in a fermentor.

Recombinant yeast (S. cerevisiae) cells containing vectors for expression of Rs-AFP2 (Y38G) and Rs-AFP2 (V39R), respectively, were grown for 7 days in a fermentor.
Mutational Analysis of a Plant Defensin

I

| Reference | 1 | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 |
|-----------|---|---|----|----|----|----|----|----|----|----|----|
| Rs-AFP1   | 17 |  |    |    |    |    |    |    |    |    |    |
| Rs-AFP2   | 17 |  |    |    |    |    |    |    |    |    |    |
| Rs-AFP3   | 17 |  |    |    |    |    |    |    |    |    |    |
| Rs-AFP4   | 17 |  |    |    |    |    |    |    |    |    |    |
| At-AFP1   | 17 |  |    |    |    |    |    |    |    |    |    |
| At-AFP2   | 17 |  |    |    |    |    |    |    |    |    |    |

II

| Antibody | 14 |  |    |    |    |    |    |    |    |    |    |
|-----------|----|---|----|----|----|----|----|----|----|----|----|
| Ab-amylase | 14 |  |    |    |    |    |    |    |    |    |    |
| Ab-β-casein | 14 |  |    |    |    |    |    |    |    |    |    |
| Ab-D-dextrin | 14 |  |    |    |    |    |    |    |    |    |    |

III

| Index | 40 | 40 | 40 | 41 |
|-------|----|----|----|----|
| Slu2  | RVC-KMKAGFQKLRHDQNGQYVL-CGG-WGGPNGDGVY--RRQK1|
| Slu3  | RYR-RKAGFQKLRHDQNGQYVL-CGG-WGGPNGDGVY--RRQK1|
| γ1-Pur |  |  |  |  |

FIG. 1. Comparison of the complete amino acid sequences of various plant defensins. Plant defensins of the first subgroup (I) display antifungal activity against F. culmorum with concomitant increased hyphal branching. Plant defensins of the second subgroup (II) display antifungal activity without inducing morphological alterations of the fungal hyphae, whereas plant defensins of the third subgroup (III) do not show antifungal activity. Residues conserved among all plant defensins are shown in dark gray boxes. Residues conserved among the plant defensins of subgroup I or subgroup II and I but not among plant defensins of subgroup III are presented in light gray boxes. The lines above the sequence of Rs-AFP1 and underneath the sequence of γ1-Pur indicate secondary structure elements according to Refs. 37 and 11, respectively. Single lines represent β-strands and double lines α-helices. Abbreviations used are: Rs-AFP, Raphanus sativus antifungal protein; At-AFP, Arabidopsis thaliana antifungal protein; Hi-AFP, Heuchera sanguinea antifungal protein; Dm-AMP, Dahlia merkit antimicrobial protein; Ct-AMP, Clitoria ternatea antimicrobial protein; Slu, S. bicolor inhibitor of α-amylase; γ1-Pur, γ1-purothionin.

Mutational Analysis of a Plant Defensin

| Reference | 1 | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 |
|-----------|---|---|----|----|----|----|----|----|----|----|----|
| Rs-AFP1   | 17 |  |    |    |    |    |    |    |    |    |    |
| Rs-AFP2   | 17 |  |    |    |    |    |    |    |    |    |    |
| Rs-AFP3   | 17 |  |    |    |    |    |    |    |    |    |    |
| Rs-AFP4   | 17 |  |    |    |    |    |    |    |    |    |    |
| At-AFP1   | 17 |  |    |    |    |    |    |    |    |    |    |
| At-AFP2   | 17 |  |    |    |    |    |    |    |    |    |    |

II

| Antibody | 14 |  |    |    |    |    |    |    |    |    |    |
|-----------|----|---|----|----|----|----|----|----|----|----|----|
| Ab-amylase | 14 |  |    |    |    |    |    |    |    |    |    |
| Ab-β-casein | 14 |  |    |    |    |    |    |    |    |    |    |
| Ab-D-dextrin | 14 |  |    |    |    |    |    |    |    |    |    |

