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# A Short Review of Anti-Rust Fungi Peptides: Diversity and Bioassays

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Pucciniales are fungal pathogens of plants that cause devastating rust diseases in agriculture. Chemically-synthesized pesticides help farmers to control rust epidemics, but governing bodies aim at limiting their use over the next decade. Defense peptides with antimicrobial activities may help to innovate a next generation of phytosanitary products for sustainable crop protection. This review comprehensively inventories the proteins or peptides exhibiting a biochemically-demonstrated antifungal activity toward Pucciniales (*i.e.*, anti-rust proteins or peptides; hereafter 'ARPs'), and also analyses the bioassays used to characterize them. In total, the review scrutinizes sixteen publications, which collectively report 35 ARPs. These studies used either *in vitro* or *in planta* bioassays, or a combination of both, to characterize ARPs; mostly by evaluating their ability to inhibit the spore germination process *in vitro* or to inhibit fungal growth and rust disease development *in planta*. Also, the manuscript shows that almost no mode of action against rust fungi was elucidated, although some might be inferred from studies performed on other fungi. This short review may serve as a knowledge and methodological basis to inform future studies addressing ARPs.

**Keywords: interaction, defense protein, recombinant protein, mechanism, antifungal**

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## RUST FUNGI IMPOSE A HEAVY PESTICIDE TOLL ON AGRICULTURE

Rust fungi (Pucciniales) are fast-evolving plant pathogens that can infect key crops and threaten global food security (Aime et al., 2018; Figueroa et al., 2020; Duplessis et al., 2021). Farmers notably rely on the use of chemically-synthesized fungicides to control rust epidemics (Oliver and Hewitt, 2014; Cook et al., 2021). Due to the suspected or proven toxicity of those products, governing bodies aim at rapidly limiting their use in agriculture. For instance, the European Commission "Farm to Fork" (F2F) strategy aims at reducing by 50% the use of chemically-synthesized pesticides by 2030 (European Commission, 2020). Therefore, modern agriculture urgently seeks alternatives to chemically-synthesized pesticides. In this context, research and innovation actors aim at developing new tools and solutions for sustainable crop protection, notably by exploiting both our knowledge of the plant immune system and naturally-occurring antimicrobial molecules (Dangl et al., 2013; Moscou and van Esse, 2017; Schwinges et al., 2019; Chen et al., 2021).

## DEFENSE PROTEINS AND PEPTIDES ARE EFFECTIVE MICROBE KILLERS

Some proteins or peptides can directly kill microbes; mostly thank to their cationic property which leads to an interaction with negatively charged membranes of microbes (Tam et al., 2015). For instance, plants possess large protein families referred to as 'pathogenesis-related' (PR), whose members exhibit consistent antimicrobial activities (Van Loon and Van Strien, 1999; Van Loon et al., 2006). Such proteins and peptides may provide innovators with molecular chassis to develop active substances for the next generation of biopharmaceuticals and phytosanitary products (Haney et al., 2019). In agriculture, optimized and vectorized anti-microbial proteins or peptides may assist the development of biological pesticides (aka biopesticides) (Montesinos et al., 2012; Schwinges et al., 2019; Li et al., 2021). Such amino acid-based biopesticides would have the advantage of being residue-free and less likely to display harmful effects towards consumers and ecosystems (Kumar et al., 2021). In such a context, we need to better understand the diversity of defense proteins and peptides and how they function.

This study aimed at building a knowledge and methodological basis to assist future studies addressing proteins or peptides that exhibit an antifungal activity against rust fungi (*i.e.*, anti-rust peptides or proteins; hereafter ARPs). To this end, we first performed a systematic analysis of the literature to build a comprehensive list of ARPs. Importantly, this analysis considered only the studies that used the exogenous application of a purified ARP on a rust fungus *in vitro* or *in planta* to evaluate its anti-rust activity (*i.e.*, direct anti-rust evidence); it thus disregarded the studies that used non-biochemical approaches (e.g., genetic approaches using protein over-expression *in planta*). Then, we analyzed the bioassays used to characterize ARPs, in order to build a portfolio of methods and approaches that could be used in future studies, and surveyed the limited information available about the ARP modes of action. The review ends by discussing key peptide features that should be considered in future studies.

