

1 The blast effector Pwl2 is a virulence factor that modifies the cellular localisation of host protein 2 HIP43 to suppress immunity.

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Abstract

The rice blast fungus *Magnaporthe oryzae* secretes a battery of effector proteins during host infection to suppress plant immunity and promote disease. Among these effectors, the MAX effector-Pwl2 was first identified as a host specificity determinant for infection of weeping lovegrass (*Eragrostis curvula*). However, its biological activity has remained unknown. Here we show that the *PWL2* gene is ubiquitous in all host-limited forms of *M. oryzae* and has undergone substantial copy number expansion indicating that it is a core effector of the blast fungus. We used CRISPR/Cas9-mediated gene editing to delete all three copies of *PWL2* and generate a *pwl2* null mutant in *M. oryzae* strain Guy11. This resulted in gain-of-virulence towards weeping lovegrass, but a reduction in the severity of disease symptoms on rice. To further investigate the virulence mechanism of Pwl2, we showed that transgenic rice and barley lines constitutively expressing *PWL2* display suppression of reactive oxygen species generation and increased susceptibility to infection by *M. oryzae*. Also, we identify the barley and rice heavy metal-binding isoprenylated protein HIPP43 as a target of the Pwl2 effector. Transgenic lines overexpressing HIPP43 exhibit attenuated defense responses and increased susceptibility to blast infection, consistent with the hypothesis that Pwl2 binds HIPP43 to modulate immunity. This binding is coupled to changes in cellular localisation of these proteins. Taken together, our results provide evidence that Pwl2 is a virulence factor in *M. oryzae* that acts by suppressing host immunity through binding and re-localising HIPP43.

Introduction

Plant pathogens secrete virulence proteins called effectors during infection to suppress the plant immune system and facilitate invasion of host tissue. This process, called effector-triggered susceptibility, enables pathogens to manipulate plant metabolic processes, overcome plant immunity and successfully colonise host plants (1). Fungal pathogens, such as the devastating blast fungus *Magnaporthe oryzae* (which destroys enough rice to feed 60 million people every year) deploy an extensive battery of effectors, with more than 500 identified to date (2, 3), but very few are functionally characterised (4). However, effectors are also recognised by plant intracellular nucleotide-binding leucine rich repeat (NLR) immune receptors to activate disease resistance. For example, in rice, the paired NLRs RGA5/RGA4, Pik-1/Pik-2, and Piks-1/Piks-2 confer resistance to *M. oryzae* strains that secrete AVR1-CO39/AVR-Pia, AVR-Pik or AVR-Mgk1 effectors, respectively (5-9). Each of these proteins are members of the *Magnaporthe* Avr and ToxB-like (MAX) effector family that are sequence unrelated, but structurally conserved (10), and are overrepresented among effectors recognised by rice NLR immune receptors (6, 7, 11, 12). Pwl2 was first identified as a host specificity determinant that controls pathogenicity towards weeping lovegrass (*Eragrostis curvula*), a widely grown forage grass and was recently shown to be recognised in barley by the Mla3 immune receptor (13, 14) While *PWL2* has been widely studied and used as an important tool to understand effector secretion and delivery, the molecular mechanisms by which Pwl2 interacts with the host have remained unknown, with little research into the virulence function of this effector.

A companion paper Zdrzałek *et al* (15) identifies Pwl2 as a MAX effector, and recent studies of MAX-effector perception by NLRs have identified host small heavy metal-associated (sHMA) domain containing proteins to be the likely targets of several of these effectors. Small HMA proteins are highly expanded across plant species with putative functions ranging from heavy metal detoxification, to potential metallochaperones and other yet to be characterised functions (16-18). These proteins can be broadly separated into two families, heavy metal-associated plant proteins (HPPs), and heavy-metal associated isoprenylated plant proteins (HIPPs), of which the latter possess a C-terminal isoprenylation motif (CaaX, where ‘a’ represents an aliphatic residue and ‘X’ is any amino acid) important for anchoring to

membranes (19). The rice sHMA protein Pi21 is a disease susceptibility factor, with rice lines expressing this protein exhibiting delayed defence responses to rice blast (19, 20). This has led to the use of loss-of-function alleles of *pi21*, as a recessive form of rice blast resistance in breeding programs (20). Furthermore, differences in host sHMA protein repertoires have been linked to host-specific adaptive evolution of the APikL2 effector family, while studies of AVR-Pik and its target OsHIPP20 suggests that effector binding may stabilize the host protein (21, 22). Importantly, sHMA protein domains have also been found integrated as non-canonical immune sensory domains in some NLR proteins, such as the rice Pik-1/2 and Pia (RGA5/4) receptors, and are essential for recognition of their cognate *M. oryzae* effectors (6, 8, 9, 11). The integration of sHMA proteins into immune receptors further underlines the relevance of sHMA proteins in pathogen virulence. However, the exact function of sHMA proteins in plants and their link to disease susceptibility in plant-pathogen interactions is still poorly understood, obscuring the specific function of MAX effectors that target these proteins.

In this study, we investigate the biological function of *PWL2* and its role in fungal pathogenesis. We were motivated to understand how a ubiquitous effector like Pwl2 functions during a susceptible interaction between *M. oryzae* and its host. We demonstrate that the *PWL2* gene is ubiquitous across *M. oryzae* lineages and has undergone significant gene duplication and expansion in copy number, with many rice blast isolates possessing 3-5 copies, making its functional analysis challenging. Using CRISPR-Cas9 gene editing, we generated a *pwl2* null mutant and verified the role of Pwl2 as a host specificity determinant towards weeping lovegrass and barley lines containing *Mla3*. We constructed transgenic rice and barley lines constitutively expressing *PWL2* and show that Pwl2 suppresses PAMP-triggered immunity through attenuation of the ROS burst, resulting in increased susceptibility to rice blast. Finally, we reveal that Pwl2 targets the sHMA protein HIPP43 in both rice and barley and show that transgenic plants overexpressing HIPP43 have reduced immune responses. We also show that Pwl2 co-localizes and alters sub-cellular localisation of HIPP43 in *Nicotiana benthamiana*. Taken together, our study provides evidence that Pwl2 plays a role in defence suppression by binding and re-localising HIPP43 to facilitate colonization of plant tissue by the blast fungus.

Results

Pwl2 is an *in-planta* effector, expressed during infection and localizes to the BIC.

