Wheat PR-1 proteins are targeted by necrotrophic pathogen effector proteins

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SUMMARY

Recent studies have identified that proteinaceous effectors secreted by *Parastagonospora nodorum* are required to cause disease on wheat. These effectors interact in a gene-for-gene manner with host dominant susceptibility loci, resulting in disease. However, whilst the requirement of these effectors for infection is clear, their mechanisms of action remain poorly understood. A yeast-two-hybrid library approach was used to search for wheat proteins that interacted with the necrotrophic effector SnTox3. Using this strategy we identified an interaction between SnTox3 and the wheat pathogenicity-related protein TaPR-1-1, and confirmed it by *in planta* co-immunoprecipitation. PR-1 proteins represent a large family (23 in wheat) of proteins that are up-regulated early in the defence response, however their function remains elusive. Interestingly, the *P. nodorum* effector SnToxA has recently been shown to interact specifically with TaPR-1-5. Our analysis of the SnTox3-TaPR-1 interaction demonstrated that SnTox3 can interact with a broader range of TaPR-1 proteins. Based on these data we utilised homology modeling to predict, and validate, regions on TaPR-1 proteins that are likely to be involved in the SnTox3 interaction. Precipitating from this work, we identified that a PR-1 derived defence signalling peptide from the C-terminus of TaPR1-1, known as CAPE1, enhanced the infection of wheat by *P. nodorum* in an SnTox3-dependent manner, but played no role in ToxA-mediated disease.

Collectively, our data suggest that *P. nodorum* has evolved unique effectors that target a common host-protein involved in host defence, albeit with different mechanisms and potentially outcomes.
INTRODUCTION

The fungus *Parastagonospora nodorum* is the causal agent of Septoria nodorum blotch (SNB), an economically damaging global disease of wheat (Solomon *et al*., 2006). *P. nodorum* is a necrotrophic pathogen that thrives on dead or dying tissue to facilitate disease (Lewis 1973; Oliver and Ipcho, 2004). Until recently, the dogma for necrotrophic pathogens like *P. nodorum* had been that they relied on the secretion of lytic and degradative enzymes to cause necrosis. However, recent findings have demonstrated that at least some necrotrophic pathogens secrete effector molecules, which specifically interact with host susceptibility proteins in a gene-for-gene manner to infect the host and cause disease (Tuori *et al*., 1995; Walton 1996; Lorang *et al*., 2007; Friesen *et al*., 2008; Oliver and Solomon 2010; Liu *et al*., 2012).

One of the better-characterised necrotrophic effectors is the secondary metabolite victorin from *Cochliobolus victoriae*, the causal agent of victoria blight on oat. Victorin was first discovered when oats containing the resistance gene to *Puccinia coronate*, *Pc-2*, were widely planted in order to provide rust resistance (Meehan and Murphy, 1946; Litzenberger 1949). However, these oats also contained the susceptibility gene to victorin, *Vb*, allowing the spread of victoria blight. Subsequent studies have shown that *Pc-2* and *Vb* cannot be genetically separated, and are thought to be encoded by the same locus. As such, the deployment of germplasm harbouring increased resistance to *P. coronate* rendered oats more susceptible to *C. victoriae* (Walton 1996). The mechanistic understanding of this interaction was greatly advanced when it was demonstrated that *C. victoriae* is also able to infect *Arabidopsis thaliana*. Subsequent studies identified the resistance gene *LOCUS ORCHESTRATING VICTORIN EFFECTS1 (LOV1)* as being
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Analysis of changes of the transcriptome, proteome and metabolome of a SnTox3-susceptible wheat line upon infiltration with purified SnTox3 protein revealed a reprogramming of the host and an induction of a plethora of defence responses (Winterberg et al., 2014). Many proteins involved in photosynthesis and some enzymes involved in primary metabolism showed down-regulation in the presence of SnTox3. Additionally, many genes associated with plant defence responses showed strong induction at 24 and 48 hours post-infiltration (hpi) including components of the jasmonic acid (JA) and phenylpropanoid pathways, along with 13 proteins classified as pathogenesis-related (PR) proteins (Winterberg et al., 2014).

However, despite knowing the requirement for these effectors to cause disease, we know little about their mechanisms of action. In an attempt to dissect the ToxA-Tsn1 system, several yeast-2-hybrid (Y2H) analyses have been published, with each reporting a different interacting protein in the host. In 2007, two studies found that ToxA interacted with plastocyanin and the chloroplast ToxABP protein (Manning et al., 2007; Tai et al., 2007). For ToxABP1, it was postulated that it could be involved in trafficking ToxA from the plasma membrane to the chloroplast, which may affect the normal function of the chloroplasts and promote the induction of reactive oxygen species (ROS) (Manning et al., 2007). Plastocyanin has also been suggested to play a role in promoting cell death, as plants in which plastocyanin was silenced using virus-induced gene silencing (VIGS) showed localised cell death (Tai et al., 2007). This is likely to be due to an interruption of electron flow, which may generate ROS, causing cell death. More recently, another study reported that ToxA, from P. nodorum, interacted with the wheat pathogenicity-related PR-1-5 protein (TaPR-1-5) (Lu et al., 2014). Subsequent
mutational analysis identified several residues on both ToxA and TaPR-1-5 that are required for interaction, as well as the induction of necrosis (Lu et al., 2014).

In this study, we have attempted to better understand the function of the SnTox3 effector through a protein interaction analysis. The resulting data show that SnTox3 interacts with a broad range of TaPR-1 proteins, in a mechanism different from that involving ToxA and TaPR-1-5. The finding that both ToxA and SnTox3 interact with wheat TaPR-1 proteins is suggestive of the critical nature of these interactions and implies that TaPR-1 proteins play a key role in mediating disease outcomes in the P. nodorum – wheat interaction.

RESULTS

SnTox3 interacts with a wheat pathogenicity related-1 protein

To identify host targets of the proteinaceous P. nodorum effector SnTox3, a Y2H approach was undertaken. To this end, the susceptible wheat variety BG220 was infiltrated with purified SnTox3 protein. A Y2H library was generated from a combination of RNA prepared from SnTox3 infiltrated wheat at 0, 6, 12, 24, 36 and 72 hpi, as well as a non-infiltrated sample. SnTox3 lacking the signal peptide (SnTox3ΔSP) was screened as bait against this Y2H library. From this library screen, several host proteins were identified that interacted with SnTox3-interacting. One of the candidate proteins, TaPR-1-1, was isolated from multiple prey clones and, was consequently chosen for further analysis. The longest prey clone for TaPR-1-1 was missing 51 amino acids from the N-terminus (ΔN-TaPR-1-1) (Figure S1), while the shortest prey sequenced contained only the last 50 amino acids. The full-length gene was amplified from BG220 cDNA and tested for direct interaction with SnTox3 using the Y2H system.
This confirmed interaction of the full-length TaPR-1-1 with SnTox3 (Figure 1a). No auto-activation of the reporter genes for either SnTox3 or TaPR-1-1 was detected when co-transformed with an empty vector control (Figure 1a) and protein expression was confirmed by western blot (Figure S2a and b).