III

| Index | 40 | 40 | 40 | 41 |
|-------|----|----|----|----|
| Slu2  | RVC-KMKAGFQKLRHDQNGQYVL-CGG-WGGPNGDGVY--RRQK1|
| Slu3  | RYR-RKAGFQKLRHDQNGQYVL-CGG-WGGPNGDGVY--RRQK1|
| γ1-Pur |  |  |  |  |

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**RESULTS**

Conception and Production of Rs-AFP2 Variants—Proteins belonging to the family of plant defensins have been purified and sequenced from a range of taxonomically divergent plant species, while others have been identified via cDNA sequencing (9). Fig. 1 represents a comparison of the complete amino acid sequences of 12 different plant defensins whose antifungal activity against F. culmorum has been assayed in our laboratories (8, 14, 17, 33). In terms of biological activity, three groups of plant defensins can be discerned: group I, those who are inhibitory to F. culmorum and cause increased hyphal branching; group II, those inhibitory to F. culmorum without causing hyphal deformations; and group III, those not affecting growth of F. culmorum at concentrations below 100 μg/ml. Rs-AFP2, the protein studied in this work, belongs to the first group.

As can be seen from the alignment of the sequences in Fig. 1, the pattern of cysteines is totally conserved in all the se- sequences, as is the glycine residue at position 34 (numbering relative to the studied protein Rs-AFP2). Those residues are important secondary structure elements and are part of the cysteine-stabilized αβ motif characterized by the sequences...
CXXXC, GXC, and XCX (X stands for any amino acid) (34). Other well conserved residues are the serine at position 8, the glycine at position 13, and the glutamate at position 29. Those conserved residues were not considered for substitution in the present study, since it is likely that they play a role in determining the structure of the peptide.

A number of amino acid residues were found to be fully conserved among the antifungal plant defensins (group I and II) but subject to non-conservative changes in plant defensins devoid of antifungal activity (group III). Those residues, namely Gln-5, Thr-10, Gly-16, and Ala-31, were considered to be suitable candidate residues for site specific mutational analysis. Lys-44 and Tyr-48, which are conserved in all group I and II plant defensins, except Dm-AMP1 and Hs-AFP1, respectively, were also retained for mutational analysis. In addition, amino acids that are conserved in group I but not in group II could be important for causing the typical morphological deformation of fungal hyphae, which is characteristic for group I plant defensins. Those residues comprise Tyr-38, Phe-40, and Gly-16, and the glutamate at position 29. Those residues were considered for substitution in the present study.

For the production of the different Rs-AFP2 variants with the desired amino acid substitution, the Rs-AFP2 coding sequence was mutated site-specifically by PCR, fused in frame to the yeast mating factor α1 (MFA1) promoter and prepro-sequence (20, 21) and subsequently transferred to yeast via a yeast shuttle vector. The different Rs-AFP2 analogues were purified from the yeast culture supernatant by a combination of ion-exchange chromatography and reversed-phase chromatography. Using this approach, we have previously shown that wild-type Rs-AFP2 can be produced in a correctly processed and bioactive form in yeast (20). In total, 19 Rs-AFP2 variants were produced and purified in this way (see Table II). The purity of the preparations was assessed by SDS-PAGE analysis. All Rs-AFP2 variants migrated essentially as single bands and were recognized by anti-Rs-AFP1 antiserum on immunoblots prepared from SDS-PAGE gels, confirming their identity as variants of Rs-AFP2 (Fig. 2). In addition, all purified proteins were recognized by anti-Rs-AFP1 antiserum on immunoblots prepared from SDS-PAGE gels, confirming their identity as variants of Rs-AFP2 (results not shown).

Two Rs-AFP2 variants, Rs-AFP2(Y38G) and Rs-AFP2(V39R) with a substitution of the tyrosine at position 38 by glycine and of valine at position 39 by arginine, respectively, were purified on a large scale from 15-liter fermentation cultures of the appropriate recombinant yeast strains. Circular dichroism spectroscopic studies were performed on these variants as well as on authentic Rs-AFP2.

The circular dichroism spectrum of Rs-AFP2 was recorded in a scan from 190 to 280 nm for seed-purified Rs-AFP2 (solid lines), Rs-AFP2Y38G (broken lines), and Rs-AFP2V39R (dotted line). The protein concentration was 0.5 mg/ml.

