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# Pn-AMPs, the hevein-like proteins from *Pharbitis nil* confers disease resistance against phytopathogenic fungi in tomato, *Lycopersicum esculentum*

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**Abstract**

The antifungal activity of hevein-like proteins has been associated with their chitin-binding activities. Pn-AMP1 and Pn-AMP2, two hevein homologues from *Pharbitis nil*, show in vitro antifungal activities against both chitin and non-chitin containing fungi. Purified Pn-AMPs retained antifungal activities only under non-reducing conditions. When *Pn-AMP2* cDNA was constitutively expressed in tomato (*Lycopersicon esculentum*) plants under the control of CaMV35S promoter, the transgenic plants showed enhanced resistance against both the non-chitinous fungus *Phytophthora capsici*, and the chitin-containing fungus *Fusarium oxysporum*. Thus, the chitin component in the fungal cell wall is not an absolute requirement for Pn-AMP's antifungal activities. These results when considered together suggest that *Pn-AMPs* have the potential for developing transgenic plants resistant to a wide range of phytopathogenic fungi.


*Keywords:* *Lycopersicon esculentum*; Solanaceae; *Pharbitis nil*; Convolvulaceae; Pn-AMP; Hevein-like proteins; Antifungal activity; Fungal resistance

## 1. Introduction

Effective and sustained control of fungal pathogens is an important issue in agricultural systems and the global losses caused by pathogens are estimated to be about 12% of the potential crop production, despite the continued release of new resistant cultivars and fungicides. Many fungi are continually becoming resistant to existing fungicides, with a few of the latter even being withdrawn from the market for environmental and safety reasons. Further, fungi often lower crop quality by producing toxins that affect human and animal health. Additional methods of disease control are therefore highly desirable.

One of the most important objectives in modern agriculture is to develop strategies for utilization of natural host plant resistance mechanisms to control diseases. As an extension of these objectives, recombinant DNA, gene transformation and molecular biological techniques provide the potential to "engineer" traits for resistance

*Abbreviations:* AMP; antimicrobial protein; Pn-AMP; *Pharbitis nil*-AMP; SDS–PAGE; sodium dodecyl sulfate–polyacrylamide gel electrophoresis; $IC_{50}$; concentration of protein required for 50% inhibition of fungal growth.

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that are effective against both specific and broad spectrum of pathogens. Therefore, significant effort has been directed toward the identification of antifungal proteins and their expression in transgenic plants (Broglie et al., 1991; Collinge et al., 1993; Florack and Stiekema, 1994; Yun et al., 1997; Koo et al., 1998; Garcia-Omeldo et al., 1998; Gao et al., 2000).

Plants produce a wide number of antifungal compounds as part of pre-existing and developmentally regulated defense barriers, and also as components of the defense response induced upon pathogen infection. The best studied among them are several classes of proteins having anti-microbial properties which include chitinases (Melchers et al., 1993; Collinge et al., 1993), glucanases (Garcia-Omeldo et al., 1998), ribosome inactivating proteins (Bolognesi et al., 2002; Chen et al., 2002), defensins (Lehrer et al., 1991; Selsted et al., 1993) and a variety of glycine/cysteine-rich small molecular weight proteins (Florack and Stiekema, 1994; Terras et al., 1995; Yun et al., 1997; Epple et al., 1997). Transgenic plants constitutively expressing several of these proteins have shown enhanced resistance to a number of pathogens (Jach et al., 1995; De Bolle et al., 1996; Epple et al., 1997; Munch-Garthoff et al., 1997; Fagoaga et al., 2001; Oldach et al., 2001; Chen et al., 2002).

Hevein and hevein-like small molecular weight anti-microbial proteins (AMPs) have been reported from rubber tree latex (Broekaert et al., 1990), seeds of amaranth (Broekaert et al., 1992) and *Pharbitis nil* (Koo et al., 1998), fruits of elderberry (Van Damme et al., 1999), leaves of sugar beet (Nielsen et al., 1997), *Arabidopsis* (Potter et al., 1993) and bark of *Eucommia ulmoides* (Huang et al., 2002). All of these AMPs are peptides containing 40–43 amino acid residues and possess a characteristic putative cysteine/glycine rich chitin-binding domain. Hence, it has been proposed that these proteins bind to chitin in the cell wall and disrupt cell wall synthesis. However, the hevein-like anti-microbial proteins from *P. nil* (Pn-AMPs) exhibit considerable antifungal activity against a broad spectrum of fungi, including those that do not contain chitin in their cell walls (Koo et al., 1998).

