Description

Utilization of an antifungal protein from Streptomyces tendae against plant pathogenic fungi

The present invention relates to the use of an antifungal protein (AFP) having a molecular weight of approx. 10 kDa from Streptomyces tendae against plant pathogenic fungi from the Ascomycetes family.

Antifungal proteins have been described for some time, some of these proteins also being termed PR proteins (plant pathogenesis-related proteins) in the case of plants (Bol, J.F. & Linthorst, H.J.M. (1990), Annu. Rev. Phytopathol., 28, 113). These proteins are formed by plants under stress conditions, for example in the case of viral or fungal infection, inducing an active defense mechanism of the plant which is termed "induced resistance" (Lindhorst, H.J.M., (1991) Cri. Rev. Plant Sci., 10(2), 123). Some of these proteins have, for example, a chitinase activity or β-1,3-glucanase activity.

Proteins with an antifungal activity have already been detected from microorganisms too, some of these proteins also showing chitinase activity or β-1,3-glucanase activity. Other proteins, in contrast, act via an interaction of the fungal cell wall.

For example, an antifungal protein (AFP) having a size of approx. 10 kDa which is active against the fungal species Paecilomyces varriotii, Byssoclamis nivea, Pencillium puberulum and Eupenicillium terrum has been isolated from Streptomyces tendae. However, the action was restricted to these species. Other species such as, for example, Paecilomyces carneus, Paecilomyces lilacenus, Penicillium chrysogenum or Penicillium claviforme are not inhibited. In addition, the use of

AFP is not justified commercially either since the fungal species mentioned are not plant pathogenic.

The object of the present invention was therefore to find an antifungal protein which is active against plant pathogenic fungi.

Surprisingly, it has now been found that AFP from Streptomyces tendae is active against plant pathogenic fungi from the Ascomycetes family.

The subject-matter of present the invention is therefore the use of an antifungal protein (AFP) with a molecular weight of approx. 10 kDa from Streptomyces tendae against plant pathogenic fungi from the Ascomycetes family, in particular against Botrytis cinera.

The antifungal protein mentioned contains a typical amino-terminal signal sequence, i.e. a hydrophilic N-terminus is followed by a hydrophobic transmembrane region, which cause extracellular secretion. Moreover, two forms are known, namely a shorter form with a molecular weight of approx. 9 860 Da and a longer form with a molecular weight of approx. 10 300 Da.

Since AFP is secreted by Streptomyces tendae into the culture medium, it can be isolated for example directly from the Streptomyces tendae culture filtrate, following methods known to the skilled worker. As an alternative, AFP can be produced by genetic engineering, the gene isolated preferably being cloned into a so-called multicopy vector, for example plJ702 (Hopwood, D.A. et al. (1985) Genetic Manipulation of Streptomyces. A Laboratory Manual. Norwich, U.K.) and using this construction to transform a suitable strain, for example the non-nikkomycin-producing strain Streptomyces tendae NP9. An example of an AFP-encoding

nucleic acid is shown in SEQ ID No. 1. When this preferred transformant was grown, for example, for 7 days in 200 ml of nutrient solution, approx. 6 mg of AFP were obtained, which corresponds to 30 mg of AFP/l. The production of AFP by genetic engineering via a preferably controlled fermentation, is therefore especially preferred.

For the use in accordance with the invention, AFP can be applied directly for example in the form of a formulation together with preferably at least one additional auxiliary. To this end, for example, plants attacked by fungi or endangered by fungal attack are sprayed with the formulation described. The AFP-concentration of the formula is generally from approx. 10 µg/ml up to approx. 500 mg/ml. Suitable auxiliaries are, for example, protease inhibitors, stabilizers such as glycerol, sucrose, salts, solvents or generally known substances from the field of crop protection.

So-called transgenic plants which are capable of producing AFP themselves are another possibility of providing protection against fungal infection.

Another embodiment is therefore the use according to the invention of AFP, the AFP being formed in a transgenic plant. The transgenic plant is, preferably, genetically engineered maize, cotton, potato, banana, Arabidopsis, casava, tobacco, oilseed rape (canola), potato, sugar beet, cereals such as, for example, wheat, barley or oats, strawberries, vegetables such as, for example, cabbage, legumes such as, for example, peas or beans, tomato, lettuce or melon.