### Table II

**Antifungal activity of Rs-AFP2 variants**

| Peptide variant | IC₅₀ value on Fusarium culmorum in μg/ml | SMF– | SMF+ |
|----------------|----------------------------------------|------|------|
| Rs-AFP2 (seed) | 2.7 ± 0.6 | 8.5 ± 2.7 |
| Rs-AFP2 (yeast) | 2.9 ± 0.8 | 8.1 ± 2.5 |
| Series 1 | | |
| Rs-AFP2(Q5M) | 4.1 ± 0.2 | 5.4 ± 1.2 |
| Rs-AFP2(I46G) | 11 ± 4.2 | >100 |
| Rs-AFP2(G16M) | 2.2 ± 0.3 | 5.0 ± 0.9 |
| Rs-AFP2(A31W) | 30 ± 5.0 | >100 |
| Rs-AFP2(Y38G) | 42 ± 17 | >200 |
| Rs-AFP2(F40M) | 16 ± 6.7 | 54 ± 13 |
| Rs-AFP2(P41R) | 100 ± 15 | >200 |
| Rs-AFP2(K44Q) | 3.5 ± 0.4 | 36 ± 9 |
| Rs-AFP2(Y48I) | 9.3 ± 1.0 | 11 ± 2.0 |
| Series 2 | | |
| Rs-AFP2(P7R) | 6.8 ± 2.4 | 8.8 ± 1.0 |
| Rs-AFP2(G9R) | 3 ± 0.5 | 3.3 ± 0.6 |
| Rs-AFP2(S12R) | 3.5 ± 1.0 | 20 ± 6.0 |
| Rs-AFP2(126R) | 7.2 ± 0.8 | 9.6 ± 3.7 |
| Rs-AFP2(L28R) | 6.4 ± 1.4 | >100 |
| Rs-AFP2(N37R) | 28 ± 0.3 | 7.0 ± 1.8 |
| Rs-AFP2(V39D) | 4.0 ± 0.2 | 32 ± 0.3 |
| Rs-AFP2(A32V) | 4.2 ± 2.5 | 18 ± 5.2 |
| Rs-AFP2(A46R) | 12 ± 2.4 | >40 |
| Rs-AFP2(F49R) | 22 ± 4.8 | 23 ± 3.0 |

**Fig. 2. SDS-PAGE analysis of Rs-AFP2 variants.** One-μg amounts of Rs-AFP2 variants were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. The molecular masses of the markers are indicated in kDa.
Rs-AFP2(V39R) was virtually identical to that of Rs-AFP2, indicating that neither the substitution itself nor the way the variant was synthesized in yeast had imposed alterations of backbone secondary structure elements (Fig. 3). Rs-AFP2-(Y38G) had a circular dichroism spectrum which was almost identical to that of Rs-AFP2, except for a slightly decreased steepness of the Δε drop in the 190–208 nm region (Fig. 3).

Antifungal Activity of Rs-AFP2 Variants—The purified Rs-AFP2 substitution variants were assessed for their antifungal activity against F. culmorum in two different media: a low ionic strength medium called SMF− (17), and the same medium supplemented with 1 mM CaCl₂ and 50 mM KCl, called SMF+. The presence of salts in the test medium, especially salts with divalent cations, is known to reduce the specific antifungal activity of Rs-AFP2 (17). Seed-purified as well as yeast-expressed wild-type Rs-AFP2 served as controls in the assays. The results of these comparative tests, expressed as IC₅₀ values, are presented in Table II. Most of the variants of the first series (substitutions by corresponding Slto2 residues) showed no or only a minor decrease of their antifungal activity in medium SMF−, except Rs-AFP2(A31W), Rs-AFP2(Y38G), and Rs-AFP2(P41Δ), which showed a substantial decrease in antifungal potency. In SMF+, the medium with added salts, a significant decrease in antifungal activity was observed for the following Rs-AFP2 analogues of the first series: Rs-AFP2(T10G), Rs-AFP2(A31W), Rs-AFP2(Y38G), Rs-AFP2(F40M), Rs-AFP2(P41Δ), and Rs-AFP2(K44Q). On the other hand, the substitutions Q5M and G16M resulted in a slight but significant increase in antifungal potency, especially noticeable in medium SMF+, whereas the substitution Y48I had little or no effect on the antifungal activity.