**Confirmation of interaction in planta**

To confirm the interaction of SnTox3 and TaPR-1-1 in planta, co-immunoprecipitation experiments were conducted following transient expression of the proteins in *Nicotiana benthamiana*. In initial experiments, the signal peptide from *N. tabacum* PR-1 (Nt-PR-1sp) was fused to TaPR-1-1ΔSP. The rationale behind the use of the Nt-PR-1sp was to promote correct trafficking of the TaPR-1 protein in *N. benthamiana*. *Agrobacterium tumefaciens* strains expressing SnTox3ΔSP:myc and NtPR-1sp-TaPR-1-1:GFP were mixed and co-infiltrated into leaves. The proteins were then pulled down using magnetic GFP-Trap_M. SnTox3ΔSP:myc associated with NtPR-1sp-TaPR-1-1:GFP using an *in planta* Co-IP method, but not with the empty:GFP vector control (Figure 1b). The interaction between NtPR-1sp-TaPR-1-1:GFP and SnTox3 was also evident when using the wheat PR-1sp on SnTox3 (TaPR-1sp-SnTox3:myc) (Figure S3). Collectively, the Co-IP and Y2H experiments indicate that TaPR-1-1 is a target of the *P. nodorum* effector protein SnTox3.

**N141 in TaPR-1-1 is not required for interaction with SnTox3**

In a recent report by Lu et al. (2014), the interaction between SnToxA and TaPR-1-5 was shown to depend on the presence of residue N141 in TaPR-1-5. TaPR-1-5 is a member of the group I basic TaPR-1 proteins and is highly conserved at the amino-acid level to four other basic TaPR-1 proteins, PR-1-1, -2, -3 and -4. The sequence
conservation between these 5 proteins is such that N141, along with N142 and N143 is strictly conserved (Lu et al., 2011). To determine if N141 was required for interaction with SnTox3, the N141A mutation was generated in TaPR-1-1 and assayed by Y2H. The mutants TaPR-1-1^{N142A} and TaPR-1-1^{N143A} were also included in this analysis. These assays showed that the TaPR-1-1^{N141A} mutation had no effect on the interaction with SnTox3. A slight reduction was observed in the interaction with SnTox3 for the TaPR-1-1^{N142A} and TaPR-1-1^{N143A} mutations (Figure 2a). No auto-activation of the reporter gene for any of the TaPR-1-1 mutants was observed when co-transformed with an empty vector control, and all proteins were shown to be expressed in yeast (Figure S2b). These interactions were also confirmed in planta using Co-IP. Given the results from our TaPR-1sp-SnTox3 experiments (Figure S3) the native wheat signal peptide was retained on the TaPR-1-1 proteins in these experiments. Collectively, these results indicate that the N141A, N142A or N143A mutations do not affect the interaction between SnTox3 and TaPR-1-1 in planta (Figure 2b) and suggest that the TaPR1-1-SnTox3 binding surface is different to that shown for SnToxA-TaPR-1-5 (Lu et al., 2014).

**SnTox3 interacts with different wheat PR-1 family members**

We were interested in understanding further the level of specificity and selectivity of the SnTox3-TaPR1 interaction. Lu et al. (2014) demonstrated that despite the very high sequence similarity between the basic TaPR-1-1 and TaPR-1-5 proteins (~#% at the protein level), ToxA only interacted with the latter. To determine if the interaction of SnTox3 with TaPR-1-1 was as specific, we assayed its interaction with TaPR-1-5, using the described Y2H approach (Figure 3a). Both host proteins (TaPR-1-1 and TaPR-1-5) were found to interact with SnTox3, implying that SnTox3 may bind more broadly to TaPR-1 proteins than previously described for ToxA.

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There are 23 TaPR-1-type genes in wheat identified thus far and these are divided into three major groups, based on their theoretical isoelectric point (Lu et al., 2011). As described above, SnTox3 interacts with the basic group I proteins TaPR-1-1 and TaPR-1-5. To address whether or not SnTox3 interacts with members of the different TaPR-1 groups, two members from each group were chosen as representatives and tested for interaction. TaPR-1-11 and TaPR-1-13 were chosen as they are also found within group I but have structural differences compared to TaPR-1-1 and TaPR-1-5 (Lu et al., 2011). TaPR-1-18 and TaPR-1-21 were chosen as representatives of group II, which are basic proteins with a C-terminal extension (CTE), while TaPR-1-7 and TaPR-1-16 served as representatives of group III, which are acidic proteins (Lu et al., 2011). TaPR-1-11 and TaPR-1-13 showed weak interaction with SnTox3 (Figure 3a) and no interaction was observed for the basic-CTE proteins TaPR-1-18 and TaPR-1-21. Of the acidic representatives, TaPR-1-16 showed a strong interaction with SnTox3 (Figure 3a); however, inconsistent interaction was observed for TaPR-1-7 with some yeast colonies showing weak interaction and others showing no interaction (Figure 3a and S4).

In our initial Y2H screen, we observed an interaction between the last 50 residues of TaPR-1-1 (C-terminal region (CTR)) and SnTox3. We therefore tested this region from TaPR-1-16, TaPR-1-7 and TaPR-1-18 (with and without the CTE) against SnTox3. The CTRs of both TaPR-1-16 and TaPR-1-7 showed strong interactions with SnTox3; however, no interaction was observed with the CTR of TaPR-1-18, regardless of the presence or absence of the CTE (Figure 3b). Western blot analysis showed that all TaPR-1 proteins were expressed (Figure S2c and d).
Homology modelling reveals surface areas on TaPR-1 that likely mediate SnTox3 interaction

Our data indicate that SnTox3 binds more broadly to the TaPR-1 proteins than ToxA; however, our Y2H results also indicate that there is some selectivity across the groups. With this in mind we used homology modelling to assist our comparison of the polymorphic differences between the non-interacting and interacting TaPR-1 proteins (Figure 4) and to visualise potential SnTox3 interaction sites on the TaPR-1 proteins. As the non-interacting TaPR-1-18 and -20 are ~97 % identical in sequence, we used TaPR-1-18 as a representative non-interacting TaPR-1 protein. TaPR-1-18 and the interacting proteins -1, -5 and -16 have 34 variable residues, not including the CTE (Figure 4a). The majority of these are surface-exposed and localise to surfaces that encompass most of the molecule (Figure 4b), making it difficult to narrow down potential SnTox3 interaction points. However, in our Y2H screen, we found that the smallest interacting region included the CTR of TaPR-1 interacting proteins (Figure 3b) demonstrating that the SnTox3 binding site includes this region of the protein (green section in Figure 4a, c). This reduces the differences between the interacting and non-interacting proteins to eight variable residues that localise to one continuous surface of the molecule (Figure 4c). Included in this region are two surface-exposed basic residues (K126 and R129 in TaPR-1-18) in the loop that connects the α4 helix and βC strand. These residues would likely contribute towards a positively charged surface in this region of the protein which would not be observed in the interacting PR-1 proteins. The remaining five variable residues locate to the far CTR (purple section in Figure 4a, c). In PR1 proteins, this region was recently identified to contain the sequence of a peptide, defined as CAPE1, which was shown to function in defence signalling (Chen et al., 2014).
Based on the information obtained from our homology model (Figure 4a, c) we used truncations and site-directed mutagenesis to further investigate possible binding sites of SnTox3. To investigate if the CAPE1 peptide was the binding site for SnTox3, this region was removed from TaPR-1-1, -5, -7 and -16 in the full-length proteins and the CTRs and tested for interaction with SnTox3 using Y2H. Using this assay all proteins retained the ability to interact with SnTox3 (Figure 5a) suggesting that SnTox3 is not binding to the CAPE1 sequence and that the five variable residues do not influence specificity.

