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1 **Simple and efficient heterologous expression of necrosis-inducing effectors using the**
2 **model plant *Nicotiana benthamiana***

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14 *Parastagonospora nodorum*, wheat

15

16 **Running title**

17 Necrotic effectors produced by plant expression system

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19 **Conflicts of interest**

20 The authors declare that they have no conflicts of interest.

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26 **Abstract**

27 Plant fungal pathogens cause devastating diseases on cereal plants and threaten global food
 28 security. During infection, these pathogens secrete proteinaceous effectors that promote
 29 disease. Some of these effectors from necrotrophic plant pathogens induce a cell death
 30 response (necrosis), which facilitates pathogen growth *in planta*. Characterisation of these
 31 effectors typically requires heterologous expression and microbial expression systems such as
 32 bacteria and yeast are the predominantly used. However, microbial expression systems often
 33 require optimization for any given effector and are, in general, not suitable for effectors
 34 involving cysteine bridges and posttranslational modifications for activity. Here, we describe
 35 a simple and efficient method for expressing such effectors in the model plant *Nicotiana*
 36 *benthamiana*. Briefly, an effector protein is transiently expressed and secreted into the
 37 apoplast of *N. benthamiana* by Agrobacterium-mediated infiltration. Two-to-three days
 38 subsequent to agroinfiltration, the apoplast from the infiltrated leaves is extracted and can be
 39 directly used for phenotyping on host plants. The efficacy of this approach was demonstrated
 40 by expressing the ToxA, Tox3 and Tox1 necrosis-inducing effectors from *Parastagonospora*
 41 *nodorum*. All three effectors produced in *N. benthamiana* were capable of inducing necrosis
 42 in wheat lines, and two of three showed visible bands on Coomassie-stained gel. These data
 43 suggest that *N. benthamiana*-agroinfiltration system is a feasible tool to obtain fungal
 44 effectors, especially those that require disulfide bonds and posttranslational modifications.
 45 Furthermore, due to the low number of proteins typically observed in the apoplast (compared
 46 to intracellular), this simple and high-throughput approach circumvents the requirement to
 47 lyse cells and further purify the target proteins that is required in other heterologous systems.
 48 Because of its simplicity and potential for high-throughput, this method is highly amenable to
 49 the phenotyping of candidate protein effectors on host plants.

50

51 **Abbreviations**

52 AWF: Apoplast washing fluid

53 NEs: Necrotrophic effectors

54 NtPR1sp: *Nicotiana tabacum* pathogenesis-related protein 1 signal peptide

55 SCR: small cysteine-rich

56

57 Introduction

58 Cereal production is susceptible to severe yield losses due to plant pathogens. During
 59 infection, plant pathogens secrete a repertoire of proteins called effectors into the apoplast or
 60 cytoplasm of the host cells to modulate plant processes and promote infection (Dodds and
 61 Rathjen 2010). Whilst these effectors underpin key disease mechanisms, many can be
 62 recognised by the plant, and the outcome of this interaction is broadly dependent on the the
 63 lifecycle of the specific pathogen. In biotrophic pathogens, effectors can be recognised by
 64 specific host receptors, triggering programmed cell death at the site of infection that results in
 65 plant resistance against these pathogens (Dodds and Rathjen 2010). In contrast, recent
 66 findings suggest that some necrotrophic pathogens, such as *Parastagonospora nodorum*,
 67 hijack these host resistance pathways for their own benefit (McDonald and Solomon 2018).
 68 Necrotrophic effectors (NEs) induce cell death only in the presence of host dominant
 69 susceptibility genes that strikingly resemble typical defence receptors (Lorang et al. 2012;
 70 Faris et al. 2010; Shi et al. 2016). Recognition, and the subsequent host cell death, is favoured
 71 by necrotrophic pathogens leading to plant susceptibility instead of resistance (Faris and
 72 Friesen 2020).