The *PWL2* gene is specifically expressed during the biotrophic phase of infection, peaking in expression at 36 - 48 hours post-infection (hpi) (3) (**Fig. S1A and B**). The Pwl2 effector localises in the biotrophic interfacial complex (BIC) and is clearly visible as a single bright punctum, initially at the tip of an invasive hypha and then adjacent to the bulbous, branched hyphae as they develop (**Fig. 1A and B**). The BIC is a plant membrane-rich structure and the predicted site of effector delivery (23-25), although experiments to date have not precluded that the BIC could be a site of effector sequestration by the host. To investigate Pwl2 secretion at the BIC during infection, we generated a single *M. oryzae* strain expressing two BIC-localised effectors Pwl2-mRFP and Bas1-GFP and visualised their localisation during infection of leaf sheath tissue of the susceptible rice cultivar Moukoto. The two effectors were observed as small punctate signals within the same BIC (**Fig. 1A and C**). By contrast, when two different *M. oryzae* strains, Ina168 and Guy11, expressing Pwl2-GFP and Pwl2-mRFP respectively were used to simultaneously infect rice tissue, we observed that the BIC formed by each individual invasive hypha harboured exclusively either Pwl2-GFP or Pwl2-mRFP but never both signals (**Fig. 1B and D**). This suggests that the Pwl2 effector is secreted from the BIC into host cells, especially when considered with previous reports showing the BIC to be the site of unconventional fungal secretion and clathrin-mediated endocytosis (24).

PWL2 is ubiquitous in isolates of *M. oryzae*.

The *PWL2* effector belongs to a wider gene family (13, 26), but conservation of the *PWL* family in the global rice blast population, and among different host-limited forms of *M. oryzae*, is not fully known. Therefore, we investigated *PWL* gene family conservation in publicly available genome sequences, including isolates that infect *Oryza sativa* (rice), *Eleusine* spp. (finger millet), *Hordeum vulgare* (barley), *Setaria* spp. (foxtail millet), *Triticum aestivum* (wheat), *Lolium* spp. (rye grass), *Brachiaria* spp. (signal grass), *Panicum repens* (torpedo grass) and *Eragrostis* spp. (weeping lovegrass) (27-30). We also included two close relatives of *M. oryzae* that infect crabgrass (*Digitaria sanguinalis*) and pearl millet (*Pennisetum* spp.). We used Pwl1 (BAH22184.1), Pwl2 (QNS36448.1), Pwl3 (AAA80240.1) and Pwl4 (AAA80241.1) protein sequences to query for the presence or absence of each protein-encoding gene. We observed that *PWL1* is present in group EC-II (31) of *Eleusine*-infecting isolates, one *Eragrostis*-infecting isolate and some *Oryza*-infecting isolates but absent in all the other host-specific lineages (**Fig. 1F and S2B**). Similarly, *PWL3* is found in most *Oryza*-infecting isolates, but almost completely lost in the other lineages, except for some *Setaria*, *Lolium*, and *Eleusine*-infecting isolates. *PWL4* is present in *Eleusine*, *Lolium* and *Triticum*-infecting isolates, but almost completely lost in *Oryza*-infecting isolates. We however found one *Digitaria*-infecting isolate that carry *PWL4* (**Fig. S2B**). In contrast to all other *PWL* family members, *PWL2* is found in all host-limited forms of *M. oryzae* and related *Magnaporthe* species (**Fig. 1E and S2B**). We conclude that Pwl2 is a core effector of the blast fungus.

Having determined Pwl2 is ubiquitous in isolates of *M. oryzae*, we next sought to determine how polymorphic the effector is, in different host-limited forms of *M. oryzae*. Apart from the *pwl2-2* variant that contains a single Asp-90-Asn substitution (13, 14), we found 14 new variants of Pwl2 that can be renamed Pwl2-3 to Pwl2-14. Majority of the polymorphic residues occurred at positions His87 to Ser92 (**Fig. S2A**). In comparison, we identified 2 variants of Pwl1 (renamed Pwl1-2 and Pwl1-3), 4 variants of Pwl3 and 1 variant of Pwl4 (**Fig. S3A, B and C**). From this analysis, we conclude that Pwl2 is highly polymorphic compared to other members in the family. We next tested the ability of *PWL2* variant alleles to be recognised on

known resistant hosts. A subset of the isolates carrying variants of *PWL2* BTJP4-16 (*pwl2-3*), JUM1 (*pwl2-2*), BN0293 (variant *Pwl2*), BF5 (variant *Pwl2*), VO107 (variant *Pwl2*), and PM1 (variant *Pwl2*) were used to infect barley lines that carry *Mla3* or weeping lovegrass. Some of the variants were identified in same background isolate carrying *PWL1*, one or multiple copies of *PWL2*, and it was not possible functionally characterise them using infection assays. We found BTJP4-16 (*pwl2-3*), JUM1 (*pwl2-2*) and BN0293 to be virulent on these hosts, providing evidence that these alleles are non-functional (**Fig. S2C-D**). Isolates VO107, BF5 and PM1 did not produce blast disease lesions in the presence of *Mla3* suggesting that alleles present in these isolates were either recognised by the host, or the *M. oryzae* isolates carry other effectors recognised by additional R-genes contained in barley cv. Baronesse or weeping lovegrass. (**Fig. S2E**).

Pwl2 allelic variability affecting host specificity is not associated with the MAX effector fold.

The majority of *Oryza*-, *Lolium*- and *Triticum*-infecting isolates of *M. oryzae* analysed have *PWL2* or the variant *pwl2-2* allele, which is associated with gain of virulence to *E. curvula* and *Mla3* (**Fig. S1C**) (13, 14). Our analyses of the presence of *PWL2* in *Lolium* and *Triticum*-infecting isolates from the pandemic wheat blast lineage (32) identified an allele of *PWL2*, designated *pwl2-3*, with several polymorphisms in the core effector fold: Glu-89-Gln, Lys-91-Gln and Ser-92-Ile (**Fig. 1E, S1D and S2A**). Interestingly, *pwl2-3* was also identified from *Digitaria* and *Pennisetum spp*-infecting isolates of *Magnaporthe* (**Fig. 1F, S2A and B**). In an accompanying paper by Zdrzalek *et al* (15) Pwl2 has been structurally verified as a predicted MAX effector, with a core β -sandwich fold formed of two antiparallel β -sheets, as well as a single α -helix, C-terminal to the β -sandwich fold. We therefore mapped a subset of the identified polymorphic residues of Pwl2 including *pwl2-3* to the effector crystal structure. All polymorphisms between *pwl2* alleles were found to reside in the C-terminal α -helix, a distinct region from the core MAX-fold (**Fig. 1E**). This suggests that the interface used to bind Pwl2 by the immune receptors present in resistant barley (*Mla3*) and weeping love grass, unlikely involves the MAX-fold.