To generate a transgenic plant, a nucleic acid encoding AFP, for example in the form of naked DNA, viral DNA or RNA, or in the form of plasmid DNA, is introduced into a plant cell. An example of an AFP-encoding nucleic

acid is shown in SEQ ID No. 1. However, the present invention also covers those nucleic acids which differ from the nucleic acid sequence of SEQ ID No. 1 owing to the degeneracy of the genetic code, but which encodes the same AFP amino acid sequence. Moreover, the invention covers those mutants or variants of the nucleic acid of SEQ ID No. 1 which encode an AFP protein which is active against plant pathogenic fungi of the Ascomycetes family, in particular against Botrytis cinera. These include, for example, fusion proteins of the AFP protein with other foreign proteins or an AFP protein with an N-terminally deleted methionine. Other examples of variants are nucleic acids which hybridize on the stringent conditions with the nucleic acid of SEQ ID No. 1. The stringent hybridization conditions can be determined for example by Sambrook, J. et al. in Molecular Cloning, A Laboratory Manual, 2nd Edition, Cold Spring Habour Laboratory Press, 1989. Transgenic plants are subsequently regenerated from the transformed plant cells. Preferably, an AFP-encoding nucleic acid can be introduced into the plant cell by means of recombinant agrobacteria, by electroporation, by bombardment with microparticles and/or by means of polyethylene glycol.

The infection with recombinant Agrobacterium tumefaciens bacteria in plant cells of dicots is described, for example, by Klee, H. et al. (1997) Annu. Rev. Plant Physiol. 38, 467 or EP-B2-0122791). The infection with recombinant Agrobacterium tumefaciens bacteria in plant cells of monocots is described, for example, by Ishida, Y. et al. (1996) Nature Biotech. 14, 745 with reference to maize (Zea mays L.). The agrobacteria used for this purpose have a T-DNA into which the AFP-expressing gene and, if appropriate, a suitable promoter, for example a plant phaseolin promoter (see, for example, EP-B2-0122791) or a viral 35S promoter (see, for example, Ishida, Y. et al.

(1996), above) had been inserted. The T-DNA can be transferred into plant cells for example by coculturing the recombinant agrobacteria together with immature embryos of the plant in question (see, for example, Ishida, Y. et al. (1996), above). To select the transformed plant cells, it is preferred to use T-DNA constructs which are capable of expressing, in the plant cell, a resistance gene from the AFP gene to be expressed. A suitable resistance gene is, for example, the gene encoding phosphinothricin acetyltransferase (see, for example, Ishida, Y. et al. (1996), above). Thus, successfully transformed plant cells can be selected for example by means of the corresponding antibiotic phosphinothricin. The regeneration of a plant from transformed plant cells is generally known (see, for example, Sagi et al. (1995), Nature Biotech, 13, 481-485, Ishida, Y. et al. (1996), above, or Schöpke et al. (1996), Nature Biotech, 14, 731-735.

In addition to the use of Agrobacterium tumefaciens as transformation means, other transformation methods are known and suitable, such as, for example, electroporation, the bombardment with microparticles or the use of polyethylene glycol (see, for example, Potrykos, (1991) Annu. Rev. Plant Physiol. Mol. Biol., 42, 205; Estruch, J.J. et al. (1997), Nature Biotech., 15, 137-141 or Dingermann, T. (1995) BIOforum, 18, 252). For example, casava (Schöpke et al. (1996), above) or banana (Sagi, et al. (1995), above) have already been transformed by bombarding plant cells with DNA-coated particles. In this method too, the AFP gene to be expressed is cloned into a suitable vector which preferably has a resistance gene allowing the subsequent selection of the transformed plant cells. The recombinant vector is preferably applied to tungsten particles with which, for example, suspensions of embryogenic cells are bombarded (see, for example, Sagi, et al. (1995), above). The cell suspensions

treated thus are subsequently selected for transformed plant cells using a suitable antibiotic, for example phosphinothricin, and a transgenic plant is regenerated therefrom, for example as already described above.

A considerable advantage of the present invention is that AFP is active against plant pathogenic fungi from the Ascomycetes family, in particular against Botrytis Cinera, which have already developed a resistance in particular to one or more crop protection agents.

The examples which follow and the figure are intended to describe the invention in greater detail without imposing any limitation:

Sequence description

SEQ ID No. 1 shows a nucleic acid sequence encoding an antifungal (223) protein (AFP) from Streptomyces tendae.