73 Despite recognising the important role effectors have in mediating disease, there are several
 74 challenges in understanding their function. These challenges are exemplified in studying
 75 effectors secreted by pathogens of non-model plants that lack genetic amenability. For
 76 example, most monocotyledon plants, such as economically important wheat and rice, are not
 77 compatible with the agroinfiltration method that allows transient expression of proteins in
 78 plants. Although the stable transformation has recently become possible in some of these
 79 plant species, including wheat, the approach is slow and cumbersome. To counter this,
 80 researchers have often turned to expressing their effectors of interest in model plant systems,
 81 such as *N. benthamiana*, as many effectors and their host cognate receptors are active in such
 82 systems (Vleeshouwers et al. 2008; Van Der Hoorn et al. 2000; Ma et al. 2012; Chen et al.
 83 2017). In these cases, the transient expression of the effector with the corresponding host
 84 receptor is often sufficient to produce a cell-death phenotype that allows functional
 85 characterisation of their interaction (e.g. determining required amino acids for recognition
 86 etc). However, this approach does not appear to work for NEs (particularly from
 87 monocotyledon cereal pathogens) and their dominant susceptibility genes and co-expression
 88 in model systems such as *N. benthamiana* does not lead to a visible cell death. Therefore, this
 89 lack of a visible phenotype precludes this co-expression approach from being used to study

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these interactions (as it can be with many biotrophic effectors). Consequently, as an alternative approach, NEs are often expressed using heterologous systems for characterisation and subsequent phenotyping on host plants (Zhang et al. 2017; Outram et al. 2020; Sung et al. 2021; Liu et al. 2012, 2009). Almost exclusively, NEs have been expressed by using microbial expression systems such as *Escherichia coli* and *Pichia Pastoris*.

Many known and predicted effectors are small cysteine-rich (SCR) proteins (Saunders et al. 2012; Duplessis et al. 2011; Sperschneider et al. 2015; Stergiopoulos and De Wit 2009). It has been demonstrated that the cysteines in many of these effectors form disulfide bonds providing stability for these proteins in the apoplast (Saunders et al. 2012). For instance, the Tox3 and Tox1 effectors from *P. nodorum* contains six and sixteen cysteines respectively, and at least one disulfide bond is required for their necrosis-inducing activity (Liu et al. 2012, 2009; Outram et al. 2020). Similarly, *Cladosporium fulvum* effector Avr2 has four disulfide bonds that are essential for its function of inhibiting cysteine protease Rcr3 in tomato plants (Rooney et al. 2005). Moreover, some effectors such as ToxA and Tox3 appear to contain pro-domains subsequent to their secretion signals which are thought to be important for folding and are processed prior to secretion from the fungi (Outram et al. 2020; Tuori et al. 2000; Liu et al. 2009). Recently, Outram et al., (2020) reported that the pro-domain of Tox3 was cleaved by KEX2 protease that lead to a processed protein with demonstrably higher activity indicating that some necrotrophic effectors require posttranslational modification and processing to become fully active. However, because of these requirements, such proteins are difficult to express using a bacterial system due to the absence of eukaryotic cell organelles necessary for disulfide bond formation and posttranslational modifications. Lobstein *et al.* (2012) developed a new bacterial strain, *E. coli* SHuffle, which favors cytoplasmic disulfide bond formations. This new strain allowed researchers to express effectors harbouring disulfide bonds in soluble forms (Zhang et al. 2017; Outram et al. 2020). For example, one study demonstrated that the *E. coli* SHuffle system was successful in producing four out of five SCRs that were trialled (Zhang et al. 2017). Subsequent functional studies on three of the effectors and showed they were biologically active (Zhang et al. 2017). However, yields were often low limiting subsequent functional characterisation. Another approach to express SCR effectors has been using secretion pathways in eukaryotic cells such as yeast. Using this method, the SCR effectors SnTox1 and SnTox3 were successfully expressed at low levels in secreted forms and showed activity in wheat lines harboring corresponding susceptibility genes (Liu et al. 2009, 2012).

However, in microbial expression systems, various physical parameters need to be adjusted to achieve optimal expression conditions that can vary among different strains and target proteins. For example, in the *E. coli* SHuffle system, the effect of temperature and inducer concentration were specific to a given protein when tested on seven different proteins requiring disulfide bonds (Lobstein et al. 2012). Likewise, in the *P. pastoris* system, methanol is the primary carbon source, and its concentration must be closely monitored depending on the rate of utilization by the cells; less methanol decreases productivity, whereas too much is toxic to cells (Karbalaei et al. 2020). In addition to these examples, many other parameters in both *E. coli* and *P. pastoris* most likely need optimization for every new target protein (Lobstein et al. 2012; Karbalaei et al. 2020), which can be time-consuming and laborious. This can be a significant bottleneck for laboratories who do not have substantial protein expertise or are not experienced with microbial expression systems.