Since Pn-AMPs have potential antifungal activities, transgenic tomato plant lines over-expressing Pn-AMP2 were obtained to examine whether its in situ expression imparts resistance to chitin-free *Phytophthora capsici* and/or chitin-containing *Fusarium oxysporum*. Our experiments showed that transgenic plants constitutively over-expressing Pn-AMP2 exhibited enhanced resistance to these two phytopathogens. These results confirm our earlier observations (Koo et al., 1998) that hevein-like Pn-AMPs have in vitro antifungal activity against fungi that do not contain chitin in their cell wall. Furthermore, we propose that Pn-AMP2 can be used to engineer plants to mediate effective and durable resistance in plants against phytopathogens.

## 2. Results

### 2.1. In vitro chitin-binding and antifungal activity of Pn-AMP

Pn-AMP1 (41 amino acids) and Pn-AMP2 (40 amino acids) exhibited almost the same level of antifungal activity against a broad spectrum of fungal pathogens. The amino acid sequences of Pn-AMP1 and Pn-AMP2 are identical except that Pn-AMP1 has an additional serine residue at the carboxyl terminus, and their chitin-binding domains displayed high homology with hevein proteins (Koo et al., 1998). On investigation of the chitin-binding ability of Pn-AMP1, it was found that Pn-AMP1 strongly binds to chitin. The interaction of Pn-AMP1 to chitin was so strong that elution required boiling of the complex with SDS buffer at 100 °C for 3 min (Fig. 1). Even after such heat treatment, Pn-AMP1 inhibited the growth of *Botrytis cinerea* with an $IC_{50}$ value of 16 μg/ml. On the other hand, DTT reduced Pn-AMP1 could not bind to the chitin column (Fig. 1) and showed no antifungal activity even at 150 μg/ml protein. Similar results were also obtained for Pn-AMP2, though the concentration of protein required for inhibition was ~8 fold lower than Pn-AMP1. These results collectively suggest that Pn-AMPs bind strongly to chitin, require high denaturing condition for their release, are thermostable, and that disulfide linkages are necessary for antifungal and chitin-binding activity.

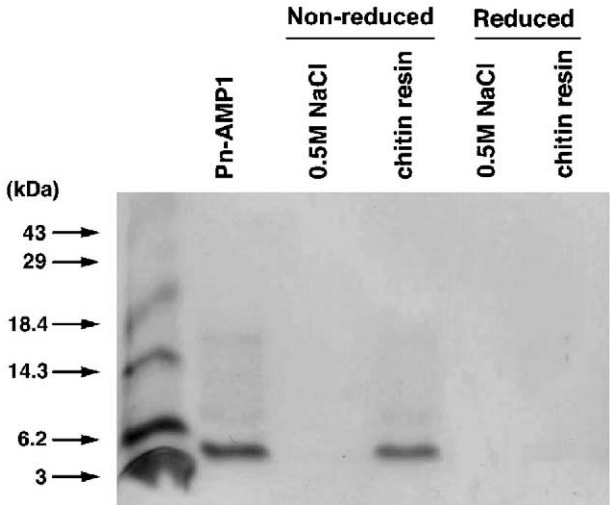


Fig. 1. Chitin-binding activity of Pn-AMPs. Purified Pn-AMP1 (100 μg) was incubated with chitin in column. After washing with 10 mM sodium phosphate buffer pH 7.0, the protein was eluted with same buffer containing 500 mM NaCl and 2% SDS respectively. The resulting supernatants were subjected to SDS–PAGE on 15% gel. Pn-AMP1: Purified Pn-AMP1, Non-reduced: Purified Pn-AMP1 directly applied onto chitin column and eluted with sodium chloride (0.5 M NaCl) and SDS, Reduced: Purified Pn-AMP1 incubated with DTT and then applied on the chitin column and eluted with sodium chloride (0.5 M NaCl) and SDS. First lane from the left is standard protein molecular weight markers.