***PWL2* occurs in high copy number in *M. oryzae* field isolates.**

The *M. oryzae* reference genome assembly (strain 70-15) has two copies of *PWL2* on chromosomes 3 and 6, annotated as MGG_04301 and MGG_13683, respectively. We observed, however, that *PWL2* copy number varies significantly in different *M. oryzae* isolates. Southern blot hybridization (**Fig. S4**), and analysis of long-read assembled genomes of the *M. oryzae* rice pathogenic strain Guy11 identified three copies of *PWL2*. A duplication of *PWL2* is present on a single contig in the Guy11 genome, whilst the third copy involves a translocation resulting in a distinct *PWL2* allele in a separate contig (**Fig. 2A**). We observed that the *PWL2* loci were associated with the occurrence of *MGR583*, *POT2* and *MAGGY* transposable elements, suggesting a possible involvement in genome translocation. To further assess *PWL2* copy number in *M. oryzae* isolates, we employed a k-mer analysis approach to determine copy number variation of *PWL2* in *M. oryzae* genomes (n = 286). Strikingly, *PWL2* copy number was high in many *M. oryzae* strains, with one isolate showing 8 copies (**Fig. 2B**). We conclude that *PWL2* is ubiquitous in *Magnaporthe* and has expanded in copy number both in *M. oryzae* and its sister species *M. grisea*.

***Apwl2* mutants show altered host range and are reduced in ability to cause rice blast disease.**

Having determined the copy number variation of *PWL2* in *M. oryzae*, we used a CRISPR/Cas9 genome editing approach to simultaneously delete the three copies of *PWL2* found in Guy11 (33). We designed a sgRNA to target *PWL2* and introduced a hygromycin phosphotransferase-encoding gene cassette (*HPH*) (**Fig. 3A**). To identify *Apwl2* null mutants, transformants were analysed by PCR using primers designed to amplify the *PWL2* coding sequence. Transformants with ectopic integrations or where not all copies of *PWL2* were deleted, gave a predicted amplicon size of 645 bp, consistent with untransformed Guy11. By contrast, in null mutants, we amplified a larger amplicon of 1.5 kb, consistent with introgression of the hygromycin resistance cassette into all three copies of *PWL2* (**Fig. 3A**). Four putative transformants were then selected for whole genome sequencing. Raw reads were aligned to the *M. oryzae* reference genome using samtools v.1.5, before converting into BAM files and visualised using IGV viewer. No reads mapping to the *PWL2* locus were identified in mutants T6 and T12 whereas fewer reads mapped to the same locus from sequenced T5 and T4 (**Fig. S5**). Regions upstream and downstream of the *PWL2* coding sequence were 100 % identical in both mutants and reference genomes. This confirmed that T6 and T12 are *Apwl2* gene deletion transformants and they were therefore selected for further analysis. We reasoned that *M. oryzae Apwl2* deletion mutants would gain virulence on weeping lovegrass (13, 26) and the barley cultivar Baroness carrying *Mla3* cultivars of barley. The *Apwl2* deletion transformants T5, T6 and T12 and Guy11 were therefore used to inoculate weeping lovegrass seedlings. We observed that the *Apwl2* mutants T6, T5 and T12 showed gain of virulence and symptoms of infection which were identical to those exhibited by a virulent G17 isolate that lacks *PWL2* (**Fig. 3B**). We conclude that T5, T6 and T12 are loss of recognition mutants of *PWL2*, and we can be confident that T6 and T12 have complete deletion of all three copies of the gene.

We next tested whether *Pwl2* has a virulence function during a compatible interaction between the blast fungus and its host. The *Apwl2* mutant T6 was infected on the susceptible rice cultivar CO39, susceptible barley lines Nigrata, Siri (*Mla8*), Golden Promise (*Mla8*), and resistant cultivar Baroness (*Mla3*). The *Apwl2* mutants showed a small but reproducible reduction in virulence on CO39 compared to wildtype Guy11, based on observed disease lesion size (**Fig. 3C and E**). Additionally, *Apwl2* produced statistically smaller lesion sizes on barley lines Siri and Golden Promise (**Fig. 3D and F**). We conclude that *PWL2* contributes to the ability of *M. oryzae* to cause blast disease.

***Pwl2* suppresses host immunity.**

Having determined that the *Pwl2* effector contributes to virulence, we aimed to gain insight into its biological function during host cell colonisation. We generated stable transgenic barley (Golden Promise) and rice (Kitaake) lines expressing *PWL2-YFP* (without its signal peptide) under control of the CaMV35S promoter. Transgenic lines expressing cytoplasmic YFP were used as controls. Transgenic plants, expressing *Pwl2* and free YFP did not show any adverse growth or fitness phenotypes (**Fig. 4A**). We first assayed *PWL2-YFP* transgenics for their response to two elicitors of PAMP-triggered immunity, flg22 and chitin. Rice and barley perceive flg22 through the pattern recognition receptor FLS2 and perceive chitin through CEBiP and CERK1 leading to immune response for example production of reactive oxygen species (ROS) (34, 35). We observed that *Pwl2* expression abolished chitin and flg22-induced ROS generation (**Fig. 4B and C**). *Pwl2* therefore contributes to virulence by suppressing PAMP-triggered immunity during compatible interactions. To investigate the effect of elevated *Pwl2* effector expression on blast infections, we infected barley plants expressing *Pwl2* with

M. oryzae Guy11. Pwl2-expressing lines were significantly more susceptible and developed blast disease symptoms earlier (2-3 dpi) compared to infection of isogenic Golden Promise plants (**Fig. 4D and E**). We conclude that Pwl2 is a modulator of plant immunity.

Pwl2 binds to the heavy metal binding domain isoprenylated protein, HIPP43.

To determine the likely target of Pwl2, transgenic barley and rice plants expressing Pwl2-YFP were used for protein extraction before performing anti-YFP co-immunoprecipitation coupled to mass spectrometry (IP-MS). This revealed a total of 791 proteins after spectral search in the *H. vulgare* (barley, cv Morex version 3) proteome database. We identified a total of 282 frequently occurring proteins in the biological replicates containing Pwl2, that were not identified in the six free-YFP control samples. To avoid focusing on false positives in the form of sticky and abundant proteins, fold-changes were calculated by first determining the average number of peptides per protein candidate, before estimating log₂ fold change compared to the control experiment. We further filtered for proteins that produced at least two peptide hits in more than half of replicates ($\geq 4/8$ biological replicates). A total of 52 proteins met this criterion and were considered as enriched in samples compared to controls and were therefore selected for further analysis. Furthermore, because Pwl2 attenuates the ROS-burst in barley transgenics lines, we focused initially on protein candidates previously reported to have roles in plant immunity or ROS generation (**Fig. 4F**). These were selected for one-to-one interactions using a yeast-two-hybrid (Y2H) assay by co-transforming constructs expressing Pwl2 and Putative Pwl2 Interacting Proteins (PIPs) identified by IP-MS (**Fig. 4F and Fig. S6**). Pwl2 showed strong binding to PIP4 (**Fig. 4G and H**). We named this protein HvHIPP43, for *Hordeum vulgare* heavy metal binding domain containing isoprenylated plant protein 43 based on homology to OsHIPP43 defined in a companion paper by Zdrzalek *et al* (15). To validate the interaction between Pwl2 and HIPP43, we carried out co-immunoprecipitation (co-IP) analysis in *N. benthamiana* and could detect HvHIPP43 interacting with Pwl2-YFP (**Fig. 4H**). Having determined that Pwl2 binds to HvHIPP43, we investigated the *PWL* gene family-encoded proteins Pwl1, Pwl3, Pwl4 and pwl2-3 variant and showed that they were all able to bind HvHIPP43 in Y2H assays, confirming that Pwl2 belongs to an effector family that binds to a host sHMA protein (**Fig. 4I**).