Here, we describe a simple and high-throughput method for heterologous expression of cysteine-rich effectors using the secretion pathway of model plant *N. benthamiana*. We demonstrate the efficacy of this method by expressing three unrelated necrosis-inducing effectors (that differ in their cysteine content) from *P. nodorum* and subsequent investigation of their activity on the host plant. Our data show that this plant expression approach can be used for rapid on-host functional screening of candidate necrotrophic effectors, especially those that require disulfide bonds and posttranslational modifications.

Materials / Methods

Plant material and growth conditions

Triticum aestivum cultivars Grandin, Corack, Calingari, and BG261 were grown in a controlled growth chamber with 250 μ E light intensity, 85% relative humidity, and photoperiod of 16-h light at 20°C/8-h dark at 12°C. *Nicotiana benthamiana* plants were grown at 22°C with 16-h light/8-h dark cycle in a growth room.

Cloning the effector proteins

The signal peptide sequences (SPs) of the effector proteins were predicted using SignalP-5.0 (Almagro Armenteros et al. 2019). The predicted SPs were replaced with *Nicotiana tabacum* PR-1 signal peptide (NtPR1sp) to promote efficient secretion of the effectors in *N. benthamiana*. These effector constructs were synthesized as gBlocks (IDT, Integrated DNA

Technology), which were cloned into pENTR/D-TOPO gateway entry vector. The entry vectors were sequence confirmed and recombined into the pB7FWG2 gateway destination vector carrying C-terminus tag (GFP) fusion. To prevent the tag fusion to the effectors, we kept the original stop codons in the recombinant effector constructs, which allow the effectors to be expressed without the protein tag. The sequence confirmed destination vectors were transformed by electroporation into *Agrobacterium tumefaciens* (GV3101, pMP90) for agro-infiltration assays.

Agro-infiltration assays

Agro-infiltration assays were carried out as described previously (Dagvadorj et al. 2017). Briefly, *A. tumefaciens* carrying the effector constructs were streaked on LB-agar plates with antibiotics (Spectinomycin 100 µg/ml, Gentamycin 20 µg/ml and Rifampicin 100 µg/ml) and grown for 2-3 days at 28°C. The bacterial cells on the plate were collected by scraping with 1 ml pipette tip, transferred to new 1.5 ml microcentrifuge tube and resuspended in 1 ml distilled water. The cells were centrifuged at 4000 rpm for 5 min at room temperature. The cell pellet was washed in 1 ml fresh Agro-induction buffer (AI; 10 mM MES pH 5.6, 10 mM MgCl₂) and centrifuged again under the same conditions. Finally, the pellet was suspended in 1 ml AI buffer, and the cell suspension was diluted with AI buffer until the optical density (OD) of the cell suspension was adjusted to 0.2 for infiltration experiments. To enhance transient expression level, the cell suspensions were co-infiltrated with *A. tumefaciens* containing p19 RNA silencing suppressor construct (OD of 0.1). *N. benthamiana* 4- to 6-week of age were agro-infiltrated, and the leaves were harvested at 2 days post-agroinfiltration for apoplast wash fluid extraction.

Apoplast washing fluid extraction

Apoplast washing fluid (AWF) was extracted as previously described with minor modifications (O'Leary et al. 2014). *N. benthamiana* leaves expressing the effector constructs were detached and submerged in a beaker with Milli-Q water. The beaker was placed into a desiccator and a vacuum of -80 kPa applied for 1 min. By gradually releasing the vacuum, the air within the apoplastic space was infiltrated with the water, and the infiltrated area appeared dark translucent by naked eye. This process was repeated until the whole leaf was infiltrated. Then, the leaves were wiped gently with clean tissue to remove the surface water and then, 3-4 leaves sandwiched together in parafilm sheets, rolled and placed into a 20 ml syringe, in a

50 mL Falcon tube. To isolate AWF, the Falcon tube was centrifuged at 500 g for 10 min at 4°C. The collected AWF (400-500 µL/per leaf) was transferred to microcentrifuge tubes and centrifuged again at 17,000 g for 5 min at 4°C to remove cell debris and other insoluble materials. The supernatant was transferred to a fresh tube and used immediately or stored at -80 °C.