To investigate whether the chitin-binding and antifungal activities of Pn-AMPs are mutually exclusive, their in vitro antifungal activity against *F. oxysporum* that contains chitin and *P. capsici* that lacks chitin in its cell wall was analyzed. Pn-AMPs exhibited potent antifungal activity against *P. capsici* and the activities were ~2–5 fold higher than that of *F. oxysporum*. The $IC_{50}$ for growth inhibition of *F. oxysporum* was much higher (10.0 and 2.5 μg/ml) than *P. capsici* (5.0 and 0.6 μg/ml) for Pn-AMP1 and Pn-AMP2, respectively. However, DTT reduced Pn-AMP1 and Pn-AMP2 showed no antifungal activity against *B. cinerea*, *P. capsici* and *F. oxysporum* even at protein concentrations as high as 150 μg/ml. These results indicate that the chitin in the fungal cell wall is not a requirement for the Pn-AMP's antifungal effect, although disulfide linkages are essential for both chitin binding and antifungal activity.

### 2.2. Expression of Pn-AMP2 in tomato plants

The coding sequence of the *Pn-AMP2* cDNA was cloned into the binary vector *pGA643*, under control of the CaMV35S promoter, for high-level constitutive expression (Fig. 2A). Tomato leaf discs were transformed by co-cultivation with *Agrobacterium* harboring *pnAMP2643* and kanamycin-resistant calli were regenerated. Among the primary transformants, two transgenic lines that showed highest-level expression of *Pn-AMP2* in Northern analysis, A4 and A5, were selected for assaying antifungal activity (Fig. 2B). Pn-AMP2 was partially purified from the transgenic plants and used in routine bioassay using quantitative hyphal growth inhibition of *Botrytis cinerea*. The purified Pn-AMP2 from the transgenic plants and seeds of *P. nil* showed similar levels of in vitro antifungal activity against *B. cinerea* (data not shown), suggesting that the recombinant Pn-AMP2 from transgenic plants is folded correctly.

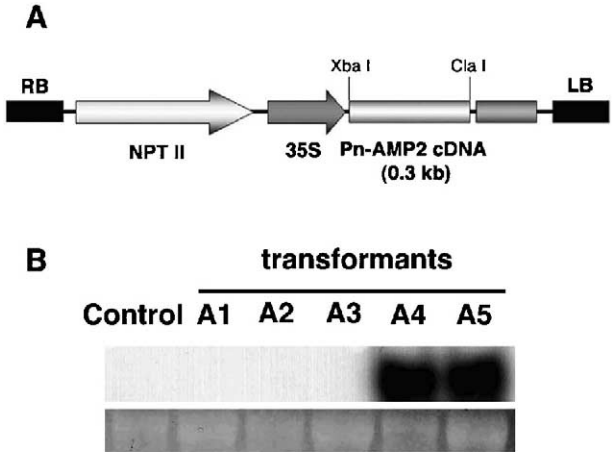


Fig. 2. Expression of Pn-AMP2 in tomato plant. (A) Physical map of the binary vector pnAMP2643 used for transformation of the tomato plants. (B) Northern blot analysis: Transgenic tomato plants transformed with *pnAMP2643* and control plant transformed with an empty vector *pGA643*, were analyzed for Pn-AMP2 expression. Equal loading of RNA (10 μg) in each lane was confirmed by pre-staining the gel with ethidium bromide (lower). RNA blots were probed with $^{32}$P-labeled Pn-AMP2 cDNA. Control: Plant transformed with an empty vector *pGA643*; A1, A2, A3: representatives of transgenic plants transformed with *pnAMP2643* with poor or no expression of Pn-AMP2 and A4, A5 with high expression.

### 2.3. Constitutive expression of Pn-AMP2 in transgenic tomato plants confers resistance against chitin and non-chitin containing phytopathogenic fungi

To determine whether *Pn-AMP2* expressed in transgenic tomato plants enhanced fungal pathogens resistance in vivo, the plants expressing *Pn-AMP2* and control plants transformed with an empty vector were grown on MS agar media in phytatrays, and challenged with agar plugs containing *P. capsici* and *F. oxysporum* hyphae. Seven days after infection, disease symptoms, such as brown colored stem rotting (necrosis), appeared on the lower part of stems in control plants but not in the transgenic plants. Fourteen days after infection, the control plants showed severe disease symptoms (Figs. 3 and 4). The control plants eventually died with severe symptoms of leaf wilting and stem rot after 21 days (data not shown). In contrast, few necrotic lesions were observed on leaves of transgenic plants challenged with *P. capsici* and *F. oxysporum* (Figs. 3 and 4). The hyphal spreading of *F. oxysporum* on agar medium with transgenic plants showed a distinct boundary from the original inoculated site. Control plants, on the other hand, showed overgrowth of fungal hyphae (Fig. 5A and B). Taken together, the results clearly suggest that overexpression of Pn-AMP2 confers enhanced resistance to *P. capsici* and *F. oxysporum*, the two major pathogens of tomato.