Pwl2 interacts with HIPP43 orthologs from other grass species.

HIPPs are expanded in plant genomes and to test whether HIPP43 has orthologs in other grass species, we generated a maximum likelihood phylogenetic tree of HMA domains from diverse grasses with high quality genomes and annotations. We found that the HIPP43 family formed a distinct clade which included orthologs from rice (*O. sativa*), wheat (*T. aestivum*), wild wheat (*Haynalidia villosa*), foxtail millet (*S. italica*), weeping lovegrass (*E. curvula*), Sorghum (*Sorghum bicolor*), *Oropetium thomaeum*, *Zea mays* and *Brachypodium distachyon* (**Figure S7 and S8**). Furthermore, we could identify copy number variation ranging from one to five copies in grasses, with barley and wheat (i.e. Triticeae lineage) having three paralogs of HIPP43, one per haploid genome. We followed this by confirming that, Pwl2 interacts with HvHIPP43 orthologs from *Triticum* (TaHIPP43), *Setaria* (SitiHIPP43), and *Eragrostis* (EcHIPP43), in Y2H assays (**Fig. 4J**). Binding of Pwl2 to rice OsHIPP43 was verified by in vitro biochemistry and a structure of the complex obtained from X-ray crystallography (see companion paper by Zdrzalek *et al* (15). We also analyzed the transcriptional profile of OsHIPP43 during infection of Guy11 on a susceptible rice line CO39 from dataset available from Yan *et al* (3). Interesting we found that, OsHIPP43 expression was up regulated during plant infection, suggesting it might play an important role in plant immune response.

Overexpression of HIPP43 suppresses PAMP-induced ROS in transgenic barley.

To investigate the biological relevance of the interaction between Pw12 and HIPP43, we generated barley transgenic lines overexpressing HIPP43. These transgenic lines were challenged with two elicitors of PAMP-triggered immunity, flg22 and chitin. Strikingly, we observed that the chitin and flg22-induced ROS burst was abolished in plants expressing HIPP43 compared to wild type and plants expressing free YFP (**Fig. 5 A, B, C and D**). Similarly, when we challenged transgenic barley plants expressing HIPP43 with *M. oryzae*, we observed increased susceptibility compared to the wildtype Golden Promise with disease lesions appearing earlier than in wild type plants. This observation is consistent with earlier results showing attenuated ROS burst in plants expressing Pw12-YFP (**Fig. 5E and F**).

Isoprenylation motif is required for HIPP43 plasmodesmata localization.

So far, there is no direct evidence for the involvement of HIPP proteins in host immunity and regulation of ROS so we reasoned that HIPP43 might bind to another host protein following interaction with Pw12. HIPP43 possesses an isoprenylation motif at its C-terminus (**Figure S9A**), and we performed live cell imaging of mCherry and YFP-tagged HIPP43 (at the N or C terminus, respectively), following transient expression in *N. benthamiana* to determine the localisation of the protein. We also tested localisation of HIPP43 without its predicted isoprenylation motif. When tagged at the N-terminus, HIPP43 mainly localises to puncta at the plasma membrane that co-localise with callose staining (aniline blue) consistent with these being sites of plasmodesmata (PD) (**Fig. 5G and H**). However, without its isoprenylation motif, PD localisation was abolished, and we observed accumulation in the nucleoplasm and plasma membrane (**Fig. S9B**). When tagged at its C-terminus (HIPP43-YFP), HIPP43 mainly localises to PD, cytoplasm, plasma membrane and chloroplasts (**Fig. S9C**).

Pw12 stabilises and alters plasmodesmata localization of HIPP43.

When we co-expressed YFP-Pw12 with mCherry-HIPP43, we observed co-localisation in the cytoplasm with the appearance of cytoplasmic mobile bodies approximately 2 µm in diameter, likely to be very large vesicles or P-bodies (**Fig. 5I**). In some cases, we also observed larger cytoplasmic mobile bodies ranging from 2-4 µm in diameter (**Fig. 5I**). We quantified the number of times HIPP43 signal was observed at PD when co-expressed with free-YFP, AVR-Pike and the effector Mep3 (3) as controls. We observed that co-localisation of YFP-Pw12 and mCherry-HIPP43 as cytoplasmic vesicles compromised PD localisation (**Fig. 5I and J**). In addition, localization of HIPP43 in large cytoplasmic mobile bodies was not observed when we co-localised HIPP43 with empty vector controls expressing YFP (**Fig. 5K and L**) or two other MAX-fold effectors, AVR-Pike (**Fig. 5M and N**) and MEP3 (**Fig. S10E and F**), as it remained localised at PDs. Moreover, the intensity of mCherry-HIPP43 fluorescence was higher when co-expressed with Pw12 compared to when expressed in the absence of Pw12, or when co-expressed with free YFP, AVR-Pike or MEP3 (**Fig. 5I and Fig. S10D**). When considered together, these results suggest that Pw12 binds to HIPP43, potentially stabilising the protein and altering its cellular location away from plasmodesmata.

Discussion

The Pwl2 effector was first identified as a host specificity determinant for infection of the forage grass species weeping lovegrass (13, 26). Its initial identification provided evidence that host range in plant pathogenic fungi was conditioned in a similar way to cultivar specificity in a gene-for-gene manner, involving dominant pathogen genes recognised by the products of cognate disease resistance genes (26). Furthermore, Pwl2 was found to belong to an expanded gene family, suggesting an important function in pathogenicity and fitness (26). However, in the following two decades, the function of Pwl2 has remained elusive despite its extensive use as a marker in cell biological studies for investigating effector regulation, secretion and delivery during plant infection (23-25).

In this study, we set out to explore the function of Pwl2 and investigate why it is such a ubiquitous effector in *Magnaporthe oryzae*. We found that the *PWL2* gene is present across all rice blast lineages, and in all publicly available genome sequences of *M. oryzae*, including each of its host-limited forms and even in sister species infecting crabgrass and pearl millet. The observation that *PWL2* and the wider *PWL* family genes have been maintained in the global blast population and occurrence of effector variants or loss-of-recognition alleles associated with certain host-adapted lineages, suggests that Pwl2 and members of this family serve an important function in pathogenesis. Furthermore, *PWL2* is present at high copy number in many *M. oryzae* isolates having undergone extensive gene duplication. This may have been facilitated by the presence of *MGR583*, *POT2* and *MAGGY* transposable elements (36, 37) flanking *PWL2* loci, providing the capacity to generate translocations and genomic instability. It is not clear whether the signatures of lineage-specific diversification are due to isolates infecting rice varieties carrying a yet unidentified resistance gene against Pwl2, or resistance genes that have evolved in different host species such as barley cultivars expressing Mla3 or *E. curvula* (14).