MS for ToxA and Tox3 protein identification

For peptide mapping of the ToxA and Tox3 proteins, AWF samples were separated on a 15% SDS-PAGE gel and stained with Coomassie blue. The proteins of interest were excised and subjected to in-gel trypsin digestion as previously described (Sung et al. 2021). Subsequent mass spectrometry data were analysed using Max-Quant software, v.1.6.0.8 (Tyanova et al. 2016). Tryptic peptides were mapped to the sequence of ToxA and Tox3 by using the trypsin enzyme digestion in semi-specific mode.

AWF infiltration into wheat leaves

AWF samples were infiltrated using a needleless syringe into the second leaf of 2-week old wheat cultivars until the infiltration zone reached 3-5 cm. The wheat leaves were checked for the induction of necrosis from one day post-infiltration (dpi) and recorded at 3 dpi.

Results

To trial the expression of SCR proteins in *N. benthamiana*, the ToxA, Tox3 and Tox1 effector proteins from *P. nodorum* were chosen (Table 1). ToxA, Tox3 and Tox1 induce necrosis on wheat lines harbouring the susceptibility genes *Tsn1*, *Snn3* and *Snn1*, respectively. The mature forms of ToxA, Tox3 and Tox1 each contain two, six and sixteen cysteine residues respectively, and their ability to induce necrosis is reliant on disulphide bond formation (Liu et al. 2012; Zhang et al. 2017; Sarma et al. 2005). In addition, ToxA and Tox3 require posttranslational modifications such as processing of pro-domain to become fully active (Outram et al. 2020; Tuori et al. 2000).

To secrete the effector proteins to the apoplast of *N. benthamiana*, all three effectors were cloned with the *Nicotiana tabacum* PR-1 signal peptide (NtPR1sp; Ohshima et al. 1987) as shown in Figure 1a. This modification promotes the proteins to follow correct secretion pathway in *N. benthamiana* (Ma et al. 2015; Breen et al. 2016; Grosse-Holz et al. 2018). The

effectors were expressed under control of CaMV 35S promoter. After two days post agro-infiltration, we isolated the apoplast washing fluid (AWF) from *N. benthamiana* leaves expressing the effectors. The proteins within extracted AWFs were resolved by SDS-PAGE for analysis (Figure 1b). Although no observable band was detected for Tox1, the AWF containing ToxA and Tox3 showed bands within the size ranges of 12-14 kDa and 17-18 kDa, respectively. These sizes are similar to the respective sizes of mature ToxA and Tox3 identified from *P. nodorum* culture filtrates, respectively. To confirm the identity of the observed proteins, the bands were excised from the gel and subjected to tryptic digest MS. As expected, 'Band1' and 'Band2' in Figure 1b were positively identified as ToxA and Tox3, respectively (Figure 1c). Our analysis of the samples revealed that pro-domain of ToxA and Tox3 could not be detected, whereas C-terminal region of ToxA and Tox3 appeared to be intact (Figure 1c). Together, these results suggest that mature forms of ToxA and Tox3 were present in the apoplast of *N. benthamiana*.

To test biological activity of the effectors, the AWFs containing each of ToxA, Tox3, and Tox1 were infiltrated into wheat cultivars carrying *Tsn1*, *Snn3* and *Snn1* susceptibility genes (Grandin, Corack, and Calingari), respectively (Figure 2). Our results showed that the ToxA, Tox3 and Tox1 AWFs were able to induce necrosis in a genotype specific manner. Although we could not detect Tox1 band in protein gel analysis, we did observe chlorosis and necrosis development in the *Snn1* wheat cultivar (Calingari) infiltrated with AWF with Tox1 (Figure 2). A slight chlorotic response was also evident on the *snn1* cultivar Corack upon infiltration of the Tox1 AWF although a minor non-specific reaction for this effector has been previously noted when purified from *E. coli* (Zhang et al. 2017). Collectively, these results suggest that *N. benthamiana* expressed and secreted ToxA, Tox3 and Tox1 are biologically active.

Discussion

In this study, we showed that fungal SCR effectors can be expressed and processed the using *N. benthamiana*-agroinfiltration system. Three unrelated SCR effectors, differing in their cysteine content, were successfully produced as active forms. In comparison to other methods currently used in the field, the approach we presented here is simple and efficient, which may facilitate the identification of putative NEs by screening for cell-death phenotype on host plants. This method may also be an alternative for researchers who struggle with expressing SCR effectors using microbial expression systems. In line with this, a recent study showed

that using agroinfiltration, the apoplast targeted NLP2 (necrosis and ethylene-inducing peptide-like protein) was successfully expressed and induced necrosis in legume plants including field pea, faba bean and lentil; but not in chickpea (Debler et al. 2021). This further corroborates that plants can serve as a heterologous expression system to produce biologically active fungal effectors in secreted form.