## 3. Discussion

This study demonstrates that the expression of the antifungal peptide Pn-AMP2 in tomato enhances the plant's defense against *P. capsici* and *F. oxysporum*. It further shows that the chitin component in the fungal cell wall is not an absolute requirement for the antifungal activity of Pn-AMPs. However both chitin binding and antifungal activity are abolished under reduced conditions, indicating that intra-molecular disulfide linkages are essential for Pn-AMP function.

One of the striking features of Pn-AMPs is their potent antimicrobial activity on a broad range of phytopathogenic fungi, including those that do not have chitin in their cell walls. Pn-AMPs show the highest homology to the hevein class of proteins and contain cysteine/glycine rich chitin-binding domain well conserved among the chitin binding proteins (Jach et al., 1995; Munch-Garthoff et al., 1997; Koo et al., 1998; Chen et al., 2002; Huang et

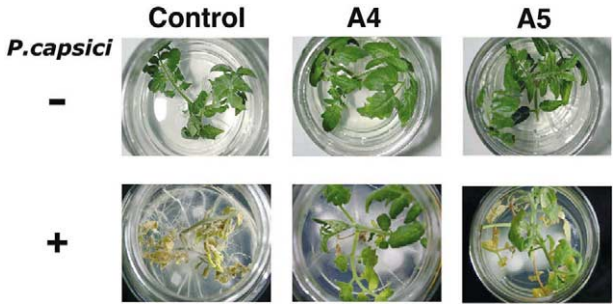


Fig. 3. Pn-AMP2 confers resistance against *P. capsici* in transgenic tomato plants. Two agar plugs of *P. capsici* hyphae were placed in phytatrays containing growing tomato plants transformed with *pnAMP2643* or *pGA643*. Photographs were taken 2 weeks after inoculation. Three independent experiments show similar results.

al., 2002). All the eight cysteines in Pn-AMPs are involved in disulfide linkages, providing an unique folded structure (Koo et al., 1998). However, the precise mechanism of fungal-growth inhibition by Pn-AMPs and chitin-binding lectins remains unclear. Since chitin-binding lectins are small proteins with no enzymatic activity, it is hypothesized that these proteins penetrate the fungal cell and affect chitin-containing components important for cell wall synthesis (Broekeart et al., 1990,1992; Florack and Stiekema, 1994; Thevissen et al., 1999). Our data reveal that *P. capsici*, non-chitin containing fungus, is highly sensitive to Pn-AMP similar to the chitin containing fungus *F. oxysporum*. Earlier, we also demonstrated that fluorescein isothiocyanate (FITR)-conjugated Pn-AMPs are preferentially localized to the septa and hyphal tip region of both the chitin containing fungus *B. cinerea* and the non-chitin containing fungus *Phytophthora parasitica* (Koo et al., 1998). Further, in our investigations on the mechanism of action of Pn-AMPs using *Saccharomyces cerevisiae* as a model system, it was observed that the growth inhibiting activity of Pn-AMPs is mediated via cell wall glycoproteins instead of chitin (unpublished data). Therefore, these results collectively suggest that chitin in the fungal cell wall is not essential for Pn-AMP interaction and its

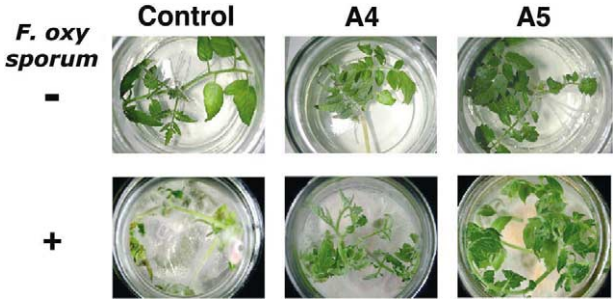


Fig. 4. Pn-AMP2 confers resistance against *F. oxysporum* in transgenic tomato plants. Two agar plugs of *F. oxysporum* hyphae were placed in phytatrays containing growing tomato plants transformed with *pnAMP2643* or *pGA643*. Photographs were taken 2 weeks after inoculation. The results are representative of three different trials.