In TaPR1-18 three polymorphic residues, K126, R129 and A141 (Figure 4a, c) remain within the CTRΔCAPE1 region. To investigate their effect on SnTox3 interaction, we generated single mutations; TaPR-1-18K126A, TaPR-1-18R129S and TaPR-1-18A141N and a double mutant TaPR-1-18K126A+R129S, based on the sequence of the interacting protein TaPR-1-1. We also tested a double mutant, TaPR-1-18A141N+G142N which would restore the triple asparagines at 141, 142 and 143 found in TaPR-1-1. These mutations were made within the full-length gene of TaPR-1-18, (which includes the CTE) and within the CTRΔCTE and tested for interaction with SnTox3 using Y2H. All mutations failed to promote an interaction between full-length TaPR-1-18 and SnTox3 in Y2H (Figure 5b), despite being expressed (Figure S2e). However, when investigating the SnTox3 interaction with mutations in the TaPR-1-18 CTRΔCTE the mutations did change the binding specificity. While TaPR1-18 CTRΔCTE does not interact with SnTox3 (Figure 3b), modelling-based individual and double mutations, did promote interactions between the CTRΔCTE of TaPR1-18 and SnTox3. The strongest interacting mutant was the double mutant K126A + R129S, in which the mutations appeared to have a somewhat additive effect compared to that of the single mutants K126A and R129S.
alone (Figure 5c). The A141N mutation also results in interaction with SnTox3, but the interaction appears slightly weaker, according to Y2H, than for the single mutants K126A and R129S. However, the TaPR-1-18 A141N, G142N mutation, which restores the asparagines seen in TaPR-1-1 and TaPR-1-5, appears to weaken the interaction compared to the single A141N mutant (Figure 5c). Western blots confirmed the expression of all TaPR-1 proteins (Figure S2e).

**PR-1 derived CAPE1 peptide enhances SnTox3-mediated disease**

The data above provide strong evidence for the interaction of SnTox3 and members of the TaPR-1 protein family. Dissecting the role of these interactions though is somewhat confounded by the poorly understood function of PR-1 proteins. A promising advance in the field has been the recent discovery of the CAPE1 defence signalling peptide embedded within PR-1 proteins (Chen *et al.*, 2014). Given the interaction of PR-1 proteins with the two necrotrophic effectors, we investigated whether or not the CAPE1 peptide plays a role in the *P. nodorum* – wheat interaction.

To assess if CAPE1-dependent defence signalling does play a role in disease, the peptide encoded by TaPR-1-1 was synthesized and infiltrated into the 2nd leaf of 2-week old Corack (*Snn3*) seedlings (Figure 7). Corack is an Australian elite cultivar that has been previously demonstrated to be only susceptible to SnTox3 (not ToxA or Tox1) (Tan *et al.*, 2014). Two hours subsequent to infiltration, the leaves were infected with *P. nodorum*. After four days, the water-infiltrated leaves showed disease symptoms typical for *P. nodorum* with chlorotic and necrotic flecking evident throughout the infected area. However, when infiltrated with 300 nM CAPE1, the leaves exhibited significantly advanced disease symptoms in nearly all replicates with the infected area appearing...
highly necrotised. In the absence of the pathogen, CAPE1 itself did not induce visible symptoms. These data demonstrate that the infiltration of CAPE1 results in enhanced susceptibility of wheat to *P. nodorum* in an Snn3-dependent manner.

To determine if the observed effect of CAPE1 on disease was restricted to a SnTox3-Snn3 interaction, the same experiment was repeated using the wheat cultivar BG261. BG261 is sensitive to ToxA (*Tsn1*) but insensitive to SnTox3 (*snn3*). Like Corack, the infiltration of CAPE1 had no discernable effect on the leaves of BG261. Subsequent infections though revealed that the infiltration of CAPE1 had no impact on the severity of *P. nodorum* disease. These data suggest that the enhanced susceptibility of wheat to *P. nodorum* induced by the defence signalling peptide CAPE1 is dependent on the SnTox3-Snn3 interaction.

**DISCUSSION**

Using the Y2H approach, we show that the proteinaceous effector SnTox3 from *P. nodorum* interacts with the wheat protein TaPR-1-1. A detailed genomic mapping study by Lu *et al.* (2011) identified 23 TaPR-1 genes in wheat. Based on theoretical isoelectric points, these 23 genes encode seven acidic and 16 basic TaPR-1 proteins. These basic proteins can be further subdivided depending on the presence of a C-terminal extension (basic-CTE); six wheat proteins contain this extension. This subdivision results in three groups; basic proteins (group I), basic with a C-terminal extension (group II) and acidic proteins (group III) (Lu *et al.*, 2011). Further analysis of SnTox3 interaction with eight representative members of the TaPR-1 family revealed that SnTox3 interacts with multiple acidic and basic wheat PR-1 proteins; three showed strong interaction, three showed weak interaction and two showed no interaction (Figure 3a). These results
suggest that, unlike ToxA, SnTox3 targets multiple members of the wheat TaPR-1 family.

Whilst being extensively described and cited in the literature, the function or role of PR-1 proteins remains enigmatic. They are the most abundantly produced PR proteins in plants upon pathogen attack, as demonstrated in model plant species (up to 2% of the total leaf protein in tobacco, for example) (van Loon and van Kammen, 1970; van Loon and van Strien, 1999; Lu et al., 2011). As such, they have attracted considerable interest in understanding their role in plant-pathogen interactions. PR-1 gene expression has long been used as a reliable marker of the activation of hypersensitive response (HR)-mediated defence pathways and the establishment of salicylic acid (SA)-mediated disease resistance in diverse plant species, but their biological role has remained elusive (van Loon et al., 2006). PR-1 proteins have been suggested to have antifungal activity or play a role in host defence signalling and cell death (van Loon et al., 2006). However, much of the evidence published to date has been circumstantial. It is important to note though that whilst their function may be the source of conjecture, PR-1 proteins are secreted suggesting that they are likely to be co-localised with Tox3 (also secreted during infection) (Carr et al., 1987).

In this study, we demonstrate that PR-1 is a target for the necrotrophic effector SnTox3, which has been shown to cause cell death of wheat leaves (Liu et al., 2009). Lu et al. (2014) also used Y2H to show that the SnToxA effector protein interacts with TaPR-1-5. Together, these studies imply that the interaction of SnToxA and SnTox3 with these pathogenicity-related proteins plays a key role in mediating the outcome of disease. There are clearly differences though in these interactions. Lu et al. (2014)
showed that SnToxA interaction is specific to TaPR-1-5, as no interaction with TaPR-1-1 was detected. This is in contrast to SnTox3, which appears able to interact with multiple TaPR-1 proteins. It was also demonstrated that the asparagine residue 141 (N141) in TaPR-1-5, which appears to be in a surface loop region, was essential to the interaction with SnToxA (Lu et al., 2014). The mutation of this residue had no affect on the interaction of TaPR-1-1 and SnTox3. These data suggest that although both effector proteins appear to target TaPR-1 proteins, there are significant differences in the interaction and possibly also the outcome of these interactions.