This approach has several advantages compared with existing methods used to express necrotrophic effectors. Firstly, the plant signal peptide allows proteins to be trafficked through secretion pathways such as the endoplasmic reticulum and Golgi facilitating the formation of disulfide bonds and other posttranslational modifications. This facilitates the correct folding of SCR proteins are therefore more likely to be soluble compared to cytoplasmic-expressed proteins. Secondly, the apoplast can be separated mechanically from the plant cells, which enables the resolution of the mature proteins in the apoplast from the unprocessed forms inside the cell. This is not the case in cytosolically expressed proteins if crude extract used for the functional studies. Thirdly, the apoplast of the plant cell contains significantly less proteins and molecules than in the cytosol. Therefore, isolated apoplast wash fluid with target protein has much less background or contaminating proteins compared to proteins expressed in the cytosol for functional studies. Furthermore, the reduced protein content in the apoplast simplifies subsequent purification of the target protein if required. Although yeast eukaryotic systems can provide these advantages, various parameters influence the optimal expression, which can differ for any given protein. Thus, the optimization process is more challenging than compared with the *N. benthamiana*-agroinfiltration system.

However, the limitations of this method must be acknowledged. This approach may not be suitable for certain effectors, specifically those that trigger rapid cell-death in *N. benthamiana*. Several studies reported that effectors caused cell-death in *N. benthamiana* when expressed with their respective signal peptide (Kettles et al. 2017; Dagvadorj et al. 2017; Fang et al. 2016; Ma et al. 2015). For instance, eleven out of 119 putative effectors from *Ustilaginoidea virens* induced cell-death in *N. benthamiana*; however, the cause of the cell-death was unknown. In other studies, a group of effectors from *Zymoseptoria tritici* (Kettles et al. 2017), XEG1 effector from *Phytophthora sojae* (Ma et al. 2015), and PstSCR1 effector from *Puccinia striiformis* (Dagvadorj et al. 2017) triggered cell-death in *N. benthamiana*, which appear to be dependent on immune receptors such as NbSER3/NbBAK1. These results suggest that some effectors can activate basal immunity

resulting the plant cell-death. For these types of effectors, non-plant systems may be more suitable.

Our study shows that *N. benthamiana*-agroinfiltration system can serve as an expression system for fungal cysteine-rich effectors. The expression of three unrelated effectors using this system displays production of high and biologically active effectors. This method can be upscaled and combined with a typical protein purification procedure to purify the protein of interest with little efforts. More importantly, this system applicable for expressing almost any protein (with some exceptions) resides in an extracellular space of the cell.

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References

- Almagro Armenteros, J. J., Tsirigos, K. D., Sønderby, C. K., Petersen, T. N., Winther, O., Brunak, S., von Heijne, G., and Nielsen, H. 2019. SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nat. Biotechnol.* 37:420–423
- Breen, S., Williams, S. J., Winterberg, B., Kobe, B., and Solomon, P. S. 2016. Wheat PR-1 proteins are targeted by necrotrophic pathogen effector proteins. *Plant J.* 88:13–25
- Chen, J., Upadhyaya, N. M., Ortiz, D., Sperschneider, J., Li, F., Bouton, C., Breen, S., Dong, C., Xu, B., Zhang, X., Mago, R., Newell, K., Xia, X., Bernoux, M., Taylor, J. M., Steffenson, B., Jin, Y., Zhang, P., Kanyuka, K., Figueroa, M., Ellis, J. G., Park, R. F., and Dodds, P. N. 2017. Loss of *AvrSr50* by somatic exchange in stem rust leads to virulence for *Sr50* resistance in wheat. *Science.* 358:1607–1610
- Dagvadorj, B., Ozketen, A. C., Andac, A., Duggan, C., Bozkurt, T. O., and Akkaya, M. S. 2017. A *Puccinia striiformis* f. sp. *tritici* secreted protein activates plant immunity at the cell surface. *Sci. Rep.* 7:1141