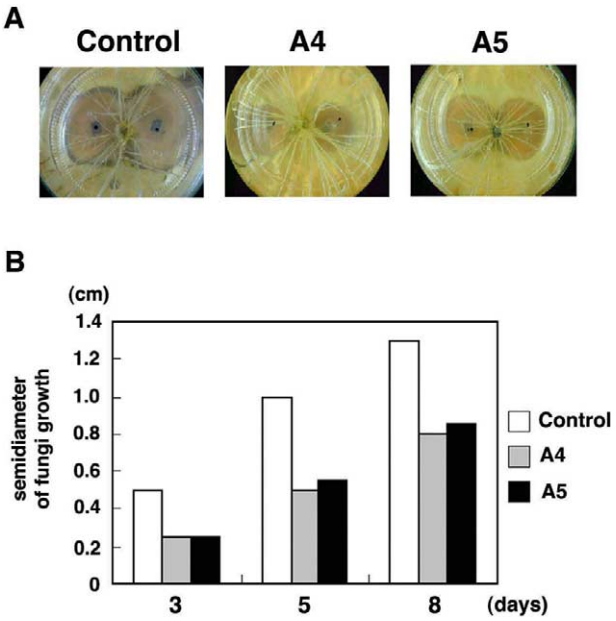


Fig. 5. Transgenic tomato plants expressing Pn-AMP2 inhibit fungal growth. (A) Two agar plugs of *F. oxysporum* hyphae were placed in phytatrays growing tomato plants transformed with pGA643 as control and A4, A5-transgenic plants transformed with *pnAMP2643*. Photographs were taken from the bottom of the phytatrays to show the hyphal growth of the fungi. (B) The hyphal semi-diameters were measured after 3, 5 and 8 days of incubation. The results are mean value of three independent experiments.

antifungal activity. Similar to some of the hevein-like AMPs, Pn-AMPs are highly basic proteins with pI ~8.0. The correlation between the antifungal activity and cationic nature of proteins suggests that antifungal activity might be related to electrostatic interactions between proteins and negatively charged membrane phospholipids of fungi. Further divalent cation like $Ca^{++}$ severely reduces such antifungal activity of basic AMPs (Koo et al., 1998; Huang et al., 2002). The induced electrostatic changes may eventually result in pore formation or a specific membrane domain interaction and inhibit the fungal growth, as suggested earlier for thionins (Florack and Stiekema, 1994; Thevissen et al., 1999).

Plants synthesize pathogenesis-related proteins in response to microbial challenge. Among these are the chitinases and glucanases, which hydrolyze fungal cell walls and exhibit strong in vitro antifungal activity (Collinge et al., 1993; Melchers et al., 1993; Munch-Garthoff et al., 1997). However, an Oomycete, such as *P. capsici*, contains little or no cell wall chitin and therefore, is unlikely to be affected by chitinases expressed in plants (Broglie et al., 1991; De Bolle et al., 1996). Cysteine-rich antimicrobial peptides are yet another group of plant antifungal agents that include thionins, plant defensins, knottin-type peptides such as the Mj-AMPs from *Mirabilis jalapa* (Florack and Stiekema, 1994; De Bolle et al., 1996; Yun et al., 1997; Garcia-Omeldo et al., 1998; Thevissen et al., 1999), and

the hevein type peptides like Ac-AMPs from *Amaranthus caudatus* (Potter et al., 1993; Koo et al., 1998), with potential antifungal activity. However, constitutive over-expression of an *Ac-AMP* in transgenic tobacco plants conferred limited resistance to *Botrytis cinerea* and *Alternaria longipes* (De Bolle et al., 1996). In this paper, we show that transgenic plants constitutively over-expressing Pn-AMP2 exhibit enhanced resistance to *P. capsici* and *F. oxysporum*, the two major phytopathogens of tomato. The results further demonstrate that the Pn-AMP2 seems to undergo ordered folding in the transgenic plants to a biologically active form. Also, the disulfide bridges are crucial for the chitin binding and antifungal activity. We demonstrate for the first time that Pn-AMP2, a hevein-like protein, over-expressed in transgenic plants, defends plants from both chitin and non-chitin containing fungal pathogens. These results collectively suggest that Pn-AMPs, with broad and potent antifungal activity, are novel candidate genes for developing transgenic plants with enhanced resistance to a wide range of phytopathogenic fungi in coming years.