We were able to apply CRISPR/Cas9 gene editing in *M. oryzae* to functionally characterise genes occurring in multiple copies through the generation of *Δpwl2* mutants. Our analysis confirmed the previously reported host specificity function of Pwl2, but also revealed it is important for blast disease, with *Δpwl2* mutants showing reduced virulence compared to the isogenic wild type strain. Pwl2 therefore plays an important role during host tissue invasion, consistent with the observation of smaller and fewer disease lesions in susceptible interactions with *Δpwl2* mutants.

Using discovery proteomics, we identified putative Pwl2-interacting proteins, including an isoprenylated small HMA protein, HIPP43, which contains a predicted C-terminal isoprenylation motif. We found that all proteins encoded by the *PWL* gene family can bind HIPP43, including HIPP43 homologs from wheat, rice, weeping lovegrass and foxtail millet. Multiple MAX effectors bind sHMA protein domains, although most previous studies have focused on the incorporation of such domains into paired NLR immune receptors, leading to disease resistance (7, 22, 38, 39). HMA domains are known to be involved in biotic/abiotic stress responses, transport of metals and metal detoxification, and their expression can be organ-specific or tissue-specific within roots, leaves, or stems (40-42). Moreover, sHMAs can localise to the nucleus, plasma membrane, cytoplasm, or plasmodesmata (41, 43, 44), suggesting they might be involved in numerous cellular processes. Consistent with this, sHMAs are expanded in plant genomes with more than 45, and more than 50 sHMA domain-encoding genes occurring in *Arabidopsis* and rice respectively (45). However, little is known about the function of sHMA proteins in plants, especially during plant/microbe interactions, even though they have been reported to act as susceptibility factors (20, 21). For example, deletion mutants of OsHIPP05 (*Pi21*), a proline rich HMA domain protein-encoding gene in rice leads to rice blast resistance (20). Consequently, *pi21* is a recessive resistance gene that

has been successfully deployed to control blast disease (20). Similarly, gene silencing or deletion mutants of *TaHIPP1* or *AtHMAD1* in wheat and *Arabidopsis* provide enhanced resistance against *Puccinia striiformis* f. sp. *tritici* and *Pseudomonas syringae* DC3000, respectively (46, 47). In addition, deletion mutants of *AtHIPP27* in *Arabidopsis* lead to increased resistance against cyst nematode (48). However, it is not clear why deletion of sHMA protein-encoding genes impacts immunity. Therefore, the observation that Pwl2 interacts with HIPP43 is revealing, especially as Pwl2 binding appears to enhance its activity, or stability, rather than inhibiting its function. This is consistent with the observation that over-expression of HIPP43 enhances blast susceptibility in rice and barley, respectively.

The crystal structure of the Pwl2/HIPP43 complex (presented in the companion paper by Zdrzałek *et al.* (15)) demonstrates that Pwl2 uses an expansive interface to mediate binding to HIPP43, largely using elements of the MAX fold to interact with the β -sheet of the host target. Interestingly, the polymorphic residues found between Pwl2 variants, Pwl2, pwl2-2 and pwl2-3 are not located at the HIPP43 binding interface, but rather located away from the MAX fold on the C-terminal helix of Pwl2. As these polymorphisms are determinants for Pwl2 recognition in resistance plants carrying functional cognate resistance gene, it suggests the interface used for immune recognition of Pwl2 in both barley Mla3 lines and resistant weeping love grass are likely spatially distinct from the interface used by Pwl2 to bind to HMA host targets.

This observation contrasts with an emerging model where host immune receptors can utilize binding interfaces spatially similar as to those found between effectors and their host targets (38, 39). Indeed, Zdrzałek *et al.* (15) show that if HIPP43 is incorporated into the Pik-1 NLR, in place of its naturally occurring integrated HMA domain, there is potential to gain immune responses to Pwl2. Therefore, we hypothesize Pwl2 may have additional host targets with spatially distinct interfaces from that observed with HIPP43. If true, this may explain differences in Pwl2 HIPP43-binding and immune recognition sites. This suggests immune recognition may have evolved to recognize Pwl2 through interfaces shared with the other undetermined host targets. However, to test this hypothesis, structural information for the natural recognition of Pwl2 by host resistance genes, such as Mla3, or genetic evidence for additional Pwl2 host targets is required.

Finally, we show Pwl2 interaction with HIPP43 is important for fungal virulence. A *Δpwl2* null mutant, lacking all three copies of the gene, produced smaller disease lesions that were also fewer in number than caused by the isogenic wild type *M. oryzae* strain Guy11. Moreover, when Pwl2 is over-expressed in transgenic rice or barley, it abolished chitin or flg22-elicited generation of ROS. We also found that over-expression of HIPP43 in rice and barley had a similar effect, suggesting that Pwl2-HIPP43 interaction results in attenuated generation of ROS and reduced immune responses. Consistent with these observations, over-expression of Pwl2 or HIPP43 leads to increased susceptibility to *M. oryzae*. How this potential enhancement of HIPP43 activity is stimulated by Pwl2 is not completely clear, but we found that transient co-expression of both Pwl2 and HIPP43 sequestered HIPP43 away from plasmodesmata, with the two proteins co-localizing in the cytoplasm and in large mobile vesicular bodies. The sub-cellular localization of Pwl2 and HIPP43 was distinct when separately expressed, with Pwl2 accumulating in the cytoplasm and mobile bodies while HIPP43 localized to the membrane, and particularly to plasmodesmata. Deletion of the isoprenylation motif abolishes PD localization showing the C-terminal isoprenylation motif is important for plasmodesmatal (PD) localization. This suggests that disrupting PD localization may lead to HIPP43 translocation to other organelles, which may in-turn influence immune signaling.

Localization of HIPPs at PD has been previously reported. HIPP7 and HIPP26 localise at PD, and this localisation can be altered by mutations in the isoprenylation motif (44, 49). Although characterised HIPPs through their HMA domains are thought to be metallochaperones, there

are no studies that link metal detoxification and immunity. Some studies suggest that there may be a connection between concentration of metal ions, such as iron, copper, cadmium and zinc and plasmodesmatal permeability (50). This might explain why a plant pathogen effector targets a plasmodesmatal localized HIPP. Both the HMA domain and the isoprenylation regions of HIPPs have been shown to be important in plant immunity. For example, a mutation targeting the proline-rich region of *pi21* is sufficient to lead to gain of resistance against *M. oryzae* (20), while interfering with the isoprenylation of HIPP1-V from wild wheat (*H. villosa*) leads to loss of resistance against *Blumeria graminis* f. sp. *tritici* accompanied by reduced localization to PD. Interestingly, HIPP1-V interacts with the E3-ligase CMPG1 at the plasma membrane leading to resistance against powdery mildew in an isoprenylation-dependent manner (51). This interaction has been reported to activate expression of genes involved in ROS generation and salicylic acid biosynthesis, suggesting that HIPP1-V is required for PTI regulation (51). An *A. thaliana* HIPP3 has also been proposed to be involved in regulation of the pathway involved in salicylic-dependent plant immunity via LSD1, PAD4 and SAG101 (40).

