

Brief Communication

First report of antifungal activity conferred by non-conventional peptides

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Peptides are generally composed of 2–100 amino acid residues and well known as key regulators of many physiological processes (Tavormina *et al.*, 2015; Wang *et al.*, 2020a). Conventional peptides have been found to exhibit pronounced antifungal activities and are relatively safe for the environment and human health (Marcos *et al.*, 2008; Ribeiro *et al.*, 2013). Therefore, antifungal peptides, as novel fungicides, are promising alternatives for combating the increased incidence of antibiotic resistance in plant pathogenic microbes. Recently, non-conventional peptides (NCPs), a novel class of peptides derived from previously unannotated CDSs, have attracted significant attention (Wang *et al.*, 2020b). Studies have demonstrated that NCPs play essential roles in various biological processes (Khitun *et al.*, 2019; Plaza *et al.*, 2017). However, antifungal activity of NCPs has not been reported to date.

Fungal plant pathogens comprise an important group of microorganisms that continuously cause substantial economic losses in agricultural production around the world (Moller and Stukenbrock, 2017). To explore the antifungal effect of NCPs against plant pathogenic fungi, 246 NCPs were synthesized accordingly (Bioyears, Wuhan, China). These NCPs were identified from the leaves of maize inbred line B73 by an integrated peptidogenomic pipeline under normal growth condition at the three-leaf stage (Wang *et al.*, 2020b). They are derived from intergenic regions, untranslated regions (UTRs), introns, junctions and out-of-frame exons (Figure 1a). The pathogens *Bipolaris zeicola*, *Curvularia lunata*, *Fusarium graminearum* and *Bipolaris maydis* were tested in this study. These pathogens cause major fungal diseases in cultivated crops worldwide. The antifungal activity of these NCPs against four pathogenic fungi was evaluated by the mycelium growth rate method (Agarwal *et al.*, 2001), as shown in Figure 1a. In brief, NCP solutions were added

to the potato dextrose agar (PDA) medium. A 5 mm plug of fungal inoculum from the edge of fungal colony was placed at the centre of the PDA medium and incubated at 28 °C in the dark. The diameter of the mycelial colony and hyphal morphology were recorded at 12, 24 and 36 hours post inoculation (hpi). Each treatment was performed in triplicate. The mycelium growth inhibition rate was determined by measuring the colony diameters and calculated by the following equation: $I\% = [(C-d) - (T-d)] / (C-d) \times 100\%$.

According to the results of the test for the colony diameter of four fungi with NCP treatments and the control, 25 of 246 NCPs inhibited hyphal growth to varying degrees. Of these 25 NCPs, one was derived from an out-of-frame exon, three from introns and 21 from intergenic regions. The molecular weights of these NCPs ranged from 800 to 2194 Da, with lengths between 7 and 19 amino acids (Figure 1b). These results indicate the widespread existence of antifungal NCPs, regardless of peptide length, molecular weight and genomic location. To further investigate the characteristics of antifungal NCPs, the mycelial growth of each pathogen fungi after NCP treatments was analysed. The result showed that 23 NCPs inhibited the mycelial growth of *B. zeicola*, and seven, four and five NCPs inhibited the mycelial growth of *C. lunata*, *F. graminearum* and *B. maydis*, respectively (Figure 1b, c). In addition, approximately 39.1% (9/23), 71.4% (5/7), 75.0% (3/4) and 80.0% (4/5) of the NCPs produced the highest inhibition rate at 24 hpi (Figure 1d, e).

Moreover, we found that 19 NCPs demonstrated high antifungal activity against only one pathogenic fungus (Figure 1b). For example, P499 and P1060 had inhibition effects on *C. lunata* and *F. graminearum*, with the highest inhibition rates of 33.0% at 12 hpi and 29.1% at 24 hpi, respectively (Figure 1c, e). The other 17 NCPs inhibited only the mycelial growth of *B. zeicola*, with the highest inhibition rate of 76.6% (Figure 1e). These results indicate the specific antifungal properties of the NCPs. These NCPs may interact with specific intracellular targets of each pathogen fungus to induce the destruction and hydrolysis of the cell membrane or cell wall, thus leading to the inhibition of fungus growth. However, the detailed mechanism is not clear, and further study is needed. Notably, six NCPs showed broad-spectrum antifungal activity and inhibited the growth of three or four fungi (Figure 1b). Among these NCPs, the intergenic peptide (P1858) and intronic peptide (P1867) led to pronounced decreases in the diameter of the mycelial colony for four evaluated fungi (Figure 1c). The inhibition rate of P1858 was highest at 36