In contrast to what was expected, most of the Rs-AFP2 variants of the second series (arginine substitutions) did not show an enhanced antifungal activity compared to Rs-AFP2. In some cases, an even lower antifungal activity was observed, possibly caused by the unfavorable presence of a positive charge at that position or by the absence of a residue necessary for interaction with the fungal target. The largest decrease in antifungal activity was observed for substitution variants Rs-AFP2(L28R) and Rs-AFP2(I46R), whereas variants Rs-AFP2(S12R), Rs-AFP2(I42R), and Rs-AFP2(I49R) showed only a modest reduction in antifungal activity. However, two Rs-AFP2 variants, namely Rs-AFP2(G9R) and Rs-AFP2(V39R), were about 2-fold more active than wild-type Rs-AFP2 when assessed in SMF+.

The antifungal activity of Rs-AFP2(V39R) purified from a large scale culture of recombinant yeast was further characterized in SMF with increasing Ca²⁺ or K⁺ concentrations and compared with that of authentic Rs-AFP2 (isolated from seed) as well as yeast-purified Rs-AFP2. As is shown in Fig. 4, the antifungal activity against F. culmorum of Rs-AFP2(V39R) was less reduced by the presence of cations in the growth medium in comparison with wild-type Rs-AFP2. Indeed, in the presence of 5 mM CaCl₂ and at a concentration of 10 μg/ml, Rs-AFP2(V39R) caused complete inhibition of the growth of F. culmorum, whereas wild-type Rs-AFP2 was basically inactive under the same conditions. At 10 μg/ml, wild-type Rs-AFP2 was fully active against F. culmorum only when the CaCl₂ concentration was equal or lower than 1.25 mM. Likewise, the activity of wild-type Rs-AFP2 was drastically reduced in the presence of 100 mM KCl, whereas Rs-AFP2(V39R) was still fully inhibitory to F. culmorum at this KCl concentration.

The potency of Rs-AFP2(V39R) relative to authentic Rs-AFP2 was also assessed on a set of seven different phytopathogenic fungi in three media differing in ionic strength: SMF−, SMF including 1 mM CaCl₂ and 50 mM KCl (SMF+), and SMF including 5 mM CaCl₂ and 50 mM KCl. As can be seen from the data presented in Table III, the relative antifungal activity of the variant was dependent on the test organism. On three fungi (F. culmorum, N. hematococca, and V. dahliae), Rs-AFP2(V39R) was more active than Rs-AFP2. In the medium SMF+, for

![Fig. 4. Cation sensitivity of the antifungal activity of variant Rs-AFP2(V39R) compared to wild-type Rs-AFP2.](image)

**Table III**

| Fungus         | IC₅₀ values (µg/ml) | SMF | SMF + 1 mM CaCl₂ | SMF + 5 mM CaCl₂ |
|----------------|---------------------|-----|------------------|------------------|
|                | Rs-AFP2(V39R)       |     | Rs-AFP2       | Rs-AFP2(V39R)   | Rs-AFP2       | Rs-AFP2(V39R)   |
| A. brassicola  | 3.2                 | 2.5 | >50             | 50               | >100           | >100             |
| A. pisi        | 1.9                 | 2.0 | >50             | >50              | >100           | >100             |
| B. cinerea     | 1.8                 | 1.6 | >50             | >50              | >100           | >100             |
| F. culmorum    | 2.1                 | 2.2 | 4.6             | 2.3              | 22             | 7                |
| N. hematococca | 2                   | 2.0 | 48              | 9                | >100           | 62               |
| P. beta        | 0.9                 | 1.4 | 14              | 40               | 27             | 70               |
| V. dahliae     | 1                   | 0.4 | 11              | 2.3              | 50             | 6                |
instance, Rs-AFP2(V39R) was about 2-, 5-, and 5-fold more potent than Rs-AFP2 against *F. culmorum*, *N. hematococca* and *V. dahliae*, respectively. As in this medium neither Rs-AFP2 nor Rs-AFP2(V39R) inhibited growth of *A. brassicicola*, *A. pisi*, or *B. cinerea* at concentrations below 50 μg/ml, the highest concentration tested, no difference in antifungal potency could be observed for these fungi. However, on *P. betae*, Rs-AFP2(V39R) was less potent than Rs-AFP2. The differences in antifungal potency between Rs-AFP2(V39R) and Rs-AFP2 were always more pronounced in the SMF media with added salts than in the low ionic strength medium SMF–.