Clearly, a key question to arise from this study pertains to the role of this interaction and indeed that of PR-1 proteins themselves. Previously, a definitive role for the SnToxA-TaPR-1-5 protein interaction could not be demonstrated by Lu et al (2014); however, infiltration experiments indicated that the binding of SnToxA and TaPR1-5 was required to cause necrosis, suggesting a putative role for PR-1 proteins in cell death (Lu et al., 2014). Interestingly, a recent peptidomic study has cast some light on the role of PR-1 proteins. The CAPE1 peptide was identified to originate from the far C-terminus of the tomato PR-1b protein and was shown to correspond to a damage-associated molecular pattern (DAMP) elicitor (Chen et al., 2014). CAPE1 was also induced by wounding and capable of inducing defence hormones such as JA and SA, as well as several pathogen-related marker genes; PR-2, PR-7 and PR-1b itself (Chen et al., 2014). However, CAPE1 did not induce PTI-responsive genes (e.g. WRKY53), suggesting that CAPE1 may induce systemic resistance rather than PTI (Chen et al., 2014). These authors also demonstrated that the pre-treatment of tomato leaves with CAPE1 prior to infection with 

Pseudomonas syringae pv tomato resulted in reduced disease symptoms and no development of HR (Chen et al., 2014). The CAPE1 peptide consists of 11 amino
acids (PxGNxxxxPY) and is encoded within the C-terminal region (Figure 4, 6) of the tomato PR-1b (amino acids 149-159). It was speculated that the CNYx motif immediately upstream of CAPE1 is required for cleavage from PR-1b (Chen et al., 2014). This was recently investigated using the CNYx motif on PROAtCAPE1, the precursor protein for the AtCAPE1 peptide (Chien et al., 2015). This study showed that a mutation of this CNY sequence can abolish the cleavage of the AtCAPE1 peptide (Chien et al., 2015). Analysis of this peptide sequence shows that it appears conserved in most of the wheat TaPR-1 proteins (Figure 6) and falls within our proposed SnTox3-TaPR-1 interaction surface.

Sixteen wheat TaPR-1 proteins have the CAPE1 peptide conserved, and 8 (TaPR-1-15 and all group III proteins) have a mutation in the proposed cleavage motif changing it from CNY to CSY. Whether this mutation would result in a non-functional cleavage motif is yet to be determined, as the cysteine and tyrosine residues are intact. Group II proteins have a tyrosine-to-phenylalanine substitution at the last amino acid of the peptide and contain a CTE, which would also extend the peptide (Figure 6). TaPR-1-11 has a proline-to-leucine substitution at the first amino acid of the peptide, while TaPR-1-12 is missing the glycine at position 3 of the peptide and also contains an asparagine-to-proline substitution at position 4 within the peptide. It remains to be shown whether the possible wheat PR-1 peptides have a similar function to the tomato CAPE1 in the induction of defence responses, and whether the group II proteins with CTEs create a larger peptide with a different function compared to the other CAPE1-like wheat peptides.
Given the potential conservation of this peptide within wheat and the fact that SnTox3 is targeting its precursor protein we tested to see if the interaction between the TaPR-1 proteins and SnTox3 required CAPE1. From these experiments we have shown that CAPE1 does not appear to be required for interaction with SnTox3 (Figure 5a). To investigate this interaction further we exploited homology modelling to visualise potential SnTox3-interacting sites on the TaPR-1 proteins. Based on our hits from our Y2H screen, we already speculated that the SnTox3 binding site occurs, at least in part, in the last 50 amino-acids of the CTR of the TaPR-1 proteins. From our model, and comparison between PR-1 interacting (TaPR-1-1, -5 and -16) and non-interacting proteins (TaPR-1-18), a number of potential amino acids could be further highlighted that may be important in interaction. While we were unable to induce an interaction with SnTox3 by altering these residues in the context of the full-length TaPR1-18, we were able to change the specificity of the CTR of TaPR1-18 through mutagenesis. Mutation of residues K126A and R129S, in the loop connecting the α4 helix and the βC strand, had the strongest effect on the interaction in Y2H experiments. In the TaPR-1 proteins shown to interact with SnTox3, these amino acid positions are either occupied by an aspartate/alanine or a serine/alanine, respectively. It is therefore plausible that the bulky positively charged residues at these positions in PR1-18 make interaction with SnTox3 unfavourable. Collectively, these data indicate that interaction with the CTR of PR-1 and SnTox3 is important, however, regions outside of this must also be required for interaction as we could not induce an interaction with the mutated variants of full-length PR1-18. These findings provide some insight into the mode of interaction between TaPR1 proteins and SnTox3. This would be benefited significantly by access to high-resolution structures of the respective binding partners, which we are currently pursuing.

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When considering the biological role of the interaction, we were also interested as to whether or not the PR-1 derived peptide CAPE1 played a role in Septoria nodorum blotch. Subsequent infiltration and infection experiments proved that the peptide significantly enhances the infection of wheat by *P. nodorum* and that this induced susceptibility is dependent on the SnTox3-Snn3 interaction. Whilst it has yet to be determined that the interaction of SnTox3 and PR-1 directly impacts on CAPE1 levels, it is tempting to speculate that SnTox3 interacts with PR-1 to mediate CAPE1 release, which in turn activates downstream defence signalling that involves or is hijacked by Snn3. Whilst again this is only speculative, the exploitation of host defence pathways by necrotrophic pathogens to facilitate disease has been previously demonstrated (Lorang *et al.*, 2007). Ongoing work is now underway to demonstrate that the interaction of SnTox3 and PR-1 proteins does mediate CAPE1 release.

It is also relevant to re-emphasize that CAPE1 does not enhance ToxA-mediated disease. Together with the fact that amino acids in TaPR-1 essential for interaction with ToxA are not required for interaction with SnTox3, and that ToxA only interacts with TaPR-1-5, these data provide further evidence that the interactions of SnTox3 and ToxA with TaPR-1 proteins are functionally distinct.

In summary, the work presented here adds valuable insight into the host targets of necrotrophic effectors. From this work and the work by Lu *et al.* (2014), it is now clear that two different effectors from *P. nodorum* are targeting the PR-1 family of proteins in wheat, albeit through potentially different mechanisms. This suggests that the PR-1 family are key targets for the manipulation of the host defence system to aid in *P. nodorum* infection. Studies are now ongoing to further functionally dissect his protein.
interaction and to also understand how the CAPE1 peptide is involved in facilitating disease.