The change in cellular localization of HIPP43 induced by Pwl2 may therefore inhibit its function in immunity or lead to a new role that enhances disease susceptibility. Future work will be necessary to see if HIPP43 directly regulates ROS generation or acts in a more indirect manner through binding other signaling components involved in PTI. By dislocating HIPP43 from the PD, Pwl2 may attenuate a role for HIPP43 in PD immunity. Pwl2 is a highly mobile effector and has been shown to move into neighbouring rice cells ahead of *M. oryzae* hyphal growth (23). This has been suggested to be a step to prepare un-invaded cells for fungal colonisation. Its role may therefore be to alter the sub-cellular localisation, concentration and stability of HIPP43, enabling more rapid tissue colonisation by the blast fungus.

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Materials and methods

Fungal strains, growth conditions and infection assays

Fungal isolates were routinely grown on complete medium (CM) at 24°C with a controlled 12 h light and dark cycle for up to 12 days (52). *H. vulgare*, *E. curvula* and *O. sativa* plants were grown for 7, 14 and 21 days respectively in 9 cm diameter plastic plant pots or seed trays. Conidia were recovered from 10-day old cultures using a sterile disposable plastic spreader in 3 mL sterile distilled water. The conidial suspension was filtered through sterile Miracloth and centrifuged at 5000 x g for 5 min at room temperature and adjusted to a final concentration of 1×10^5 conidia mL⁻¹ in 0.2 % gelatin. The spore suspension was used for spray or leaf drop infections assays. After spray inoculation, the plants were covered in polythene bags and incubated in a controlled plant growth chamber at 24°C for 48 h with a 12 h light and dark cycle, and 85% relative humidity, before removing the polythene bags. The inoculated plants were incubated for 3 more days before scoring the lesions. For each treatment 10 leaves were collected before counting typical disease lesions that are characterised by ellipsoid necrotic lesions with a grey centre, on the leaf surface (53). Each experiment was repeated minimum of three times, yielding consistent outcomes.

Leaf infection assay and live-cell imaging

Rice leaf sheaths from 4 weeks old susceptible cultivars Moukoto or CO39 were inoculated with 4 mL of a suspension at 5×10^4 of conidia mL⁻¹ in distilled water using a micropipette as described in Kankanala et al 2007 (25). The inoculated leaf sheaths were incubated at 24°C for at least 24 h before a thin layer of the inner leaf sheath was dissected using a blade and mounted on a glass slide. Live cell imaging was carried out on an IX81 motorized inverted microscope (Olympus, Hamburg, Germany) for conventional and differential interference contrast (DIC) microscopy using the Photometrics CoolSNAP HQ2 camera (Roper Scientific, Germany). Images were analyzed using ImageJ. For Leica SP8 laser confocal microscopy, settings were as follows; GFP, YFP and RFP/mCherry tagged proteins were excited using 488, 514 and 561 nm laser diodes and the emitted fluorescence detected using 495-550, 525-565 and 570-620 nm respectively. The auto-fluorescence from chlorophyll was detected at 650-740 nm.

Generation of fungal transformation plasmids

Single or multiple DNA fragments were cloned into fungal transformation vector using In-Fusion HD Cloning Kit (Clontech, USA). Briefly, fragments from cDNA, genomic DNA or synthesized DNA were amplified using primers that introduce a 15bp overhang complementary to sequences at the restriction sites of the destination vector or adjacent insert fragments. This allows the ends to fuse by homologous recombination during cloning. Positive transformation was accessed using colony PCR and constructs sequenced then analysed on SnapGene. A list of primers is provided in Table S3.

RNA isolation, RNA sequencing and analysis

To study *in-planta* gene expression profile of *PWL2* and other effectors, leaf drop infection assays were carried out on a susceptible rice line Moukoto and samples collected at 24 and 72 hpi. Infected plant material was ground into fine powder using a sterile nuclease-free mortar & pestle containing liquid nitrogen. RNA was isolated from *M. oryzae* mycelia inoculated or rice leaves using QIAGEN RNeasy Plant Mini Kit according to manufacturer's instructions. The RNA quality was determined on a NanoDrop spectrophotometer (Thermo Scientific, UK) and Agilent 2100 Bioanalyser using an RNA nano-chip kit (Agilent Technologies, UK). Library preparation was carried out using Illumina® sequencing TruSeq Stranded Total RNA Library

Prep Kit before sequencing of 100 bp paired ends reads using Illumina Genome Analyser GXII platform by the Exeter Sequencing Service (University of Exeter). *M. oryzae* specific reads were recovered as unmapped reads after mapping to the *O. sativa* L. ssp. japonica genome (IRGSP-1.0) (54). To determine differentially genes expression, reads were then mapped to transcripts from the *M. oryzae* 70-15 reference genome (55) or a laboratory strain KE002 using TopHat2 splice site-aware aligner (56). Differential expression analysis was carried out using DESeq2 packages (57).

Generation of Cas9-sgRNA targeted gene deletion

A sgRNA was designed for CRISPR-Cas9 genome editing using an online tool E-CRISP <http://www.e-crisp.org/E-CRISP/>. A 20-nucleotide sequence was selected at the *PWL2* locus (not including the PAM NGG-sequence). The sgRNA was first synthesised using the EnGen sgRNA synthesis kit New England Biolabs (NEB #E3322) before mixing with the Cas9-NLS to form the RNP complex. The mixture was incubated at room temperature for 10 min before fungal transformation. *M. oryzae* protoplast were generated as described by Talbot *et al* (52). The RNP complex together with the donor template was mixed with Guy11 protoplasts re-suspended in 150 µL of STC to a concentration of 1×10^8 and incubated at room temperature for 25 min before adding 60% PEG. Successful transformants were selected on complete media (CM) agar containing Hygromycin B to a final concentration of 200 µg mL⁻¹.

Southern blot blotting

This procedure was adapted from the protocol of Southern (58) with adjustments as described by Sambrook *et al* (59). Fungal genomic DNA (15 µg) was digested with the appropriate restriction enzyme overnight at 37°C. The digested genomic DNA was then separated by gel electrophoresis. The DNA embedded in the agarose gel was de-purinated by immersion in 0.25 M HCl before transferring the DNA onto Hybond-NX membrane (Amersham Biosciences). The membrane was exposed to UV light in a BLX-254 cross linker (Bio-Link®) to fix and immobilise the DNA. The membrane was then probed overnight using a DIG-labelled PCR product in freshly prepared hybridization buffer (25-50ng mL) in a 50 mL falcon tube. Chemiluminescent detection of DIG-labelled DNA was carried using CDP-Star solution (Roche) before detection.