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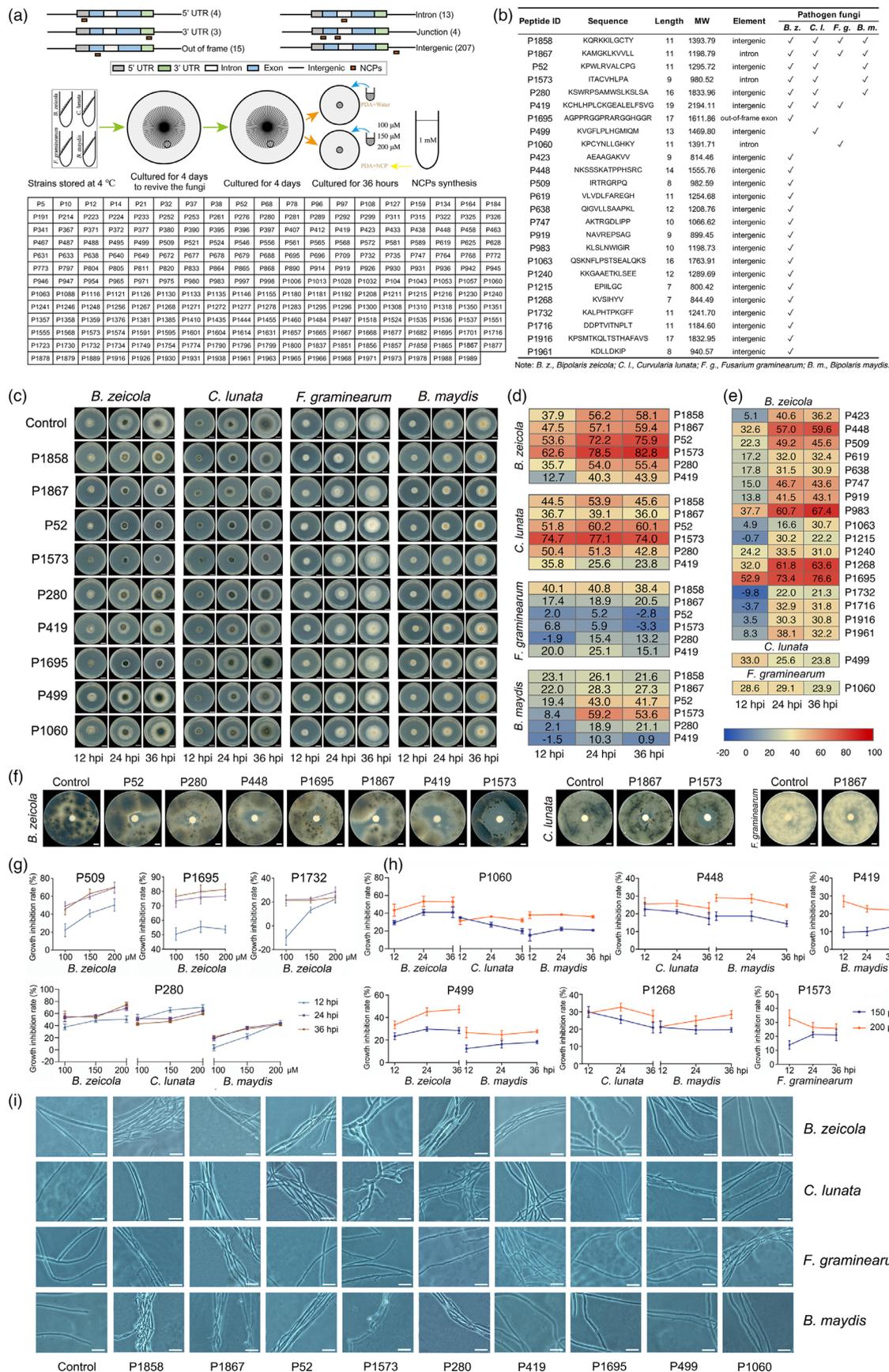