EXPERIMENTAL PROCEDURES

Plant, bacteria and fungal growth conditions

SnTox3-sensitive wheat (*Triticum aestivum* genotype BG220) was grown as previously described (Solomon *et al*., 2006). *Triticum aestivum* genotypes Corack and BG261 were grown in a controlled environment chamber with a 16 h day, 8 h night cycle with 20 °C day temperature and 12 °C night temperature. Light intensity was at 250 μE with 85 % relative humidity. *Nicotiana benthamiana* plants were grown in a constant temperature room at 22 °C on a light/dark cycle of 16 h light 8 h dark. *Escherichia coli* cultures were grown in either Luria-Bertani (LB) broth or on agar plates overnight in 37 °C with appropriate antibiotics for plasmid selection. *Agrobacterium tumefaciens* cultures were grown in either Luria-Bertani (LB) broth or on agar plates at 28 °C for two days with appropriate antibiotics for plasmid selection. *P. nodorum* isolate SN15 was grown for 2 weeks at 22 °C in a 12-12 h dark and light cycle.

Cloning and vectors

pUC57_SnTox3ΔSP was generated by GenScript Inc. and used as a cloning platform. SnTox3ΔSP was digested from the vector using the restriction enzymes EcoRI and BamHI for cloning into the Clontech Y2H DNA-binding domain vector pGBK7. TaPR-1-1 was amplified from BG220 cDNA using the primers PR-1-1_F and PR-1-1_R which included the signal peptide (Table S1). The full-length gene was ligated into the Clontech Y2H activation domain vector pGADT7.
SnTox3ΔSP was re-amplified for Gateway® cloning (Invitrogen) using the primers: Tox3_gate_F, Tox3_gate_R and Tox3-ST_gate_R, (Table S1). attB recombination sites were added by a second PCR using the attB1 and attB2 primers (Table S1) to all sequences and recombined into pDONR201 vector using BP Clonase II (Invitrogen). NtPR-1sp-TaPR-1-ST and TaPR1sp-SnTox3 were synthesised by GeneArt® (Applied Biosystems) with attB recombination sites present allowing BP clonase II (Invitrogen) to recombine it into pDONR201. LR clonase II (Invitrogen) was used to recombine SnTox3ΔSP and TaPR1sp-SnTox3 into the plant expression vector pGWB17 (SnTox3ΔSP:myc and TaPR1sp-SnTox3:myc) (Nakagawa et al., 2007). LR clonase II (Invitrogen) was used to recombine NtPR-1sp-TaPR-1 into the plant expression vector pB7FWG2.0 (NtPR-1sp-TaPR-1:GFP) (Karimi et al., 2002). All plasmids were transformed into E. coli by electroporation, sequenced, and plasmids with confirmed inserts were transformed into Agrobacterium tumefaciens strain GV3101, pMP90 cells by electroporation. Positive transformants were used for in planta Nicotiana benthamiana assays. pB7FWG2.0_empty vector was also transformed by electroporation into A. tumefaciens strain GV3101, pMP90.

TaPR-1-1 was amplified out of the Y2H vector, pGADT7, with the primers PR-1-1_gate_F and PR-1-1_gate_R (Table S1), which contain partial AttB sites. Full AttB recombination sites were added by a second PCR using the attB1 and attB2 primers (Table S1). TaPR-1-1 was then recombined into pDONR201 vector using BP clonase II (Invitrogen). TaPR-1-1 mutations were made by mutating the pDONR201_TaPR-1-1 plasmid using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) as per the manufacturer’s instructions. The mutation primers used were PR-1-1 N141A sense, PR-1-1 N141A antisense, PR-1-1 N142A sense, PR-1-1 N142A

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antisense, PR-1-1 N143A sense and PR-1-1 N143A antisense. Primer sequences are shown in Table S1. The mutated PR-1-1 proteins were then placed into the pGADT7 vector which has the addition of Gateway sites (Engelhardt et al., 2012), and into the plant expression vector pB7FWG2.0 (NtPR-1sp-TaPR-1:GFP) (Karimi et al., 2002) using LR clonase II reactions (Invitrogen).

gBlocks of the full-length TaPR-1-5, TaPR-1-7, TaPR-1-11, TaPR-1-13, coTaPR-1-16, TaPR-1-18 and coTaPR-1-21 were synthesised with their signal peptides and with attB recombination sites on the ends by Integrated DNA Technologies (IDT). In the case of TaPR-1-16 and TaPR-1-21 these gBlocks were codon-optimised for *Saccharomyces cerevisiae* in order to allow synthesis of the gBlock. These ds linear DNA gBlocks allowed each gene to be recombined into the pDONR201 vector by use of BP clonase II (Invitrogen). These genes then underwent recombination into the Y2H activation domain vector, pGADT7, which has the addition of Gateway sites (Engelhardt et al., 2012) by LR clonase II (Invitrogen). The gBlocks were used as template to amplify the last 50 amino acids of TaPR-1-7, coTaPR-1-16 and TaPR-1-18 using the primers PR-1-7Ct_F and PR-1-7_R, PR-1-16Ct_F and PR-1-16_R respectively. PR-1-18 was cloned with and without the C-terminal extension (CTE) using PR-1-18Ct_F and PR-1-18_R and PR-1-18-CTE_R (Table S1). These genes were ligated into the Clontech Y2H activation domain vector pGADT7.

Amplification of the full-length and last 50 amino acids (Ct) of TaPR-1-1, TaPR-1-5, TaPR-1-7 and coTaPR-1-16 without the CAPE1 peptide was done using the following primer combinations. TaPR-1-1-CAPE1 was cloned with PR-1-1_F and PR1-1-CAPE1_R,
TaPR-1-1Ct-CAPE1 was cloned with PR-1-1Ct_F and PR-1-1-CAPE1_R, TaPR-1-5-CAPE1 was cloned with PR-1-5_F and PR-1-5-CAPE1_R, TaPR-1-5Ct-CAPE1 was cloned with PR-1-5Ct_F and PR-1-5-CAPE1_R, TaPR-1-7-CAPE1 was cloned with PR-1-7_F and PR-1-7-CAPE1_R, TaPR-1-7Ct-CAPE1 was cloned with PR-1-7Ct_F and PR-1-7-CAPE1_R, coTaPR-1-16-CAPE1 was cloned with coPR-1-16_F and coPR-1-16-CAPE1_R, coTaPR-1-16Ct-CAPE1 was cloned with coPR-1-16Ct_F and coPR-1-16-CAPE1_R (Table S1). These genes were ligated into the Clontech Y2H activation domain vector pGADT7.

TaPR-1-18 mutants were generated using the using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) as per the manufacturer’s instructions using the pGADT7_TaPR-1-18 plasmid as the template. The following mutation primers were used; PR-1-18 K126A sense, PR-1-18 K126A antisense, PR-1-18 R129S sense, PR-1-18 R129S antisense, PR-1-18 K126A, R129S sense, PR-1-18 K126A, R129S antisense, PR-1-18 A141N sense, PR-1-18 A141N antisense and PR-1-18 A141N, G142N sense, PR-1-18 A141N, G142N antisense. Primer sequences are shown in Table S1. The amplification of the C-termini without the CTE from the TaPR-1-18 mutant sequences was conducted using the TaPR-1-18Ct_F and TaPR-1-18-CTE_R primers (Table S1) with above pGADT7_TaPR-1-18 mutants as templates.