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- 313 Debler, J. W., Henares, B. M., and Lee, R. C. 2021. Agroinfiltration for transient gene
314 expression and characterisation of fungal pathogen effectors in cool □ season grain
315 legume hosts. *Plant Cell Rep.*
- 316 Dodds, P. N., and Rathjen, J. P. 2010. Plant immunity: towards an integrated view of plant–
317 pathogen interactions. *Nat. Rev. Genet.* 11:539–548
- 318 Duplessis, S., Cuomo, C. A., Lin, Y. C., Aerts, A., Tisserant, E., Veneault-Fourrey, C., Joly,
319 D. L., Hacquard, S., Amselem, J., Cantarel, B. L., Chiu, R., Coutinho, P. M., Feau, N.,
320 Field, M., Frey, P., Gelhaye, E., Goldberg, J., Grabherr, M. G., Kodira, C. D., Kohler,
321 A., Kues, U., Lindquist, E. A., Lucas, S. M., Mago, R., Mauceli, E., Morin, E., Murat,
322 C., Pangilinan, J. L., Park, R., Pearson, M., Quesneville, H., Rouhier, N., Sakthikumar,
323 S., Salamov, A. A., Schmutz, J., Selles, B., Shapiro, H., Tanguay, P., Tuskan, G. A.,
324 Henrissat, B., Van de Peer, Y., Rouze, P., Ellis, J. G., Dodds, P. N., Schein, J. E., Zhong,
325 S., Hamelin, R. C., Grigoriev, I. V., Szabo, L. J., and Martin, F. 2011. From the Cover:
326 Obligate biotrophy features unraveled by the genomic analysis of rust fungi. *Proc Natl*
327 *Acad Sci U S A.* 108:9166–9171
- 328 Fang, A., Han, Y., Zhang, N., Zhang, M., Liu, L., Li, S., Lu, F., and Sun, W. 2016.
329 Identification and Characterization of Plant Cell Death-Inducing Secreted Proteins From
330 *Ustilaginoidea virens*. *Mol. Plant. Microbe. Interact.* 29:405–416
- 331 Faris, J. D., and Friesen, T. L. 2020. Plant genes hijacked by necrotrophic fungal pathogens.
332 *Curr. Opin. Plant Biol.* 56:74–80
- 333 Faris, J. D., Zhang, Z., Lu, H., Lu, S., Reddy, L., Cloutier, S., Fellers, J. P., Meinhardt, S. W.,
334 Rasmussen, J. B., Xu, S. S., Oliver, R. P., Simons, K. J., and Friesen, T. L. 2010. A
335 unique wheat disease resistance-like gene governs effector-triggered susceptibility to
336 necrotrophic pathogens. *Proc. Natl. Acad. Sci.* 107:13544–13549
- 337 Friesen, T. L., Stukenbrock, E. H., Liu, Z., Meinhardt, S., Ling, H., Faris, J. D., Rasmussen,

Dagvadorj and Solomon 2021

338 J. B., Solomon, P. S., McDonald, B. A., and Oliver, R. P. 2006. Emergence of a new
339 disease as a result of interspecific virulence gene transfer. *Nat. Genet.* 38:953–956

340 Grosse-Holz, F., Madeira, L., Zahid, M. A., Songer, M., Kourelis, J., Fesenko, M., Ninck, S.,
341 Kaschani, F., Kaiser, M., and van der Hoorn, R. A. L. 2018. Three unrelated protease
342 inhibitors enhance accumulation of pharmaceutical recombinant proteins in *Nicotiana*
343 *benthamiana*. *Plant Biotechnol. J.* 16:1797–1810

344 Van Der Hoorn, R. A. L., Laurent, F., Roth, R., and De Wit, P. J. G. M. 2000.
345 Agroinfiltration is a versatile tool that facilitates comparative analyses of *Avr9/Cf-9*-
346 induced and *Avr4/Cf-4*-induced necrosis. *Mol. Plant-Microbe Interact.* 13:439–446

347 Karbalaei, M., Rezaee, S. A., and Farsiani, H. 2020. *Pichia pastoris*: A highly successful
348 expression system for optimal synthesis of heterologous proteins. *J. Cell. Physiol.*
349 235:5867–5881

350 Kettles, G. J., Bayon, C., Canning, G., Rudd, J. J., and Kanyuka, K. 2017. Apoplastic
351 recognition of multiple candidate effectors from the wheat pathogen *Zymoseptoria tritici*
352 in the nonhost plant *Nicotiana benthamiana*. *New Phytol.* 213:338–350