**Effect of Rs-AFP2 Variants on Ca2+ Uptake by Fungi**—Although the precise molecular target of Rs-AFP2 on fungal hyphae is not yet known, recent work in our laboratory has shown that Rs-AFP2 causes very rapid ion fluxes, including increased Ca2+ uptake, when added to fungal hyphae (15). To investigate whether the ability of Rs-AFP2 to stimulate Ca2+ uptake in fungi is linked to its antifungal effect, 45Ca2+ uptake was measured in *F. culmorum* treated with different concentrations of either Rs-AFP2, the virtually inactive variant Rs-AFP2(Y38G), and the variant with increased antifungal potency, Rs-AFP2(V39R). The medium used for this test consisted of half-strength potato dextrose broth supplemented with 1 mM MgCl2 and 50 mM KCl. As shown in Fig. 5, Rs-AFP2 caused a dose-dependent increase of 45Ca2+ uptake, which at a dose of 100 μg/ml reached a level that was about 20-fold higher relative to water-treated controls. At the same dose, Rs-AFP2(V39R) stimulated 45Ca2+ uptake by over 50-fold, and the higher 45Ca2+ uptake stimulation of Rs-AFP2(V39R) versus wild-type Rs-AFP2 was observed over the whole concentration range tested. In marked contrast, however, addition of the variant Rs-AFP2(Y38G) with impaired antifungal properties resulted in 45Ca2+ uptake rates that fluctuated around the levels observed for water-treated control cultures.

**DISCUSSION**

A structure-activity analysis of Rs-AFP2, a plant defensin from radish causing growth inhibition of fungal hyphae (17), was carried out in order to investigate which residues are important for antifungal activity of the peptide. Candidate amino acid residues were considered to be those conserved among plant defensins exhibiting antifungal activity but not among those devoid of antifungal activity as outlined in Fig. 1. Following this rationale, we have chosen to produce a series of nine Rs-AFP2 analogues in which particular amino acid residues were changed to the corresponding residue of the plant defensin SIα2, which does not display antifungal activity. Residue Pro-41 was deleted rather than substituted as the loop encompassing this residue is shorter in SIα2 than in Rs-AFP2. A second series of Rs-AFP2 variants was aimed at increasing the net positive charge (at physiological pH) of Rs-AFP2 by substituting particular residues by an arginine at various non-conserved positions along the Rs-AFP2 sequence. This approach was inspired by the fact that Rs-AFP2, which has a higher net positive charge than Rs-AFP1, has a 2–30-fold higher activity relative to Rs-AFP1 (17).

Wild-type and variant peptides were produced in yeast and purified using identical chromatographic procedures. After the last purification step consisting of reversed phase chromatography, the different peaks were assayed for antifungal activity in order to identify the elution position of the Rs-AFP2 variant. All peptides showed similar retention times. When analyzed by

![FIG. 5. Stimulation of 45Ca2+ uptake in *F. culmorum* by Rs-AFP2 variants. Yeast-purified Rs-AFP2 (circles), Rs-AFP2(Y38G) (squares), and Rs-AFP2(V39R) (triangles) were added to a suspension of *F. culmorum* (∼1 mg biomass dry weight/ml) in half-strength potato dextrose broth supplemented with 1 mM MgCl2 and 50 mM KCl, and 45Ca2+ uptake was measured 30 min upon addition of the proteins. Values are expressed as relative (in %) to 45Ca2+ uptake of a suspension treated with water and are means of four replicates.](https://example.com/fig5)

![FIG. 6. Stereoview of Rs-AFP1 with indication of the position of residue Ala-31. The atoms of residue Ala-31 are shown as black balls, while the atoms of the disulfide linkages are shown as white balls.](https://example.com/fig6)
The presence of glycine in the type VI b-sheet. The conformation of the other variants was not verified, but the absence of free thiol groups indicated that the disulfide bridges had formed.