**Yeast-two-hybrid library screen and specific transformations**

A Y2H library was generated using the Make Your Own “Mate & Plate™” Library System (Clontech) following the manufacturer’s instructions. SnTox3 was heterologously expressed in *Pichia pastoris* and isolated as previously described (Liu *et al.*, 2009;
Winterberg et al., 2014). Culture filtrates containing SnTox3 was infiltrated into *T. aestivum* cultivar BG220 (Liu et al. 2009). Material was harvested at 0, 6, 12, 24, 36 and 72 hpi along with un-infiltrated leaves. RNA was extracted from these samples and each underwent cDNA synthesis as described in the Make Your Own "Mate & Plate™" Library System. 3 µl of each ds cDNA sample was combined to create the library using the previously stated kit. pGBK7_SnTox3ΔSP was transformed into the Y2HGlold yeast strain following the manufacturer’s instructions of the Yeastmaker™ Yeast Transformation System (Clontech).

All subsequent small-scale transformations to investigate specific interactions also followed the Yeastmaker™ Yeast Transformation System protocol or followed the Frozen-EZ Yeast Transformation II Kit™ (ZymoResearch) as per the manufacturer’s instructions. The yeast library screen of SnTox3 against the generated wheat BG220 library was performed using the Matchmaker™ Gold Yeast Two-Hybrid System (Clontech) following the manufacturer’s instructions.

**Transient gene expression in *N. benthamiana***

Positive *A. tumefaciens* transformants were grown for 2 d at 28°C in LB supplemented with appropriate antibiotics. Rifampicin 50 µg/ml and gentamycin 25 µg/ml were added to all *A. tumefaciens* cultures. Kanamycin 50 µg/ml and hygromycin 50 µg/ml were added to the pGWB17 cultures (Nakagawa et al. 2007) while spectinomycin 100 µg/ml was added to the pB7FWG2.0 cultures (Karimi et al., 2002). Kanamycin 50 µg/ml was added to the silencing suppressor culture pJL3-p19 (Voinnet et al., 2003). For plant inoculations, all *A. tumefaciens* cultures were resuspended to OD600 = 1. Cultures
carrying SnTox3ΔSP:myc or TaPR-1sp-SnTox3:myc were mixed 1 : 1 with cultures carrying NtPR-1-1-TaPR-1:GFP or GFP:empty vector to a final OD600 of each strain of 0.5. OD600 = 0.1, pJL3-p19 was also added to each mixed culture. The mixed cultures were infiltrated with a 1 ml syringe without a needle through the abaxial leaf surface superficially wounded with a needle. One plant was used per mixture with two leaves per plant being infiltrated. Leaves were harvested 3 dpi.

Co-immunoprecipitation experiments and immunoblot analyses

300 mg of leaf tissue was used per sample. Total proteins were extracted as previously described (Engelhardt et al., 2012). Briefly, samples were ground in liquid N2 and resuspended in GTEN buffer containing 0.15% Nonidet P-40 substitute (Amresco®), 1 mM PMSF (Amresco®), 1 % protease inhibitor cocktail with EDTA (Amresco®) and 1 % PVPP. To immunoprecipitate GFP fusion proteins, magnetic GFP-Trap_M (Chromotek) was used, following the manufacturer’s protocol with the addition of filtering the supernatant after the 10 min centrifugation through a 0.2 µm filter before proceeding to an additional 30 min centrifugation.

Protein extraction from yeast cells was performed as described by Kushnirov (2000). The presence of recombinant proteins was monitored by western blot analysis. Briefly, extracted proteins were resolved by 11 % SDS-PAGE as previously described (Sambrook et al., 1989) and proteins were transferred to PVDF membranes (Bio-Rad) using the Mini Trans-Blot® system (Bio-Rad) as previously described (Sambrook et al., 1989). To detect fusions containing cMyc, an α-c-Myc antibody (A14) from rabbit (Santa Cruz Biotechnology, Inc.) was used. GFP-fusions were detected using an α-GFP (FL) antibody from rabbit (Santa Cruz Biotechnology, Inc.). These antibodies were detected
by an anti-rabbit IgG HRP from goat (Sigma-Aldrich®). The HA-fusions were detected using anti-HA High Affinity from rat (Roche) followed by an anti-rat IgG HRP from goat (Sigma-Aldrich®). Protein bands were detected on the immunoblot using ECL substrate (Bio-Rad) and visualised by ImageQuant4000 (GE Healthcare).

**Phylogenetic analysis**

Protein alignments were generated using the software package Geneious v6.1.7 (www.geneious.com).

**Wheat PR-1 modelling**

The PR-1-18 model was generated using the automated webserver ModWeb (http://salilab.org/modweb) (Eswar 2003). The template for the PR-1-18 model was tomato PR1 protein P14a (PDB entry 1cfe) (Fernández et al., 1997), which shares ~55% sequence identity across the 140 residues that were modeled. Structural analysis and figure preparation was performed with PyMOL (http://www.pymol.org/; DeLano Scientific LLC).

**Wheat infection assays**

Syringe infiltrations of the 2nd leaf of 2-week-old wheat seedlings were conducted with either H2O or 300 nM CAPE1 peptide. The CAPE1 peptide was derived from the C-terminus of TaPR-1-1 was synthesised and purified to >99 % purity by Mimotopes. *P. nodorum* isolate SN15 was inoculated onto the same area where infiltration had occurred using a spore concentration of 1 x 10^6 as previously described (Tan et al. 2008).
ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION LEGENDS

Figure S1. Protein alignment of PR-1 proteins. Alignment of the partial PR-1 sequences identified by Y2H (dN-TaPR-1-1 and CTR) against BR34 TaPR-1-1 taken from NCBI.

Figure S2. Confirmation of protein expression. Protein extraction from yeast cultures to confirm expression in Y2H experiments.

Figure S3. In planta confirmation of SnTox3 – TaPR-1-1 interaction. GFP co-immunoprecipitation undertaken in N. benthamiana.

Figure S4. SnTox3 interaction with TaPR-1-7. Y2H assay screening SnTox3ΔSP against TaPR-1-7.

Table S1. Primer sequences

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REFERENCES


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the vacuolar tobacco proteins AP24, chitinase and β-1,3-glucanase in transgenic plants. *Plant Mol. Biol.* **21**, 583–593.


FIGURE LEGENDS

Figure 1. Identification and confirmation of the SnTox3 host interactor TaPR-1-1.

(a) Y2H assay using SnTox3ΔSP and full-length TaPR-1-1 proteins. Serial dilutions from cell suspensions of a single yeast colony expressing bait and prey plasmids are shown. Growth on –Leu,Trp indicates the presence of both plasmids in the yeast colony and growth on QDO/X/A media shows interaction of the two proteins. Serial dilution indicates the strength of interaction. Top panels (Lam/T-antigen) show a negative control interaction, second row (p53/T-antigen) shows a positive control interaction. SnTox3ΔSP was co-transformed with TaPR-1-1 and empty vector, in rows three and four, respectively. TaPR-1-1 was also co-transformed with empty vector, in the final row. (b) In planta confirmation of SnTox3 – TaPR-1-1 interaction. GFP co-immunoprecipitation undertaken in N. benthamiana. Transient expression via A. tumefaciens in N. benthamiana leaves of SnTox3ΔSP:myc (33 kDa) with NtPR1sp-TaPR1-1:GFP (44 kDa) and SnTox3ΔSP:myc with empty:GFP (27 kDa). Protein extracts were incubated with GFP_Trap_M beads. The beads were separated magnetically, washed and the captured proteins were eluted from the beads. Samples were analysed by western blot and probed with both GFP and myc specific antibodies. Top panel
represents input proteins. Bottom panel represents proteins captured during GFP IP. (CS) = Coomassie stain shows protein loading. Numbers on the left indicate molecular weight marker.