353 Liu, Z., Faris, J. D., Oliver, R. P., Tan, K. C., Solomon, P. S., McDonald, M. C., McDonald,
354 B. A., Nunez, A., Lu, S., Rasmussen, J. B., and Friesen, T. L. 2009. SnTox3 acts in
355 effector triggered susceptibility to induce disease on wheat carrying the *Snn3* gene.
356 *PLoS Pathog.* 5:e1000581

357 Liu, Z., Zhang, Z., Faris, J. D., Oliver, R. P., Syme, R., and McDonald, M. C. 2012. The
358 cysteine rich necrotrophic effector SnTox1 produced by *Stagonospora nodorum* triggers
359 susceptibility of wheat lines harboring *Snn1*. *PLoS Pathog.* 8:e1002467

360 Lobstein, J., Emrich, C. A., Jeans, C., Faulkner, M., Riggs, P., and Berkmen, M. 2012.
361 Erratum to: SHuffle, a novel *Escherichia coli* protein expression strain capable of
362 correctly folding disulfide bonded proteins in its cytoplasm. *Microb. Cell Fact.* 11:1–16

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- 363 Lorang, J., Kidarsa, T., Bradford, C. S., Gilbert, B., Curtis, M., Tzeng, S. C., Maier, C. S.,
364 and Wolpert, T. J. 2012. Tricking the guard: Exploiting plant defense for disease
365 susceptibility. *Science*. 338:659–662
- 366 Ma, L., Lukasik, E., Gawehns, F., and Takken, F. L. W. 2012. The use of Agroinfiltration for
367 transient expression of plant resistance and fungal effector proteins in *Nicotiana*
368 *benthamiana* leaves. *Methods Mol. Biol.* 835:61–74
- 369 Ma, Z., Song, T., Zhu, L., Ye, W., Wang, Y., Shao, Y., Dong, S., Zhang, Z., Dou, D., Zheng,
370 X., Tyler, B. M., and Wang, Y. 2015. A *Phytophthora sojae* glycoside hydrolase 12
371 protein is a major virulence factor during soybean infection and is recognized as a
372 PAMP. *Plant Cell*. 27:2057–2072
- 373 McDonald, M. C., and Solomon, P. S. 2018. Just the surface: advances in the discovery and
374 characterization of necrotrophic wheat effectors. *Curr. Opin. Microbiol.* 46:14–18
- 375 O’Leary, B. M., Rico, A., McCraw, S., Fones, H. N., and Preston, G. M. 2014. The
376 infiltration-centrifugation technique for extraction of apoplastic fluid from plant leaves
377 using *Phaseolus vulgaris* as an example. *J. Vis. Exp.* :e52113
- 378 Ohshima, M., Matsuoka, M., Yamamoto, N., Tanaka, Y., Kano-Murakami, Y., Ozeki, Y.,
379 Kato, A., Harada, N., and Ohashi, Y. 1987. Nucleotide sequence of the PR-1 gene of
380 *Nicotiana tabacum*. *FEBS Lett.* 225:243–246
- 381 Outram, M. A., Sung, Y.-C., Yu, D., Dagvadorj, B., Rima, S. A., Jones, D. A., Ericsson, D.
382 J., Sperschneider, J., Solomon, P. S., Kobe, B., and Williams, S. J. 2020. The crystal
383 structure of SnTox3 from the necrotrophic fungus *Parastagonospora nodorum* reveals a
384 unique effector fold and insights into Kex2 protease processing of fungal effectors.
385 *bioRxiv*. :2020.05.27.120113
- 386 Rooney, H. C., Van’t Klooster, J. W., van der Hoorn, R. A., Joosten, M. H., Jones, J. D., and
387 de Wit, P. J. 2005. Cladosporium Avr2 inhibits tomato Rcr3 protease required for Cf-2-

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388 dependent disease resistance. *Science*. 308:1783–1786

389 Sarma, G. N., Manning, V. A., Ciuffetti, L. M., and Karplus, P. A. 2005. Structure of Ptr
390 ToxA: An RGD-Containing Host-Selective Toxin from *Pyrenophora tritici-repentis*.
391 17:3190–3202

392 Saunders, D. G. O., Win, J., Cano, L. M., Szabo, L. J., Kamoun, S., and Raffaele, S. 2012.
393 Using hierarchical clustering of secreted protein families to classify and rank candidate
394 effectors of rust fungi. *PLoS One*. 7:e29847