## A CATALOG OF 35 ARPS WITH NOTICEABLE PROPERTIES

To identify ARPs, we performed a systematic literature survey on the Web of Science by performing searches with combinations of key words such as "exogenous peptide", "antimicrobial activity", "Pucciniales", "rust", or "inhibition germination". This survey identified sixteen papers, published between 1996 and 2021 (**Table 1**). Collectively, these papers explicitly reported 35 different ARPs with an antimicrobial activity towards eleven Pucciniales species (**Table 1**; **Figure 1A**). Three rust species served as models in more than three papers: the Asian soybean rust fungus *Phakopsora pachyrhizi* (*Phakosporaceae*; five papers, twelve ARPs reported) (Fang et al., 2010; Vasconcelos et al., 2011; Brand et al., 2012; Lacerda et al., 2016; Schwinges et al., 2019), the white pine blister rust fungus *Cronartium ribicola* (*Cronartiaceae*; three papers; six ARPs reported) (Jacobi et al., 2000; Zamany et al., 2011; Liu et al., 2021) and the wheat leaf rust fungus *Puccinia triticina* (*Pucciniaceae*; three papers; seven ARPs reported) (Barna et al., 2008; Alfred et al., 2013; Wang et al., 2020) (**Figure 1A**). The eight remaining rust species were addressed in less than two papers, and belong either to the *Pucciniaceae* or to the *Melampsoraceae* families (Corrêa et al., 1996; Mathivanan et al., 1998; Rauscher et al., 1999; Dracatos et al., 2014; Petre et al., 2016).

In total, 18 ARPs originate from plants, 10 derive from animals or fungi, and 7 are synthetic peptides (**Table 1**). Overall, the 35 ARPs grouped into two categories: small and large ARPs; comprising less or more than 50 amino acids, respectively (**Figure 1B**). ARPs globally display variable predicted isoelectric points, ranging from 4.95 to 12.6. The nine large ARPs carry an N-terminal signal peptide for secretion, and all but two belong to a well-defined plant pathogenesis related (PR) protein family (Van Loon and Van Strien, 1999). Small ARPs were obtained by chemical synthesis (performed in house or by a company to which the peptide was purchased), and large ARPs were obtained *via* the chromatographic purification of protein extracts from fungal, yeast, or bacterial cultures (**Figure 1B**).

Among the 35 ARPs, some present noticeable properties or activities that could be exploited for crop protection. For instance, RISP (Rust Induced Secreted Protein), a large ARP from poplar, showed a targeted activity that inhibits Pucciniales growth without affecting the growth of other fungi and bacteria (Petre et al., 2016). RISP could thus represent an active compound that controls rust epidemics without altering beneficial microbe communities, which may help achieve sustainable, integrated crop protection (Hacquard et al., 2017). Also, some ARPs display high stability that may be critical to withstand harsh field conditions (such as UV exposure, light and temperature variations, rain washing, and interaction with microflora and microbiota). Indeed, four ARPs are thermostable (PuroA; PuroB, RISP and chitinase EC 3.2.1.14); meaning that they remain stable and functional despite being exposed to high temperatures (Mathivanan et al., 1998; Alfred et al., 2013; Petre et al., 2016). Furthermore, some ARPs could effectively protect leaves from rust infection for weeks by stably remaining on the leaf surface (Petre et al., 2016; Schwinges et al., 2019).

## ARP STUDIES COMBINE *IN VITRO* AND *IN PLANTA* APPROACHES

To better understand the approaches used to evaluate ARP properties and activities, we analyzed the material and method sections of the sixteen studies reported in the **Table 1**. The studies used two main approaches: *in vitro* or *in planta* (**Table 1**, **Figure 1C**). *In vitro* approaches mostly evaluate the ability of purified peptides to inhibit the spore germination process, as the obligate biotrophic nature of most *Pucciniales* prevents the use of *in vitro* growth inhibition assays commonly used with