**Figure 2.** TaPR-1-1 mutational analysis. (a) Y2H assay testing SnTox3ΔSP against TaPR-1-1 and three TaPR-1-1 mutations N141A, N142A and N143A for interaction. Serial dilutions from cell suspensions of a single yeast colony expressing bait and prey plasmids are shown. Growth on –Leu,Trp indicates the presence of both plasmids in the yeast colony and growth on QDO/X/A media shows interaction of the two proteins. Serial dilutions indicate the strength of interaction. TaPR-1-1 mutants were also screened against empty vector indicating no auto-activation of reporter genes. (b) In planta confirmation. GFP co-immunoprecipitation undertaken in *N. benthamiana*. SnTox3ΔSP:myc (33 kDa) transiently expressed via *A. tumefaciens* in *N. benthamiana* leaves, with each of the following proteins: TaPR-1-1 N141A:GFP, TaPR-1-1 N142A:GFP, TaPR-1-1 N143A:GFP (43 kDa) and empty:GFP (27 kDa). Protein extracts were incubated with GFP_Trap_M beads. The beads were separated magnetically, washed and the captured proteins were eluted from the beads. Samples were analysed by western blot and probed with both GFP and myc specific antibodies. Top panel represents input proteins. Bottom panel represents proteins captured during GFP IP. (CS) = Coomassie stain shows protein loading. Numbers on the left indicate molecular weight markers.

**Figure 3.** SnTox3 interaction with different TaPR-1 proteins. (a) Y2H assay screening SnTox3ΔSP against seven TaPR-1 proteins: TaPR-1-5, TaPR-1-11, TaPR-1-13, TaPR-1-18, TaPR-1-21, TaPR-1-7 and TaPR-1-16. (b) Y2H assay screening SnTox3ΔSP against the CTRs of three TaPR-1 proteins; TaPR-1-7, TaPR-1-16 and TaPR-1-18 with
and without the CTE. Serial dilutions from cell suspensions of a single yeast colony expressing bait and prey plasmids is shown. Growth on –Leu,Trp indicates the presence of both plasmids in the yeast colony and growth on QDO/X/A media shows interaction of the two proteins. Serial dilutions indicate the strength of interaction. TaPR-1 proteins were also screened against empty vector indicating no auto-activation of reporter genes.

Figure 4. Homology model of TaPR-1-18 shown in cartoon representation. (a) Multiple sequence alignment of TaPR-1-18, -1, -5, -16. The position of the secondary structure elements in the TaPR-1-18 model is shown on top of the alignment and labeled according to the nomenclature used for tomato PR-1b (Fernandez et al., 1997). Surface accessibility of residues is shown at the bottom of alignment; dark blue – exposed, light blue – partially exposed, white – buried. Numbers in green indicate the cysteine pairing and residues unique to TaPR-1-18 are represented with a blue background. The alignment was formatted using ESPript 3.0 (Robert & Gouet 2014). The triple asparagine residues that are important for ToxA-TaPR-1-5 binding (Lu et al., 2014) are highlighted with *. Highlighted are the residues in the smallest-interacting region identified for TaPR-1-1 by Y2H (green) and the CAPE1 peptide (magenta). (b) Surface representation of the TaPR-1-18 model (top and bottom rotated 180° around the x-axis). Residues unique to TaPR1-18 are highlighted in blue and localise to surfaces that encompass most of the molecule. (c) Cartoon representation of the TaPR1-18 model. Green highlights the smallest-interacting region identified for TaPR-1-1 by Y2H and the CAPE1 peptide is displayed in magenta. Residues unique to TaPR-1-18
compared to TaPR-1-1, -5, -16 within the smallest-interacting region are displayed as sticks and labeled.

**Figure 5.** Investigation of the SnTox3 interaction site on TaPR-1. (a) Y2H assay screening SnTox3ΔSP (Tox3) against four full-length proteins without their CAPE1: TaPR-1-1ΔCAPE1, TaPR-1-5ΔCAPE1, TaPR-1-7ΔCAPE1 and TaPR-1-16ΔCAPE1 and the same four TaPR-1 proteins using only the C-terminal region (CTR) without their CAPE1 peptide: TaPR-1-1CTRΔCAPE1, TaPR-1-5CTRΔCAPE1, TaPR-1-7CTRΔCAPE1 and TaPR-1-16CTRΔCAPE1. (b) Y2H assay screening SnTox3ΔSP (Tox3) against the full-length TaPR-1-18 mutants: TaPR-1-18K126A, TaPR-1-18R129S, TaPR-1-18K126A+R129S, TaPR-1-18A141N and TaPR-1-18A141N+G142N. (c) Y2H assay screening SnTox3ΔSP (Tox3) against the CTR of TaPR-1-18K126A and TaPR-1-18R129S, TaPR-1-18K126A+R129S, TaPR-1-18A141N and TaPR-1-18A141N+G142N each without the C-terminal extension (CTE). Serial dilutions from cell suspensions of a single yeast colony expressing bait and prey plasmids is shown. Growth on –Leu,Trp indicates the presence of both plasmids in the yeast colony, growth on QDO/X/A media shows interaction of the two proteins. Serial dilutions indicate the strength of interaction. TaPR-1 proteins were also screened against empty vector (empty) indicating no auto-activation of reporter genes.