## 4. Experimental

### 4.1. Materials, strains and plasmids

Tomato (*L. esculentum*), Korean cultivar, Seo-Gwang, was grown in a growth chamber (Conviron) under 16 h light and 8 h dark cycle at 25 °C. *Botrytis cinerea*, *Escherichia coli* XL1-Blue MRF and *Agrobacterium tumefaciens* LBA4404 were the organisms used in this study. The pathogenic fungi, *Phytophthora capsici* and *Fusarium oxysporum*, used in this experiment were gifts from Dr. Wang Yuanchao, Nanjing Agricultural University, China.

### 4.2. Chitin-binding activity

Pn-AMP1 and Pn-AMP2 were purified from *Pharbitis nil* as described earlier (Koo et al., 1998). Chitin-binding ability of purified Pn-AMP1 was examined by applying 100 μg of homogeneous Pn-AMP1 onto a column containing 2 ml of regenerated chitin (Sigma, USA) equilibrated with 10 mM sodium phosphate buffer, pH 7.0. After incubating for 10 min at 4 °C, the column was washed successively with 4 bed volumes of 10 mM sodium phosphate buffer, pH 7.0 and with the same buffer containing 0.5 M NaCl. Finally, Pn-AMP1 was eluted from chitin by adding 1 ml SDS-sample buffer and incubating at 100 °C for 3 min. The presence of Pn-AMP1 was analyzed by SDS-PAGE. To evaluate whether these disulfide linkages are essential for chitin binding and antifungal activity, Pn-AMP1 was reduced by incubating with 10 mM DTT for 30 min at 37 °C.

### 4.3. Bioassay for antifungal activity

Antifungal activities of Pn-AMP1 and Pn-AMP2 were assayed by microspectrophotometry as described earlier (Broekaert et al., 1992). Fungal strains were maintained in potato dextrose agar or V8 agar medium at 25 °C. Fungal spores or hyphal fragments were collected as described elsewhere (Duvick et al., 1992). Various concentrations of Pn-AMP1 or Pn-AMP2 (20 μl) were mixed with 80 μl of appropriate growth medium (half-strength potato dextrose broth) containing $10^4$ fungal spores or with 100 fungal hyphal fragments/ml/well in a 96-well microplate. Percent growth inhibition was calculated from the absorbance at 595 nm after 48 h incubation at 25 °C. $IC_{50}$ value was calculated as concentration of protein (μg/ml) required for 50% growth inhibition after 48 h of incubation.

### 4.4. Generation of transgenic tomato plants

To construct the plant transformation vector, the *Pn-AMP2* coding sequence (GenBank accession number, U40076) was cloned into the *Xba* I and *Cla* I sites between the CaMV35S promoter and the 5′/7′ terminator region of *pGA643* to yield *pnAMP2643*. *pnAMP2643* or *pGA643* for the control experiment was introduced into *Agrobacterium tumefaciens LBA4404* and used for transformation of tomato leaf explants by co-cultivation. The transgenic plants were regenerated under kanamycin selection (50 mg/l) as described earlier (Horsch et al., 1985).

### 4.5. Northern blot analysis

Total RNA from control and transgenic plants was isolated as described earlier (Hong et al., 1987). Total RNA (10 μg) was denatured and separated on a formaldehyde agarose gel (1.2%), and transferred to Gene Screen membrane (NEN, Boston, MA). The blot was pre-hybridized with 1% BSA, 1 mM EDTA, 7% SDS and 250 mM sodium phosphate, pH 7.2, at 68 °C for 4 h. The $^{32}$P-labelled cDNA probe containing 274 bp full coding region of Pn-AMP2 (*Bam*HI and *Eco*RI digest) was hybridized in pre-hybridization buffer at 68 °C overnight. The blot was washed twice with 2×SSC, 0.1% SDS at 68 °C for 30 min, and twice with 0.1×SSC, 0.1% SDS at 68 °C for 30 min. Hybridization signals were visualized by exposing the X-ray film for two days at −70 °C.