**TABLE 1 |** An overview of plant anti-rust peptides (ARPs).

| References | Number of ARP reported (ARP IDs) | ARPs belong to plant pathogenesis-related (PR) gene families | ARP originate from plants | Targeted rust species (rust disease) | Purified ARP obtention method | *In vitro* approach used [ARP concentration range] | *In planta* approach used [ARP concentration range] |
|---|---|---|---|---|---|---|---|
| Alfred et al., 2013 | 4 (PuroA*; Pina-R39G; PuroB*; GSP-5D) | no | yes (wheat; *Triticum aestivum*) | *Puccinia striiformis* f. sp. *tritici* (wheat yellow rust or wheat stripe rust)*; Puccinia triticina* (wheat leaf rust) | chemical (solid-phase synthesis) | yes (1, 3) [0.01-1 mg/mL] | yes (4, 6) [0.01-1 mg/mL] |
| Barna et al., 2008 | 1 (PAF; *Penicillium* antifungal protein) | no | no (fungus *Penicillium chrysogenum)* | *Puccinia triticina (*wheat leaf rust) | cellular (chromatographic purification of *P. chrysogenum* culture extract) | yes (1; 2) [0.001-0.1 mg/mL] | yes (4, 6) [0.1-1 mg/mL] |
| Brand et al., 2012 | 7 (IAPs: P61458; A5LDU0; Gm0025x0067; Gm0026x00785; A3KLW0; Q7YRI0; Q9XEY7) | no | yes (soybean; *Glycine max*); no (various animals species) | *Phakopsora pachyrhizi* (asian soybean rust) | chemical (solid-phase synthesis) or commercial (purchased peptides) | no | yes (5) [0.001-1 mg/mL] |
| Corrêa et al., 1996 | 5 (RGD; GRGDGSPK; RGDSPC; RGDS; GRGD) | no | no (synthetic peptides) | *Uromyces appendiculatus* (bean rust) | NA | yes (1; 2; 3) [0.01-2 mM] | no |
| Dracatos et al., 2014 | 2 (NaD1 & NaD2; Class II & I defensins, respectively) | yes (PR-12) | yes (tobacco; *Nicotiana alata*) | *Puccinia coronata* f. sp. *avenae* (crown rust)*; Puccinia sorghi* (maize common rust) | cellular (chromatographic purification following heterologous expression in the yeast *Pichia pastoris*) | yes (1; 2; 3) [0.0001-0.1 mg/mL] | yes (4) [0.1-1 mg/mL] |
| Fang et al., 2010 | 2 (Sp2 & Sp39; random 12-mer peptides) | no | yes (soybean; *Glycine max*) | *Phakopsora pachyrhizi* (asian soybean rust) | viral (phage-display) | yes (1; 2) [0.5- $1.5\times10^{13}$ virions/mL] | yes (5) [$1.5\times10^{13}$ virions/mL] |
| Jacobi et al., 2000 | 4 (Cecropin B; $(Ala^{8,13,18})$-magainin II amide; D2A21 & D4E1 synthetic membrane interactive peptides) | no | no (animal or synthetic peptides) | *Melampsora medusae* (conifer-aspen leaf rust)*; Cronartium ribicola* (white pine blister rust) | commercial (purchased peptides) | yes (1) [0.0001-0.1 mg/mL] | no |
| Lacerda et al., 2016 | 1 (Drr230a; defensin) | yes (PR-12) | yes (pea; *Pisum sativum*) | *Phakopsora pachyrhizi* (asian soybean rust) | cellular (chromatographic purification following heterologous expression in the yeast *Pichia pastoris*) | yes (1) [1-10 mg/mL] | yes (5) [1-10 mg/mL] |
| Liu et al., 2021 | 1 (PmPR10-3.1; *Pinus monticola* pathogenesis-related protein 10-3.1) | yes (PR-10) | yes (white pine; *Pinus monticola*) | *Cronartium ribicola* (white pine blister rust) | cellular (chromatographic purification following heterologous expression in the bacteria *Escherichia coli*) | yes (2) [0.1-1 mg/mL] | no |
| Mathivanan et al., 1998 | 1 (EC 3.2.1.14; chitinase*) | no | no (fungus *Fusarium chlamydosporum)* | *Puccinia arachidis* (peanut rust) | cellular (chromatographic purification from *P. chrysogenum* culture extract) | yes (1) [0.1-1 mg/mL] | no |
| Petre et al., 2016 | 1 (RISP*; rust-induced secreted protein) | no | yes (poplar; *Populus trichocarpa*) | *Melampsora larici-populina* (poplar leaf rust) | cellular (chromatographic purification following heterologous expression in the bacteria *Escherichia coli*) | yes (1; 2) [0.1-1 mg/mL] | yes (5; 6) [0.1-1 mg/mL] |
| Rauscher et al., 1999 | 1 (Pr-1a; pathogenesis-related 1a) | yes (PR-1) | yes (broad bean; *Vicia faba*) | *Uromyces fabae* (broad bean rust) | cellular (chromatographic | yes (1) (unknown) | no |