**Figure 6.** Alignment of putative wheat CAPE1-like peptides. Accession numbers and protein names are shown in column 1 with the putative cleavage site (CNYx) and peptide motif (PxGNxxxxxPY) motif in column 2. The group each protein has been placed in based on isoelectric point (Lu et al., 2011) is shown in column 3. Red characters indicate different amino acids compared to the CAPE1 peptide from tomato. The “*” symbol represents the stop codon.
Figure 7. The effect of CAPE1 pre-treatment of *P. nodorum* infection. Top panel shows leaves infiltrated with the control (H$_2$O) and 300 nM CAPE1. Corack (Snn3+) leaves on the top, BG261 (Snn3-) on the bottom. Bottom panel shows leaves infiltrated with the control (H$_2$O) and 300 nM CAPE1 and then infected with WT (SN15) *P. nodorum*. Corack leaves (top) were harvested at 4 dpi. BG261 leaves (bottom) were harvested 6 dpi as disease had not progressed well enough at four days on this cultivar.
Figure 1. Identification and confirmation of the SnTox3 host interactor TaPR-1-1. (a) Y2H assay using SnTox3ΔSP and full-length TaPR-1-1 proteins. Serial dilutions from cell suspensions of a single yeast colony expressing bait and prey plasmids are shown. Growth on -Leu,Trp indicates the presence of both plasmids in the yeast colony, growth and blue colouration on QDO/X/A media shows interaction of the two proteins. Serial dilution indicates the strength of interaction. Top panel (Lam/T-antigen) show a negative control interaction, second row (p53/T-antigen) shows a positive control interaction. SnTox3ΔSP was co-transformed with TaPR-1-1 and empty vector, in rows three and four, respectively. TaPR-1-1 was also co-transformed with empty vector, in the final row. (b) In planta confirmation of SnTox3 - TaPR-1-1 interaction. GFP co-immunoprecipitation undertaken in N. benthamiana leaves of SnTox3ΔSP:myc (33 kDa) with NIPR1sp-TaPR1-1:GFP (44 kDa) and SnTox3ΔSP:myc with empty:GFP (27 kDa). Protein extracts were incubated with GFP-Trp_M beads. The beads were separated magnetically, washed and the captured proteins were eluted from the beads. Samples were analysed by western blot and probed with both GFP and myc specific antibodies. Top panel represents input proteins. Bottom panel represents proteins captured during GFP IP (CS) = Coomassie stain shows protein loading. Numbers on the left indicate molecular weight marker.
Figure 2. TaFR-1-1 mutation analysis. (a) Y2H assay testing SnTox3SP against TaFR-1-1 and three TaFR-1-1 mutations N141A, N142A and N143A for interaction. Serial dilutions from cell suspensions of a single yeast colony expressing bait and prey plasmids are shown. Growth on -Leu,Trp indicates the presence of both plasmids in the yeast colony. Growth and blue colouration on QDO/X/A media shows interaction of the two proteins. Serial dilutions indicate the strength of interaction. TaFR-1-1 mutants were also screened against empty vector indicating no auto-activation of reporter genes. (b) Immunoprecipitation, GFP co-immunoprecipitation undertaken in N. benthamiana. SnTox3ΔSP/myc (33 kDa) transiently expressed via A. tumefaciens in N. benthamiana leaves, with each of the following proteins: TaFR-1-1 N141A,GFP, TaFR-1-1 N142A,GFP and empty-GFP (27 kDa). Protein extracts were incubated with GFP, Top, M beads. The beads were separated magnetically, washed and the captured proteins were eluted from the beads. Samples were analysed by western blot and probed with both GFP and myc specific antibodies. Top panel represents input proteins. Bottom panel represents proteins captured during GFP IP (L3 = Coomassie stain shows protein loading). Numbers on the left indicate molecular weight markers.
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**Figure 4.** Homology model of PRI-1-18 shown in cartoon representation. (a) Multiple sequence alignment of PRI-1-18, -1, -5, -16. The position of the secondary structure elements in the PRI-1-18 model is shown on top of the alignment and labeled according to the nomenclature used for tomato PRI-1 (Fernández et al., 1999). Surface accessibility of residues is shown at the bottom of the alignment: dark blue = exposed; light blue = partially exposed; white = buried; red = green indicates the cysteine pairing and residues unique to PRI-1-18 are represented with a blue background. The alignment was formatted using ESPript 3.0 (Robert & Gouet, 2014). The triple asparagine residues that are important for TauA-PRI-1-18 binding (Su et al., 2014) are highlighted with *; highlighted are the residues in the smallest interacting region identified for PRI-1-1 by Y2H (green) and the CAPET peptide (magenta). (b) Surface representation of the PRI-1-18 model. (c) Cartoon representation of the PRI-1-18 model. Green highlights the smallest-interacting region identified for TauPR-1-1 by Y2H and the CAPET peptide is displayed in magenta. Residues unique to PRI-1-18 compared to PRI-1-1, -5, -16 within the smallest-interacting region are displayed as sticks and labeled.
Figure 5. Investigation of the SnTox3 interaction site on TaPR-1. (a) Y2H assay screening SnTox3Δ55P (Tox3) against four full length proteins without their C-terminus (CTR) without their C-terminus (CTR) without their C-terminus (CTR) without their C-terminus (CTR): TaPR-1-CTRΔCAPE1, TaPR-1-CTRΔCAPE1, TaPR-1-CTRΔCAPE1, and TaPR-1-CTRΔCAPE1, and the same four TaPR-1 proteins using only the C-terminal region (CTR) without their C-terminus (CTR): TaPR-1-CTRΔCAPE1, TaPR-1-CTRΔCAPE1, TaPR-1-CTRΔCAPE1, and TaPR-1-CTRΔCAPE1. (b) Y2H assay screening SnTox3Δ55P (Tox3) against the full length TaPR-1-18 mutants: TaPR-1-18ΔCAPE1, TaPR-1-18ΔCAPE1, TaPR-1-18ΔCAPE1, TaPR-1-18ΔCAPE1, and TaPR-1-18ΔCAPE1, and TaPR-1-18ΔCAPE1 each without the C-terminal extension (CTE). Serial dilutions from cell suspensions of a single yeast colony expressing bait and prey plasmids is shown. Growth on -Leu,Trp indicates the presence of both plasmids in the yeast colony; growth and blue coloration on QDO/X/A media shows interaction of the two proteins. Serial dilutions indicate the strength of interaction. TaPR-1 proteins were also screened against empty vector (empty) indicating no auto-activation of reporter genes.

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<td>II</td>
</tr>
<tr>
<td>HQ541981 (TaPR - 1-21)</td>
<td>CNYN PPGNFGDGRPFAFLTLDAEAK *</td>
<td>II</td>
</tr>
<tr>
<td>HQ541986 (TaPR - 1-6)</td>
<td>CSYN PPGNFPGVS PY*</td>
<td>III</td>
</tr>
<tr>
<td>HQ541967 (TaPR - 1-7)</td>
<td>CSYN PPGNFPGVS PY*</td>
<td>III</td>
</tr>
<tr>
<td>HQ541968 (TaPR - 1-8)</td>
<td>CSYN PPGNFPGVS PY*</td>
<td>III</td>
</tr>
<tr>
<td>HQ541969 (TaPR - 1-9)</td>
<td>CSYN PPGNYGGS PY*</td>
<td>III</td>
</tr>
<tr>
<td>HQ541976 (TaPR - 1-16)</td>
<td>CSYN PPGNVEGVS PY*</td>
<td>III</td>
</tr>
<tr>
<td>HQ541979 (TaPR - 1-19)</td>
<td>CSYN PPGNVEGVS PY*</td>
<td>III</td>
</tr>
<tr>
<td>HQ541972 (TaPR - 1-12)</td>
<td>CSYS PPGPVV GQVPY*</td>
<td>III</td>
</tr>
</tbody>
</table>

**Figure 6.** Alignment of putative wheat CAPE1-like peptides. Accession numbers and protein names are shown in column 1 with the putative cleavage site (CNYE) and peptide motif (PGNxxxxxPY*) motif in column 2. The group each protein has been placed in based on isoelectric point (Lu et al. 2011) is shown in column 3. Red characters indicate different amino acids compared to the CAPE1 peptide from tomato. The * symbol represents the stop codon.
Figure 7. The effect of CAPE1 pre-treatment of *P. nodorum* infection. Top panel shows leaves infiltrated with the control (H₂O) and 300 nM CAPE1. Corack (Snn3+) leaves on the top, BG261 (Snn3-) on the bottom. Bottom panel shows leaves infiltrated with the control (H₂O) and 300 nM CAPE1 and then infected with WT (SN15) *P. nodorum*. Corack leaves (top) were harvested at 4 dpi. BG261 leaves (bottom) were harvested 6 dpi as disease had not progressed well enough at four days on this cultivar.