Figure 1 Effects of NCPs on pathogenic fungi. (a) NCP sources and the treatment procedures of fungal species with NCPs. The peptide IDs of synthesized NCPs in this study are shown in the lower panel. For detailed information of these NCPs, see Wang *et al.* (2020b). (b) Detailed information for NCPs showing antifungal activity against four fungal species under the treatments of NCPs at 100 μ M. (c) Images of the *in vitro* assay showing four pathogenic fungi treated with 100 μ M NCPs at 12, 24 and 36 hpi. Scale bars = 5 mm. (d) The inhibition rate (%) of NCPs with broad-spectrum antifungal activities under the treatments of NCPs at 100 μ M. (e) The growth inhibition rate of NCPs exhibited specific antifungal activity under the treatments of NCPs at 100 μ M. (f) Antifungal effects of NCPs identified by the paper disc assay. (g) Growth inhibition rate (%) of NCPs raised with an increasing concentration of NCPs. (h) Growth inhibition rate (%) of NCPs with the broader antifungal spectrum under the treatments of NCPs at 150 μ M and 200 μ M. (i) Hyphal morphology of four pathogenic fungi treated with 100 μ M NCPs. Scale bars = 20 μ m.

hpi for *B. zeicola* (58.1%) and at 24 hpi for *C. lunata* (53.9%), *F. graminearum* (40.8%) and *B. maydis* (26.1%). The highest inhibition rate of P1867 for four fungi was observed at 36, 24, 36 and 24 hpi, respectively (Figure 1d). The intergenic NCP (P52) and intronic NCP (P1573) exhibited high antifungal activities against all fungi, except *F. graminearum* (Figure 1c, d). Moreover, the diameters of *B. zeicola*, *C. lunata* and *F. graminearum* mycelial colonies decreased on medium containing P419 compared with the control, whereas *B. zeicola*, *C. lunata* and *B. maydis* were sensitive to P280, with maximum inhibition rates of 55.4%, 51.3% and 21.1%, respectively (Figure 1d). These results indicate that some NCPs can exhibit broad-spectrum antifungal activity against pathogenic fungi, similar to the characteristics and properties of some conventional peptides (Ribeiro *et al.*, 2013).

Furthermore, the antifungal activities of seven NCPs were validated by another method described by Woo *et al.* (2002). The pronounced inhibition zones against the fungi were observed after the treatments by these peptides (Figure 1f). On the other hand, by increasing the dose of NCPs with a final concentration of 150 μ M and 200 μ M, the inhibition rates of four peptides (P509, P1695, P1732 and P280) raised with an increase in the NCPs concentration (Figure 1g). Notably, the antifungal spectrum of six NCPs, including P1060, P448, P419, P499, P1268 and P1573, were broader at 150 μ M or 200 μ M than those at 100 μ M (Figure 1h). These results indicate that the antifungal activities of some NCPs occur in a dose-dependent manner.

To further explore the antifungal effect of NCPs, alterations in the morphology and growth of fungal hyphae were examined by using a light microscope at a magnification of 400 \times (10 \times ocular lens and 40 \times objective lens) (Figure 1i). Abnormal branching was observed at the rim of *B. zeicola* colonies grown on the media containing P1695 or P1573, demonstrating the inhibition of *B. zeicola* growth by these two NCPs. The addition of P1573 to the PDA medium was also found to cause abnormal branching in *C. lunata*. In addition, P1695 and P1573 caused obvious distortion and tumescence of hyphae against *B. zeicola*, and P1573 against *C. lunata*. By contrast, treatments with other NCPs caused mycelial aggregates. These results suggest that NCPs can exert antifungal activity against pathogenic fungi by inhibiting hyphal growth.

Overall, the antifungal activities of 25 NCPs were demonstrated here for the first time. These NCPs showed a wide spectrum of antifungal activity with varying efficacy against different fungal species. The NCPs may be considered as promising fungicides for combating plant fungal diseases in the future. Furthermore, these findings illustrate that NCPs have vital functional roles in plant biology, despite the fact that they

are derived from previously unannotated CDSs, including intergenic and intronic regions.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

L.W. designed the project. L.T., X.C., X.J., S.W., X.W., J.Z. and Y.Z. performed the experiments. L.W., L.T., S.W. and Y.C. analysed the data. L.W., L.T. and X.C. wrote the manuscript.

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