### 4.6. Bioassay of transgenic plants

Tomato plants were inoculated with *P. capsici* and *F. oxysporum* under aseptic conditions. We selected A4 and A5 transgenic plants with highest expression of Pn-AMP2 for resistance bioassay. Plant containing empty

pGA643 vector served as control. All of these lines were propagated into independent shoots and were rooted on hormone-free media. Two agar plugs (0.5 cm in diameter) of fungal hyphae were placed in sterile phytatrays containing tomato plants growing on half strength MS medium (Murashige and Skoog, 1962). The hyphal semi-diameters were measured after three, five and eight days after inoculation. Disease symptoms were scored 10 days after infection and indexed.

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## References

Bolognesi, A., Polito, L., Lubelli, C., Barbieri, L., Parente, A., Stirpe, F., 2002. Ribosome-inactivating and adenine polynucleotide glycosylase activities in *Mirabilis jalapa* L. tissues. J. Biol. Chem. 277, 13709–13716.

Broekaert, I., Lee, H.I., Kush, A., Chua, N.H., Raikhel, N., 1990. Wound-induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree (*Hevea brasiliensis*). Proc. Natl. Acad. Sci. 87, 7633–7637.

Broekaert, W.F., Marien, W., Terras, F.R., De Bolle, M.F., Proost, P., Van Damme, J., Dillen, L., Claeys, M., Rees, S.B., Vanderleyden, J., 1992. Antimicrobial peptides from Amaranthus caudatus seeds with sequence homology to the cysteine/glycine-rich domain of chitin-binding proteins. Biochemistry 31, 4308–4314.

Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P.H., Knowlton, S., Mauvais, J., Broglie, R., 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizotocnia solani*. Science 254, 1194–1197.

Chen, Y., Vandenbussche, F., Rouge, P., Proost, P., Peumans, W.J., Van Damme, E.J., 2002. A complex fruit-specific type-2 ribosome-inactivating protein from elderberry (*Sambucus nigra*) is correctly processed and assembled in transgenic tobacco plants. Eur. J. Biochem. 269, 2897–2906.

Collinge, D.B., Kragh, K.M., Mikkelsen, J.D., Nielsen, K.K., Rasmussen, U., Vad, K., 1993. Plant chitinases. Plant Journal 3, 31–40.

De Bolle, M.F., Osborn, R.W., Goderis, I.J., Noe, L., Acland, D., Hart, C.A., Torrekens, S., Van Leuven, F., Broekaert, W.F., 1996. Antimicrobial peptides from *Mirabilis jalapa* and *Amaranthus caudatus*: expression, processing, localization and biological activity in transgenic tobacco. Plant Mol. Biol. 31, 993–1008.

Duvick, J.P., Rood, T., Rao, A.G., Marshak, D.R., 1992. Purification and characterization of a novel antimicrobial peptide from maize (*Zea mays* L.) kernels. J. Biol. Chem. 267, 18814–18820.

Epple, P., Apel, K., Bohlmann, H., 1997. Over expression of an endogenous thionin enhances resistance of *Arabidopsis* against *Fusarium oxysporum*. Plant Cell 9, 509–520.

Fagoaga, C., Rodrigo, I., Conejero, V., Hinarejos, C., Tuset, J.J., Arnau, J., Pina, J.A., Navarro, L., Pena, L., 2001. Increased tolerance to *Phytophthora citrophthora* in transgenic orange plants constitutively expressing a tomato pathogenesis related protein PR-5. Mol. Breeding 7, 175–185.

Florack, D.E., Stiekema, W.J., 1994. Thionins: properties, possible biological roles and mechanisms of action. Plant Mol. Biol. 26, 25–37.

Gao, A.G., Hakim, S.M., Mittanck, C.A., Wu, Y., Woerner, B.M., Stark, D.M., Shah, D.M., Liang, J., Rommens, C.M., 2000. Fungal pathogen protection in potato by expression of a plant defensin peptide. Nat. Biotechnol. 18, 1307–1310.

Garcia-Olmedo, F., Molina, A., Alamillo, J.M., Rodriguez-Palenzuela, P., 1998. Plant defense peptides. Biopolymers 47, 479–491.

Jach, G., Gornhardt, B., Mundy, J., Logemann, J., Pinsdorf, E., Leah, R., Schell, J., Maas, C., 1995. Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant Journal 8, 97–109.