*(Continued)*

**TABLE 1** | Continued

| References | Number of ARP reported (ARP IDs) | ARPs belong to plant pathogenesis-related (PR) gene families | ARP originate from plants | Targeted rust species (rust disease) | Purified ARP obtention method | *In vitro* approach used [ARP concentration range] | *In planta* approach used [ARP concentration range] |
|---|---|---|---|---|---|---|---|
| | | | | | purification from bean leaf extracts) | | |
| Schwinges et al., 2019 | 1 (DS01*; dermaseptin 01) | no | no (frog *Phyllomedusa genus*) | *Phakopsora pachyrhizi* (asian soybean rust) | cellular (chromatographic purification following heterologous expression in the bacteria *Escherichia coli*) | yes (3) [0.01-0.1 mg/mL] | yes (4) [0.1-1 mg/mL] |
| Vasconcelos et al., 2011 | 1 (XIP; chitinase-like xylanase inhibitor protein) | yes (PR-8) | yes (coffee; *Coffea arabica*) | *Phakopsora pachyrhizi* (asian soybean rust) | cellular (chromatographic purification following heterologous expression in the yeast *Pichia pastoris*) | yes (1) [1-10 mg/mL] | no |
| Wang et al., 2020 | 2 (TaTLP1 & TaPR1; *Triticum aestivum* thaumatin-like protein 1 & *Triticum aestivum* pathogenesis-related protein 1, respectively) | yes (PR-1 & PR-5) | yes (wheat; *Triticum aestivum*) | *Puccinia triticina* (wheat leaf rust) | cellular (chromatographic purification following heterologous expression in the bacteria *Escherichia coli*) | yes (2) [1-10 mg/mL] | no |
| Zamany et al., 2011 | 1 (Pm-AMP1; *Pinus monticola* antimicrobial peptide 1) | no | yes (white pine; *Pinus monticola*) | *Cronartium ribicola* (white pine blister rust) | cellular (chromatographic purification following heterologous expression in the bacteria *Escherichia coli*) | yes (2; 3) [0.01-0.1 mg/mL] | no |

*(*) ARP with specific properties, which are detailed in the main text.*
*(1) the study evaluated the ARP-mediated inhibition of spore germination by calculating germination rates.*
*(2) the study evaluated ARP-mediated inhibition of the growth or differentiation of germ tube or hyphae assay by calculating elongation or branching reduction.*
*(3) the study measured ARP-mediated alteration of fungal structure morphology or infection structure development by microscopy.*
*(4) the study performed ARP treatment prior to rust inoculation.*
*(5) the study performed ARP treatment concomitant to rust inoculation.*
*(6) the study performed ARP treatment after rust inoculation.*

cultivable fungi. *In planta* approaches evaluate the ability of peptide treatments to reduce the growth of the fungus on the host plant (most often on leaves). Amongst the sixteen publications, eight used only *in vitro* approaches, one used only an *in-planta* approach, and seven combined both (**Table 1**).

The most common *in vitro* assay (used in eleven studies) assesses the ability of a peptide to inhibit spore germination ('inhibition of germination assay'). In such an assay, the experimenter classically spreads spores onto an agar medium with peptides at a concentration usually ranging from 0.01 to 0.1 mg/mL, and evaluates afterward the germination rate. In addition, ten studies used *in vitro* assays to evaluate the inhibition of the elongation of germ tubes or hyphae, the development of infection structures (e.g., appressoria), or the altered morphology of fungal structures. *In planta* assays assess the ability of a peptide to inhibit fungal growth on a leaf ('infection assay'). In such an assay, the experimenter classically inoculates the rust fungus onto its host plant (or on detached leaves or leaf discs) before, during, or after treatment with purified peptides at a concentration usually ranging from 0.1 to 1 mg/mL, and evaluates afterward the appearance of disease symptoms by using a visual scoring system. Such scoring classically evaluates uredinia size and distribution, necrosis, and chlorosis depending on each pathosystem (Roelfs and Martens, 1988; Godoy et al., 2006). Noteworthy, for both *in vitro* and *in planta* approaches, two papers used electron microscopy to assess the alteration of fungal cellular structures (Rauscher et al., 1999; Lacerda et al., 2016). Such an approach reveals in detail the structural outcome of ARP treatment, but is arduous and costly to implement, explaining its seldom use.