Whole genome sequencing

Purified RNA-free DNA was obtained using Hexadecyltrimethylammonium Bromide (CTAB). Template quality was assessed using a NanoDrop spectrophotometer and Qubit. Sequencing was carried out at Exeter Sequencing services (University of Exeter, UK) and Novogene (Cambridge, UK). NEXTflex™ Rapid DNA-seq Library Prep Kit was used to prepare and index libraries before sequencing on HiSeq 2500 (Illumina) with two lanes per sample. The quality of sequencing reads was first checked using the FastQC toolkit (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/>). From the raw data (fastq files), adaptor sequences were trimmed from sequences containing adaptors and low-quality reads removed using the fastq-mcf program. The trimmed sequences were aligned to the reference genome (70-15) (55) using BWA (Burrow Wheeler Aligner) <https://github.com/lh3/bwa> (60). Bam files were visualized on IGV genome viewer to determine CRISPR gene deletion.

Copy number variation of *PWL2*

A total of 286 *M. oryzae* isolates with raw Illumina-based sequencing information were downloaded from NCBI (performed on October 16th, 2019). Copy number variation was assessed using a k-mer analysis approach using the k-mer analysis toolkit (KAT; v2.4.1). Coding sequence information for *M. oryzae* isolate 70-15 were used as template for k-mer analysis and raw Illumina reads were input. Default parameters were used including k-mer of length 27 nt. Copy number variation of individual effectors is based on average k-mer coverage as compared with the median coverage for all genes.

Phylogenetic analysis of *Magnaporthe*

Genome sequences of diverse *M. oryzae* isolates were identified based on literature review and search on NCBI (performed on August 9th, 2019). For isolates having only Illumina sequencing data, raw paired reads were downloaded from NCBI, trimmed using Trimmomatic (v0.36) using the parameters: removing of adapters with ILLUMINACLIP:TruSeq2-PE.fa:2:30:10, remove leading low quality or N based with quality below 5 for leading (LEADING:5) and trailing sequence (TRAILING:5), scan and cut reads with 4 bp sliding window below 10 (SLIDINGWINDOW:4:10), and minimum length of 36 bp (MINLEN:36). KmerGenie (v1.7048) was used to identify an optimal k-mer for genome assembly using default parameters. Genome assembly was performed using minia (v0.0.102) with default parameters. kSNP3 (v.3.021) was used to develop a phylogenetic tree of *M. oryzae* using assembled genomes as input with parameters of k-mer of 29 bp and minimum fraction of 0.4. The phylogenetic tree was generated using RAxML (v) using the General Time Reversible model of nucleotide substitution under the Gamma model of rate heterogeneity.

Generation of *Agrobacterium* transformation plasmids

Single or multiple DNA fragments were cloned into binary vector using In-Fusion HD Cloning Kit (Clontech, USA). Briefly, fragments from cDNA were amplified using primers that introduce a 15bp overhang complementary to sequences at the restriction sites of the destination vector or adjacent insert fragments. Alternatively synthesized DNA fragments were designed to have 15bp overhang complementary to sequences at the restriction sites of a binary vector pG514 customized for infusion cloning by digesting with either *Xba*I and *Pca*I or *Sac*I (New England Biolabs) to remove the *ccDB* toxin encoding gene. Positive transformation was accessed using colony PCR and constructs sequenced and analyzed on SNAP gene.

Transient expression in *Nicotiana benthamiana*

Agrobacterium tumefaciens strain *GV3101* (61) was used for transient expression. Three-week-old *N. benthamiana* leaves were infiltrated with transformed *Agrobacterium* carrying T-DNA constructs, expressing the gene of interest. Bacterial cultures were diluted to obtain a final OD 600nm of 0.4 in agroinfiltration buffer (10mM MES, 10 mM MgCl₂, 150 μM acetosyringone, pH 5.6). Leaf discs were cut from agroinfiltrated tissue 48 hpi and subjected for microscopy or used co-IP.

Plant transformation and ROS measurement

Agrobacterium tumefaciens strain *AGL1* (61) was used for plant transformation as described in (62). Positive transgenics plants were first selected on hygromycin followed by confirmation using PCR. Alternatively, leaf discs were collected from transgenic plants and analysed for copy number at iDNA Genetics Ltd (Norwich, UK) to detect hygromycin insertion events. In addition, expression of the protein was evaluated using SDS-PAGE. To measure response to

PTI elicitors, a 4-mm diameter biopsy punch (Integra™ Miltex™) was used to cut leaf discs from 5 weeks old *H. vulgare* and *O. sativa* transgenic plants. The leaf discs were transferred into 96-well-plates (Greiner Bio-One) containing 100 µL of distilled H₂O in each well and incubated overnight at room temperature. The assay was carried out by replacing the distilled H₂O in the wells by a 100 µL reaction solution of 100 µM Luminol or L-012 (Merck), 20 µg mL⁻¹ horseradish peroxidase (Merck) together with elicitors, flg22 (1 µM final concentration), chitin (1mg/mL final concentration) or without (mock). The photon count was carried out using the (HRPCS218, Photek) equipped with a 20 mm F1.8 EX DG ASPHERICAL RF WIDE LENS (Sigma Corp). Each experiment was repeated three times conducted a minimum of three times, yielding consistent outcomes.

Co-immunoprecipitation and sample preparation for mass spectrometry

Leaves were harvested from two-three weeks old barley transgenic plants and rapidly frozen in liquid nitrogen before storage or immediately ground into fine powder using a GenoGrinder® tissue homogenizer. Ground powder was quickly transferred into ice-cold 1.5 mL Eppendorf tube and 2 mL of ice-cold extraction buffer (GTEN [10% (v/v) glycerol, 25 mM Tris pH 7.5, 1 mM EDTA 150 mM NaCl, 2% (w/v) PVPP, 10 mM DTT, 1 X protease inhibitor cocktail (Sigma), 0.5% (v/v) IGEPAL added and mixed thoroughly. This was centrifuged at 3000 x g for 10 min at 4°C to recover total proteins in the supernatant and repeated twice. A volume of 2 mL of total protein was mixed with 25 µL of GFP-TRAP agarose beads (50% slurry, ChromoTek) and incubated shaking overnight at 4°C. The GFP-TRAP agarose beads were centrifuged for 2 min at 3000 x g before washing three times with wash buffer (ChromoTek). Bound proteins were recovered by resuspending the beads in loading buffer (loading dye, 10 mM DTT, H₂O) and incubated at 70°C for 10 minutes. An aliquot of the sample was run on SDS-PAGE gel and used for Western blotting (63). Recovered proteins from co-immunoprecipitation were run on an SDS-PAGE gel for approximately 1 cm. The gel was washed for 1 h in distilled water followed by 1 incubation SimplyBlue™ Safe stain. The gel was then washed three times in distilled water the region containing proteins excised using a scalpel.