Examples

Example 1:

To prepare test plates, 50 µl of a spore suspension of *Botrytis cinerae* were added to 100 ml of test agar (20 g/l malt extract, 10 g/l glucose, 2 g/l yeast extract, 0.5 ammonium sulfate, 15 g/l agar, pH 6.0). 25 ml of this were poured into Petri dishes. The final spore concentration was 10^5 spores/ml. Sterile antibiotic test disks (Schleicher und Schuell, Dassel, Frg) 0.5 cm in diameter were subsequently applied to a test plate. Then, 5, 10, 15 and 20 µl of the AFP solution (100 mg/ml lyophilized culture filtrate of *S. tendae*-transformants) were subsequently pipetted onto the test disks and the test plate was incubated for 4 days at 30°C. The diameter of the inhibitory zone was 0.7 cm

for 5 µl of AFP solution, 1 cm diameter for 10 µl, 1.3 cm for 15 µl of solution and 1.9 cm for 20 µl.

Example 2:

To prepare test plates, 50 µl of a spore suspension of *Botrytis cinerae* ZF 3629 (resistant to BCM (Benlat, Benomyl™, Hoechst Schering AgrEvo GmbH, Frankfurt, carbendazem) were added to 100 ml of test agar (20 g/l malt extract, 10 g/l glucose, 2 g/l yeast extract, 0.5 ammonium sulfate, 15 g/l agar, pH 6.0). 25 ml of this were poured into Petri dishes. The final spore concentration was 10^5 spores/ml. Sterile antibiotic test disks (Schleicher und Schuell, Dassel, Frg) 0.5 cm in diameter were subsequently applied to a test plate. Then, 5, 10, 15 and 20 µl of the AFP solution (100 mg/ml lyophilized culture filtrate of *S. tendae*-transformants) were subsequently pipetted onto the test disks and the test plate was incubated for 4 days at 30°C. The diameter of the inhibitory zone was 0.9 cm for 5 µl of AFP solution, 1.3 cm diameter for 10 µl, 1.9 cm for 15 µl of solution and 2.2 cm for 20 µl.

Example 3 - Improved production by fermentation:

In a flask equipped with a hose, 100 ml of the preculture medium (103 g/l sucrose, 20 g Tryptic Soy Broth (Oxoid, Wesel), 10 g/l $MgCl_2$ and 10 g/l yeast extract) were inoculated with an agar section of strain *S. tendae*. The culture was grown for 5 days at 28°C at 200 rpm on an orbital shaker.
10 l of the production medium (103 g/l sucrose, 20 g Tryptic Soy Broth (Oxoid, Wesel), 10 g/l $MgCl_2$, 10 g/l yeast extract, 100 µl desmophen, pH 7.5) were inoculated with 100 ml of the above-described preculture.

The fermenter employed was a Biostat E (Braun, Melsungen). Fermentation was carried out for 4 days at 28°C, 700 rpm and 0.5 vvm of air. Thereupon, fermentation was stopped, and the culture supernatant was harvested by centrifugation at 3000 rpm and 4°C. The batch was subsequently dried by lyophilization. It was possible to employ the powder directly. If 15 µl of the lyophilizate (100 mg/ml) were employed against *Botrytis cinerae* in an inhibitory-zone test, an inhibitory zone of 1.7 cm and, against *Botrytis cinerae* ZF 3629, an inhibitory zone of 2.3 cm were detected.

We claim:

1. The use of an antifungal protein (AFP) having a molecular weight of approx. 10 kDA from Streptomyces tendae against plant pathogenic fungi from the Ascomycetes family, in particular against Botrytis cinera.

2. The use as claimed in claim 1, wherein AFP is used in the form of a formulation together with preferably at least one auxiliary.

3. The use as claimed in claim 1, wherein AFP is formed in a transgenic plant.

4. The use as claimed in claim 3, wherein the transgenic plant is selected from among maize, cotton, potato, banana, Arabidopsis, casava, tobacco, oilseed rape (canola), potato, sugar beet, cereals, strawberries, vegetables, legumes, tomato, lettuce or melon.

5. The use as claimed in claim 4, wherein cereals are selected from amongst wheat, barley or oats, the vegetable used is cabbage, or the legumes are selected from amongst peas or beans.

6. The use as claimed in any of claims 3 - 5, wherein the transgenic plant comprises a nucleic acid as claimed in SEQ ID No. 1, SEQ ID No. 1 being part of the claim.

7. The use as claimed in any of claims 1 - 6, wherein the plant pathogenic fungus stated is resistant to one or more crop protection agents.