Within the first substitution series, variants that showed a clearly reduced activity on *F. culmorum* were Rs-AFP2(T10G), Rs-AFP2(A31W), Rs-AFP2(Y38G), Rs-AFP2(F40M), and Rs-AFP2(P41Δ). The importance of the residues at positions 10, 38, and 40 is underscored by our observation that substitution in antifungal activity of these variants was due to the unfavorable presence of an extra charge or to the replacement of an amino acid essential for the antifungal activity.

Remarkably, the loss in antifungal potency in all these cases was less noticeable in the low ionic strength medium than in the medium supplemented with 1 mM CaCl₂ and 50 mM KCl. This may be explained by assuming that the interaction between Rs-AFP2 and its putative receptor on fungal hyphae is based both on ionic interactions and non-ionic stereospecific interactions. Upon increasing the ionic strength of the medium, the ionic interactions with the putative receptor are weakened due to competition between Rs-AFP2 and inorganic cations. In the case where non-ionic stereospecific interactions are weakened due to an unfavorable substitution, the overall interaction is also expected to become more susceptible to ionic competition.

The two most interesting Rs-AFP2 analogues of the arginine substitution series are Rs-AFP2(G9R) and Rs-AFP2(V39R). Although these variants show no significantly increased activity on *F. culmorum* in the low ionic strength medium, their activity on this fungus is much less influenced by the presence of cations in comparison with wild-type Rs-AFP2. This is again consistent with our model, which predicts that the interaction between Rs-AFP2 and its putative receptor is based both on ionic and non-ionic interactions. Introducing an extra charged residue at positions 9 or 39 may reinforce the ionic interactions, leading to variants that are at an advantage in competing with cations for binding at the putative receptor site.

The relative antifungal potency of the arginine substitution variant Rs-AFP2(V39R) compared to Rs-AFP2 appeared to be dependent on the test fungus. Rs-AFP2(V39R) was more active on *F. culmorum, N. hematococca, and V. dahliae* (three taxonomically related fungi belonging to the family Nectriaceae), but less active on *P. betae*. This suggests that the putative receptor on hyphae of different fungal species may reveal conformational or compositional differences.

As relatively high ionic strength conditions occur in all plant cell compartments (17), Rs-AFP2 variants such as Rs-AFP2(G9R) and Rs-AFP2(V39R) displaying a decreased cation antagonism in their activity against some phytopathogenic fungi could be useful for plant transformation experiments aimed at obtaining disease-resistant crops. We have previously shown that transgenic tobacco plants expressing wild-type Rs-AFP2 are more resistant to the fungal pathogen *Alternaria longipes* than untransformed plants (8). Further enhancement of the resistance level may be achieved through the expression of either Rs-AFP2(G9R) or Rs-AFP2(V39R) in transgenic plants.

We have previously shown that Rs-AFP2 stimulates Ca²⁺ uptake by fungal hyphae, an effect that can be observed within minutes after addition of the peptide (15). This stimulation of Ca²⁺ uptake may be part of the responses triggered by the interaction of Rs-AFP2 with its putative receptor. Our results now seem to indicate that antifungal activity and ability to trigger enhanced Ca²⁺ uptake are correlated. Indeed, the variant Rs-AFP2(Y38G), which is virtually devoid of antifungal activity in presence of inorganic salts, was unable to stimulate Ca²⁺ uptake in *F. culmorum*. On the other hand, the arginine substitution variant Rs-AFP2(V39R) displaying enhanced antifungal potency caused about 2.5-fold higher Ca²⁺ uptake than Rs-AFP2. Controlled Ca²⁺ influx is believed to be essential for directing polar growth at the tip of fungal hyphae (35). For pollen tubes, which like fungal hyphae grow at their tip, it has been documented that various treatments resulting in elevated cytosolic Ca²⁺ levels invariably lead to growth arrest (36).
Fig. 8. Three-dimensional representation of Rs-AFP2 with indication of the residues affecting antifungal activity when substituted. The Rs-AFP1 molecule is represented in four orientations obtained by rotations of 90° about the vertical axis, with the ribbon presentation of the backbone at the top and the corresponding space-filling models at the bottom. The residues that caused a reduction of the antifungal activity in the high ionic strength medium are shown in dark blue (Thr-10, Leu-28, Tyr-38, Phe-40, Lys-44, Ile-46); and those which caused a reduction between 2- and 4-fold when substituted are indicated in light blue (Ser-12, Ala-42, Phe-49). The residues that enhanced the antifungal activity by more than 2-fold when substituted by arginine are marked in red (Gly-9, Val-39). Residue Ala-31, which is likely to entail major conformational changes in the backbone structure when substituted by tryptophan, and the deleted residue Pro-41 are indicated in green.

