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Brief Communication

First report of antifungal activity conferred by non-conventional peptides

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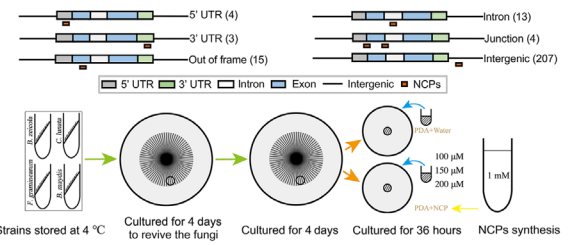
Peptides are generally composed of 2-100 amino acid residues and well known as key regulators of many physiological processes (Tavormina *et al.*, 2015; Wang *et al.*, 2020a). Conventional peptides have been found to exhibit pronounced antifungal activities and are relatively safe for the environment 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) × 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 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

(a)



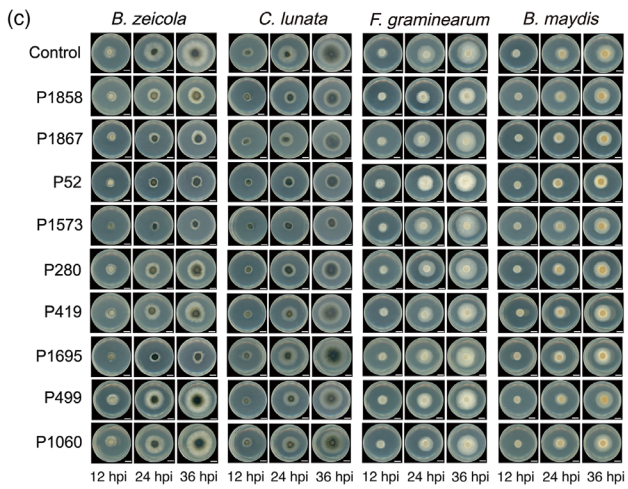
P5	P10	P12	P14	P21	P32	P37	P38	P52	P68	P78	P96	P97	P108	P127	P159	P134	P164	P184
P191	P214	P223	P224	P233	P252	P253	P261	P276	P280	P281	P289	P292	P299	P311	P315	P322	P325	P326
P341	P367	P371	P372	P377	P380	P390	P395	P396	P397	P407	P412	P419	P423	P433	P438	P448	P458	P463
P467	P487	P488	P495	P499	P509	P521	P524	P546	P556	P561	P565	P568	P572	P581	P589	P619	P625	P628
P631	P633	P638	P640	P649	P672	P677	P678	P679	P688	P695	P696	P709	P732	P735	P747	P764	P768	P772
P773	P797	P804	P805	P811	P820	P833	P864	P865	P868	P890	P914	P919	P926	P930	P931	P936	P942	P945
P946	P947	P954	P965	P971	P975	P980	P983	P997	P998	P1006	P1013	P1028	P1032	P104	P1043	P1053	P1057	P1060
P1063	P1088	P1116	P1121	P1126	P1130	P1133	P1135	P1146	P1155	P1180	P1181	P1192	P1208	P1211	P1215	P1216	P1230	P1240
P1241	P1246	P1248	P1256	P1267	P1268	P1271	P1272	P1277	P1278	P1283	P1295	P1296	P1300	P1308	P1310	P1318	P1350	P1351
P1357	P1358	P1359	P1376	P1381	P1385	P1410	P1435	P1444	P1455	P1460	P1484	P1497	P1518	P1524	P1535	P1536	P1537	P1551
P1555	P1568	P1573	P1574	P1591	P1595	P1601	P1604	P1614	P1631	P1657	P1665	P1667	P1668	P1677	P1682	P1695	P1701	P1716
P1723	P1730	P1732	P1734	P1749	P1754	P1774	P1790	P1796	P1799	P1800	P1837	P1851	P1856	P1857	*P1858*	P1865	P1867	P1877
P1878	P1879	P1889	P1916	P1926	P1930	P1931	P1938	P1961	P1963	P1965	P1966	P1968	P1971	P1973	P1978	P1988	P1989	

(b)

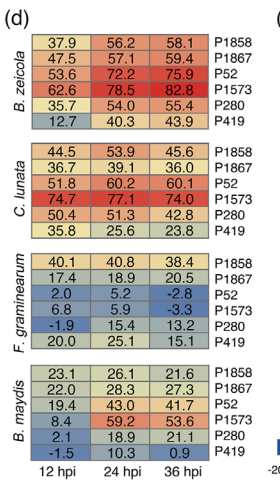
Peptide ID	Sequence	Length	MW	Element	Pathogen fungi			
					B. z.	*C. l.*	*F. g.*	*B. m.*
P1858	KQRKKILGCTY	11	1393.79	intergenic	✓	✓	✓	✓
P1867	KAMGKLKVVLL	11	1198.79	intron	✓	✓	✓	✓
P52	KPWLRVALCPG	11	1295.72	intergenic	✓	✓		✓
P1573	ITACVHLPA	9	980.52	intron	✓	✓		✓
P280	KSWRPSAMWSLKSLSA	16	1833.96	intergenic	✓	✓		✓
P419	KCHLHPLCKGEALELFSVG	19	2194.11	intergenic	✓	✓	✓	
P1695	AGPPRGGPRARGGHGGR	17	1611.86	out-of-frame exon	✓			
P499	KVGFLPLHGMIQM	13	1469.80	intergenic		✓		
P1060	KPCYNLLGHKY	11	1391.71	intron			✓	
P423	AEAAGAKVV	9	814.46	intergenic	✓			
P448	NKSSSKATPPHSRC	14	1555.76	intergenic	✓			
P509	IRTRGRPQ	8	982.59	intergenic	✓			
P619	VLVDLFAREGH	11	1254.68	intergenic	✓			
P638	QIGVLLSAAPKL	12	1208.76	intergenic	✓			
P747	AKTRGDLIPP	10	1066.62	intergenic	✓			
P919	NAVREPSAG	9	899.45	intergenic	✓			
P983	KLSLNWIGIR	10	1198.73	intergenic	✓			
P1063	QSKNFLPSTSEALQKS	16	1763.91	intergenic	✓			
P1240	KKGAAETKLSEE	12	1289.69	intergenic	✓			
P1215	EPIILGC	7	800.42	intergenic	✓			
P1268	KVSIHYV	7	844.49	intergenic	✓			
P1732	KALPHTPKGFF	11	1241.70	intergenic	✓			
P1716	DDPTVITNPLT	11	1184.60	intergenic	✓			
P1916	KPSMTKQLTSTHAFAVS	17	1832.95	intergenic	✓			
P1961	KDLLDKIP	8	940.57	intergenic	✓			