## ARP MODES OF ACTIONS REMAIN MOSTLY UNKNOWN

To better understand how ARPs function, we screened the literature to identify reported modes of actions. This screen identified only two publications that reported information pertaining to the mode of action. Firstly, Fang and colleagues (2010) reported that Sp2 and Sp39 bind to a protein with an

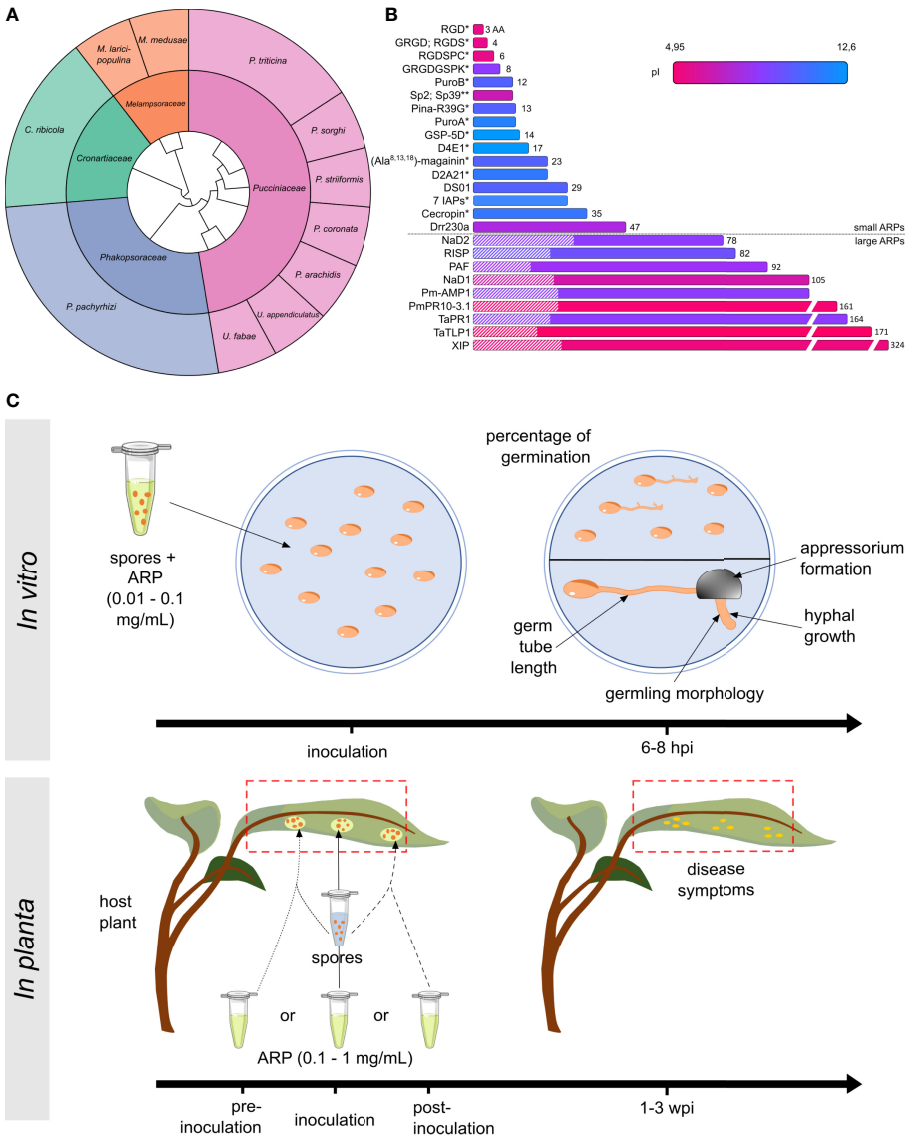


**FIGURE 1** | Overview of known anti-rust peptides (ARPs) and bioassays. **(A)** Sunburst phylogenetic tree of Pucciniales taxa and species used to evaluate ARPs activities. The inner and outer circles indicate Pucciniales families and species, respectively. The genus names are *Phakopsora*, *Cronartium*, *Melampsora*, *Puccinia*, or *Uromyces*. **(B)** Classification of the ARP according to their length in amino acid (aa: linear scale). Predicted isoelectric points (pI: color gradient) and known signal peptides for secretion (hatched area) are indicated. ARPs were i) purified by chromatographic purification from proteinaceous cellular extracts, ii) chemically synthesized or purchased (*), or iii) phage-displayed (**). Amino acid sequences for EC 3.2.1.14 and Pr-1a were not indicated in the original publications. **(C)** For *in vitro* bioassays, spores are treated with 0.01 to 0.1 mg/mL purified ARP and spread onto water agar media, and the percentage of germination or the germling morphology and development is assessed 6 to 8 hours post inoculation (hpi) (see **Supplementary Figure S1** for more details). For *in planta* bioassays, host plant (whole plant, detached leaf, or leaf disc) are treated with 0.1 to 1 mg/mL purified ARP prior to inoculation (dotted line), concomitantly with inoculation (solid line), or after inoculation (dashed line). Disease symptoms are assessed using a visual scoring system 1 to 3 weeks post inoculation (wpi). Typically, the scoring system assessed uredinia size, uredinia distribution, necrosis, or chlorosis.

apparent size of 20 kDa from germinated urediniospores of *P. pachyrhizi*; though the paper did not evaluate the biological relevance of that observation (Fang et al., 2010). Secondly, Mathivanan and colleagues (1998) used light microscopy to show that treatment with a chitinase altered the cell wall appearance of *P. arachidis* urediniospores (Mathivanan et al., 1998). The purified peptide also displayed a chitinase activity, suggesting that it exhibits its anti-rust activity by degrading polysaccharides on the spore surface. For all the other ARPs, the literature reports no known modes of action against rust fungi. However, for some ARPs, a mode of action was proposed, or could be inferred, based on assays performed with other phytopathogenic fungi that are not Pucciniales. For instance, NaD1 binds to the cell wall of *Fusarium oxysporum* hyphae and