395 Shi, G., Zhang, Z., Friesen, T. L., Raats, D., Fahima, T., Brueggeman, R. S., Lu, S., Trick, H.
396 N., Liu, Z., Chao, W., Frenkel, Z., Xu, S. S., Rasmussen, J. B., and Faris, J. D. 2016.
397 The hijacking of a receptor kinase-driven pathway by a wheat fungal pathogen leads to
398 disease. *Sci. Adv.* 2:1–9

399 Sperschneider, J., Dodds, P. N., Gardiner, D. M., Manners, J. M., Singh, K. B., and Taylor, J.
400 M. 2015. Advances and Challenges in Computational Prediction of Effectors from Plant
401 Pathogenic Fungi. *PLoS Pathog.* 11:1–7

402 Stergiopoulos, I., and De Wit, P. J. G. M. 2009. Fungal effector proteins. *Annu. Rev.*
403 *Phytopathol.* 47:233–263

404 Sung, Y., Outram, M. A., Breen, S., Wang, C., Dagvadorj, B., Winterberg, B., Kobe, B.,
405 Williams, S. J., and Solomon, P. S. 2021. PR1-mediated defence via C-terminal
406 peptide release is targeted by a fungal pathogen effector. *New Phytol.* 229:3467–3480

407 Tuori, R. P., Wolpert, T. J., and Ciuffetti, L. M. 2000. Heterologous expression of functional
408 Ptr ToxA. *Mol. Plant-Microbe Interact.* 13:456–464

409 Tyanova, S., Temu, T., and Cox, J. 2016. The MaxQuant computational platform for mass
410 spectrometry-based shotgun proteomics. *Nat. Protoc.* 11:2301–2319

411 Vleeshouwers, V. G. A. A., Rietman, H., Krensek, P., Champouret, N., Oh, S., Wang, M.,
412 Bouwmeester, K., Vosman, B., Visser, R. G. F., Govers, F., Kamoun, S., and Vossen, E.

Dagvadorj and Solomon 2021

413 A. G. Van Der. 2008. Effector Genomics Accelerates Discovery and Functional
 414 Profiling of Potato Disease Resistance and *Phytophthora Infestans* Avirulence Genes.
 415 3:e2875
 416 Zhang, X., Nguyen, N., Breen, S., Outram, M. A., Dodds, P. N., Kobe, B., Solomon, P. S.,
 417 and Williams, S. J. 2017. Production of small cysteine-rich effector proteins in
 418 *Escherichia coli* for structural and functional studies. Mol. Plant Pathol. 18:141–151
 419
 420

Figure legends

Figure 1. SCR effector proteins expression in *Nicotiana benthamiana*. (a) The schematic view of effector constructs used in this study. (b) Coomassie brilliant blue stained sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of apoplast washing fluid (AWF) isolated from *N. benthamiana* expressing various constructs. Asterisks indicate the expected size of the matured ToxA and Tox3 proteins. AWF from *N. benthamiana* expressing GFP was used for background control. (c) Tryptic in-gel digest of ToxA and Tox3 from AWF of *N. benthamiana*. Tryptic peptides recovered after in-gel digest of corresponding ToxA and Tox3 bands, mapped to the sequence of ToxA and Tox3, respectively, are shown in grey. Predicted pro-domain sequences are underlined. Peptides were analysed by MaxQuant v1.6.8.0 specifying digestion mode as “Trypsin/P”.

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446

447 **Figure 2.** Apoplast washing fluid (AWF) containing the secreted ToxA, Tox3 and Tox1
 448 trigger necrosis in wheat cultivars carrying the susceptibility genes Grandin (*Tsn1*), Corack
 449 (*Snn3*) and Calingari (*Snn1*), respectively. Wheat cultivars Corack (*tsn1*, *snn1*) and BG261
 450 (*snn3*, *snn1*) were used for genotype specificity controls, and AWF from *N. benthamiana*
 451 expressing GFP was used for AWF background control. The leaves were harvested and
 452 recorded three days after AWF infiltration.

453

454 **Table 1.** Analysis of *P. nodorum* effectors used in this study.

SCR effector	Accession number	Number of cysteines	Predicted signal peptide by SignalP 5.0	Reference
ToxA	AGE15694.1	2	1-16	(Friesen et al. 2006)
Tox3	ACR78113.1	6	1-20	(Liu et al. 2009)
Tox1	JN791682.1	16	1-17	(Liu et al. 2012)

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