The three-dimensional structure of Rs-AFP1 has been studied by two-dimensional 1H NMR, which has revealed that Rs-AFP1 consists of an α-helix (Asn-18–Leu-28) and a triple-stranded antiparallel β-sheet (β-strand 1: Lys-2–Arg-6; β-strand 2: His-33–Tyr-38; β-strand 3: His-43–Pro-50) (Fig. 6; Ref. 37). Meanwhile, the structure of Rs-AFP1 has been refined down to a root mean square deviation of 1.60 Å for all heavy atoms of the backbone, and the results of this refinement will be presented elsewhere. Since Rs-AFP1 is near-identical to Rs-AFP2, it is assumed that it adopts the same conformation. The spatial orientation of the residues affecting the antifungal activity of Rs-AFP2 upon substitution was analyzed using the high resolution structure of Rs-AFP1. According to the Rs-AFP1 model, all residues substituted in the present study do face outwards of the peptide backbone and are therefore unlikely to be essential for structure stabilization. The only exception is residue Ala-31, which is positioned at the interior face of the hairpin loop connecting the α-helix to β-strand 2 (Fig. 6). Substitution of Ala-31 by a bulky tryptophan residue in Rs-AFP2(A31W) most probably results in a conformational distortion, which might explain the drastic reduction of the antifungal activity of this variant. In addition, deletion of Pro-41, which adopts a cis-configuration in Rs-AFP1 as part of a type VI β-turn, is also likely to entail a distortion of at least the domain encompassing the second and third β-strand and the interconnecting type VI β-turn.

A graphical overview of the specific antifungal activity determined on F. culmorum of the different amino acid substitution variants when assayed in high ionic strength medium is provided in Fig. 7. When those residues affecting the antifungal activity are visualized on a three-dimensional model (Fig. 8), it becomes apparent that they all cluster into two adjacent sites. A first site is formed by the residues Tyr-38, Phe-40, Pro-41, Ala-42, Lys-44, and Ile-46. Except for Pro-41 and Lys-44, all those residues are highly hydrophobic. When Lys-44 was substituted by the neutral residue Gln, a substantial decrease of the antifungal potency was observed, suggesting that a positive charge within this predominantly hydrophobic cluster is important for the antifungal activity. This is further substantiated by the observation that the introduction of an additional positive charge within this site at position 39 resulted in enhanced antifungal activity in the presence of inorganic salts. The second site is formed by Thr-10, Ser-12, Leu-28, and Phe-49, which form a patch of contiguous residues despite their scattered positions along the Rs-AFP2 sequence (Fig. 7). Here again, introducing a positive charge within the cluster, namely at position 9, resulted in an enhanced antifungal potency in the high ionic strength medium.

The two regions important for the antifungal activity of Rs-AFP2 might constitute two sites contacting a single putative receptor. Alternatively, the presence of two sites could be indicative of two binding sites on each of two receptor molecules. The latter possibility has been proposed in a model for the interaction between the human growth hormone and its receptor (38). In the case of the human growth hormone, a mutational analysis has also revealed two domains that are involved in the interaction with the human growth hormone receptor. Each of the two domains interacts with a receptor molecule, entailing receptor dimerization, the initial trigger in the signal transduction pathway (39). The physiological meaning of the two functional sites of Rs-AFP2 will remain an open question until its putative receptor has been identified and characterized.

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