permeabilizes the plasma membrane (Van Der Weerden et al., 2008; Van Der Weerden et al., 2010) (**Supplementary Figure S1**). To conclude, ARP modes of action remain vastly unknown, though knowledge gained *via* other fungi may be relevant.

## CONCLUSION AND OUTLOOK: KEY THINGS TO CONSIDER FOR FUTURE ARP STUDIES

Overall, this short review inventoried sixteen papers that collectively reported 35 anti-rust peptides (ARPs) targeting in total 11 different rust species. It showed that the studies mainly used *in vitro* assays, sometimes complemented by *in planta* assays, to evaluate ARP properties and activities. The study also highlighted a clear knowledge gap regarding ARP modes of actions, since no explicit mode of action against rust fungi has been reported so far.

Defense proteins and peptides are growingly viewed as new active substances that can be leveraged to implement a next generation of sustainable biopesticides. In the case of ARPs, the research community crucially needs to better understand their mode of action in order to reach technology readiness levels aligned with phytosanitary implementation. Future studies could leverage the methods and technologies used in model fungi to decipher ARP modes of actions. Notably, assays that use fluorescent labels may help track ARPs and identify their binding sites on spores. Also, structure-function analyses that use truncated or site-directed mutagenized ARPs may help identify functional domains and residues important for anti-rust activity, stability, and specificity.

## AUTHOR CONTRIBUTIONS

Conception and design of the study (JL, BP, SD); data acquisition, analysis, and interpretation (JL, GD, EC, BP); manuscript drafting (JL, GD, EC); manuscript revision and editing (JL, GD, EC, BP, SD). All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fagro.2022.966211/full#supplementary-material

**Supplementary Figure 1 |** Detailed effects of anti-rust peptides (ARPs). Effects of ARPs are shown on an urediniospore that germinates by differentiating a germ tube, an appressorium, and then infection hyphae. The rectangular insert on the bottom left indicates the proposed modes of actions discussed in the main text. (a) delay of *Cronartium ribicola* urediniospore germination; (b) particularly on chitin of nascent germ tube walls; (c) only against *Puccinia coronata* f. sp *avenae*; (d) only against *Puccinia sorghi*; (e) binding to a protein from *Phakopsora pachyrhizi* without a defined mode of action.

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