Note: *B. z.*, *Bipolaris zeicola*; *C. l.*, *Curvularia lunata*; *F. g.*, *Fusarium graminearum*; *B. m.*, *Bipolaris maydis*.

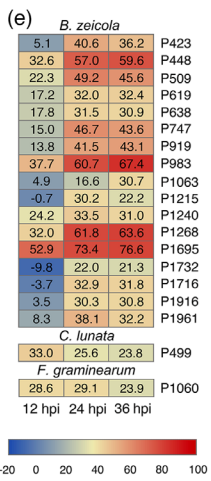
(c)



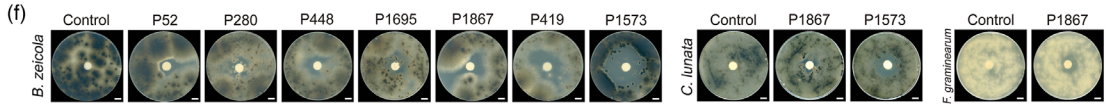
(d)



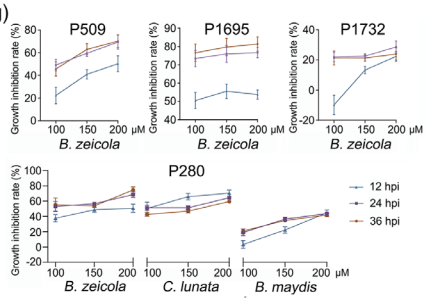
(e)



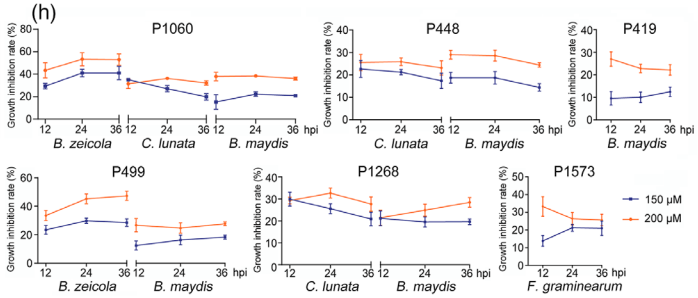
(f)



(g)



(h)



(i)

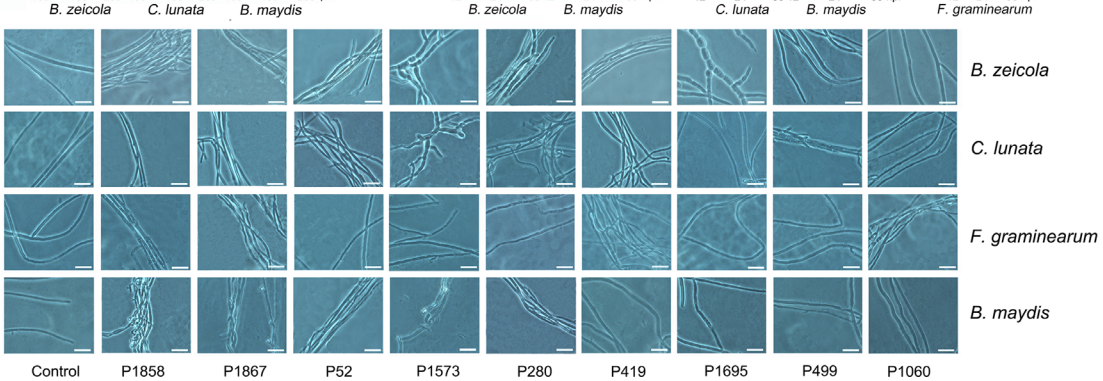


Figure 1 Effects of NCPs on pathogenic fungi. (a) NCP sources and the treatment procedures of fungal species with NCPs. The peptide IDs of synthesized NCPs in this study are shown in the lower panel. For detailed information of these NCPs, see Wang *et al*. (2020b). (b) Detailed information for NCPs showing antifungal activity against four fungal species under the treatments of NCPs at 100 μM. (c) Images of the *in vitro* assay showing four pathogenic fungi treated with 100 μM 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 μM. (e) The growth inhibition rate of NCPs exhibited specific antifungal activity under the treatments of NCPs at 100 μM. (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 μM and 200 μM. (i) Hyphal morphology of four pathogenic fungi treated with 100 μM NCPs. Scale bars = 20 μm.

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 μM and 200 μM, 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 μM or 200 μM than those at 100 μM (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 × (10 × ocular lens and 40 × 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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