Hong, J.C., Nagao, R.T., Key, J.L., 1987. Characterization and sequence analysis of a developmentally regulated putative cell wall protein gene isolated from soybean. J. Biol. Chem. 262, 8367–8376.

Horsh, R.B., Fry, J.E., Hoffmann, N.L., Eichholfz, D., Rogers, S.G., Fraley, R.T., 1985. A simple and general method for transferring genes into plants. Science 227, 1229–1231.

Huang, R.-H., Xiang, Y., Liu, X.-Z., Zhang, Y., Hu, Z., Wang, D.-C., 2002. Two novel antifungal peptides distinct with a five disulfide motif from the bark of *Eucommia ulmoides* Oliv. FEBS Lett. 521, 87–90.

Koo, J.C., Lee, S.Y., Chun, H.J., Cheong, Y.H., Choi, J.S., Kawabata, S., Miyagi, M., Tsunasawa, S., Ha, K.S., Bae, D.W., Han, C.D., Lee, B.L., Cho, M.J., 1998. Two hevein homologues isolated from the seeds of *Pharbitis nil* L. exhibit potent antifungal activity. Biochem. Biophys. Acta 1382, 80–90.

Lehrer, R.I., Ganz, T., Selsted, M.E., 1991. Defensins: endogenous antibiotic peptides of animal cells. Cell 64, 229–230.

Melchers, L.S., Sela Buurlage, M.B., Vloemans, S.A., Woloshuk, C.P., van Roekel, J.S., Peen, J., Van den Elzen, P.J., Cornelissen, B.J., 1993. Extra cellular targeting of the vacuolar tobacco proteins AP24. Chitinase and beta-1,3-glucanase in transgenic plants. Plant Mol. Biol. 21, 583–593.

Munch-Garthoff, S., Neuhaus, J.M., Boller, T., Kemmerling, B., Kogel, K.H., 1997. Expression of beta-1,3-glucanase and chitinase in healthy, stem-rust-affected and elicitor-treated near-isogenic wheat lines showing Sr5-or Sr24-specified race-specific rust resistance. Planta 201, 235–244.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum 15, 473–497.

Nielsen, K.K., Nielsen, J.E., Madrid, S.M., Mikkelsen, J.D., 1997. Characterization of a new antifungal chitin-binding peptide from sugar beet leaves. Plant Physiol. 113, 83–91.

Oldach, K.H., Becker, D., Lorz, H., 2001. Heterologous expression of genes mediating enhanced fungal resistance in transgenic wheat. Molec. Plant Micr. Interac. 14, 832–838.

Potter, S., Uknes, S., Lawton, K., Winter, A.M., Chandler, D., DiMaio, J., Novitzky, R., Ward, E., Ryals, J., 1993. Regulation of a hevein-like gene in *Arabidopsis*. Molec. Plant Micr. Interac. 6, 680–685.

Selsted, M.E., Tang, Y.Q., Morris, W.L., McGuire, P.A., Novotny, M.J., Smith, W., Henschen, A.H., Cullor, J.S., 1993. Purification, primary structures, and antibacterial activities of beta-defensins, a new family of antimicrobial peptides from bovine neutrophils. J. Biol. Chem. 268, 6641–6648.

Terras, F.R., Eggermont, K., Kovaleva, V., Raikhel, N.V., Osborn, R.W., Kester, A., Rees, S.B., Torrekens, S., Van Leuven, F., Vanderleyden, J., 1995. Small cysteine-rich antifungal proteins from radish: their role in host defense. Plant Cell 7, 573–588.

Thevissen, K., Terras, F.R., Broekaert, W.F., 1999. Permeabilization of fungal membranes by plant defensins inhibits fungal growth. Appl. Environ. Microbiol. 65, 5451–5458.

Van Damme, E.J., Charels, D., Roy, S., Tierens, K., Barre, A., Martins, J.C., Rouge, P., Van Leuven, F., Does, M., Peumans, W.J., 1999. A gene encoding a hevein-like protein from elderberry fruits is homologous to PR-4 and class V chitinase genes. Plant Physiol. 119, 1547–1556.

Yun, D.J., Bressan, R.A., Hasegawa, P.M., 1997. Plant antifungal proteins. Plant Breed. Rev. 14, 39–88.