Mass spectrometry and data processing

Protein purification, immunoprecipitation, sample preparation, liquid chromatography followed by tandem mass spectrometry (LC-MS/MS), and data analysis were previously described in full detail (64). Briefly proteins identified in immunoaffinity-enriched samples were measured with data dependent method on high resolution LC-MS systems, Orbitrap Fusion (Thermo Ltd). The acquired spectra were peak-picked and searched by Mascot search engine (Matrix Science Ltd) to identify the peptide sequences from the search space defined by the background proteome. The peptides were combined into proteins based on the principle of parsimony by the search engine. Resulting proteins were further described by quantitative values based on the number of spectra that identify them. The individual runs were combined in the Scaffold program (Proteome Software Inc.), where the data were evaluated and filtered to contain less than 1% false positives (FDR) and resulted matrix exported as a spreadsheet. The matrix of proteins detected in different samples serves as the input for an R script for further processing and visualization.

Yeast two-hybrid analysis

To clone genes of interest into bait (pGBKT7 DNA-BD cloning) vector or prey (pGADT7 activating domain) vector, using the In-Fusion HD Cloning Kit (Clontech) kit. The bait and prey vectors were digested using vector was digested using *Bam*H1 and *Eco*R1 while the gene

to be inserted was PCR-amplified using primers containing 15 bp overhangs with homology to the two ends of the digested bait vector. For transformation, a single colony Y2H gold yeast strain was mixed in 1 mL of liquid yeast extract peptone dextrose (YPD). Competent cells were prepared as described in manufactures transformation protocol (Zymo Research). Briefly, for transformation 700 ng – 1 µg of plasmids expressing Pwl2 or other effectors in pGADT7 and *HvHIPP43* or plant proteins in pGBKT7 were co-transformed in competent cells and Frozen-EZ Yeast Solution 3 added before incubation at 28°C for 1 h and transformed cells plated on selection media lacking Leucine (L) and Tryptophan (W) and incubated at 28° C for 3-5 days. To detect interactions the colonies were transferred to media lacking Leucine (L), Tryptophan (W), Adenine (A) Histidine (H), Bromo-4-Chloro-3-Indolyl a-D-galactopyranoside (X-a-gal) and 10 mM 3-amino-1,2,4-triazole (3AT) (Sigma). The plates were imaged after 60-72 incubation at 28°C. Each experiment was conducted a minimum of three times, yielding consistent outcomes.

***In-planta* co-immunoprecipitation (co-IP)**

To test interaction of protein *in planta*, genes of interest tagged with either GFP or Myc were cloned into the vector pGW514 and transformed into *Agrobacterium* strain *GV301* and co-infiltrated (OD₆₀₀ = 0.4 for effectors and OD₆₀₀ = 0.6 for HIPP43) into 3 to 4-week-old *N. benthamiana* leaves and incubated for 48 h to allow protein expression. Total protein was isolated in ice-cold extraction buffer (GTEN [10% (v/v) glycerol, 25 Mm Tris pH 7.5, 1 mM EDTA 150 mM NaCl], 2% (w/v) PVPP, 10 Mm DTT, 1 X protease inhibitor cocktail (Sigma), 0.5% (v/v) IGEPAL). Total protein was co-immunoprecipitated using the anti-GFP M2 resin (Sigma-Aldrich, St. Louis, MO) and washed 3 times using immunoprecipitation buffer before analyzing on an SDS-PAGE gel. Recovered proteins from co-immunoprecipitation were separated on an SDS-PAGE gel and transferred onto a polyvinylidene difluoride (PVDF) membrane using a Trans-Blot turbo transfer system (Bio841 Rad, Germany). Detection was performed using the appropriate antibody either anti-GFP-HRP or anti-Myc-HRP. Imaging was carried out using an ImageQuant LAS 4000 luminescent imager (GE Healthcare 844 Life Sciences, Piscataway, NJ, U.S.A.) according to manufacturer's instructions.

Sub-cellular localization and plasmodesmata quantification

Leaf discs were obtained from *N. benthamiana* plants agroinfiltrated with different construct combinations at 24 and 48 hpi and mounted on a slide immersed in perfluorodecalin and observed using X63 oil immersion lens. For Leica SP8 laser confocal microscopy, settings were as follows; GFP, YFP and RFP/mCherry tagged proteins were excited using 488, 514, and 561 nm laser diodes and the emitted fluorescence detected using 495-550, 525-565, and 570-620 nm respectively. The auto-fluorescence from chlorophyll was detected at 650-740 nm. To stain and quantify plasmodesmata, agroinfiltrated leaves were further infiltrated with 0.1% aniline (Sigma-Aldrich #415049, in PBS buffer, pH 7). Images were analyzed using ImageJ to determine the number of plasmodesmata. First, cell peripheries were divided into 20 µm sections and the number of plasmodesmata determined per 20 µm.

Phylogenetic analysis of grass HMA proteins

Proteins from *B. distachyon* (314; v3.1), *H. vulgare* (Jul2020), *T. aestivum* (RefSeqv2.1; 09-16-2020), *O. sativa* (323; v7.0), *Oropetium thomaeum* (386; v1.0), *Sorghum bicolor* (454; v3.1.1), *Setaria italica* (312; v2.2), and *Zea mays* (RefGen_V4) containing an HMA domain were identified using InterProScan (v5.59-91.0; Pfam PF00403). The HMA domain was extracted using the script QKdomain_process.py including an additional 10 amino acid sequences N and C-terminal of the Pfam boundaries (-n 10 -c 10). The non-redundant set of

HMA domains were identified using CD-HIT (v.4.8.1) with parameter -c 1.0. Structure guided multiple sequence alignment was performed using MAFFT with parameters dash, max iteration of 1000, and globalpair. Maximum likelihood phylogenetic analysis was performed using RAxML (v8.2.12) using the Gamma model of rate heterogeneity, JTT amino acid substitution model, and 1,000 bootstraps. Coding sequence was identified for the HIP43 gene family and aligned using MUSCLE (v5) translation alignment using default parameters. Maximum likelihood phylogenetic analysis was performed using RAxML (v8.2.12) using the General Time Reversible model of nucleotide substitution under the Gamma model of rate heterogeneity and 1,000 bootstraps.

Statistical analysis and Protein structure prediction using AlphaFold2

Significance difference between groups of samples was performed using GraphPad Prism 10. *P* values < 0.05 were considered significant, *****P*<0.0001, ****P*<0.001, ***P*<0.01 while values *P* values > 0.05 were considered as non-significant in an unpaired Student's t-test. Structure prediction was carried out using AlphaFold2 and the structure analyzed, and figures made using ChimeraX

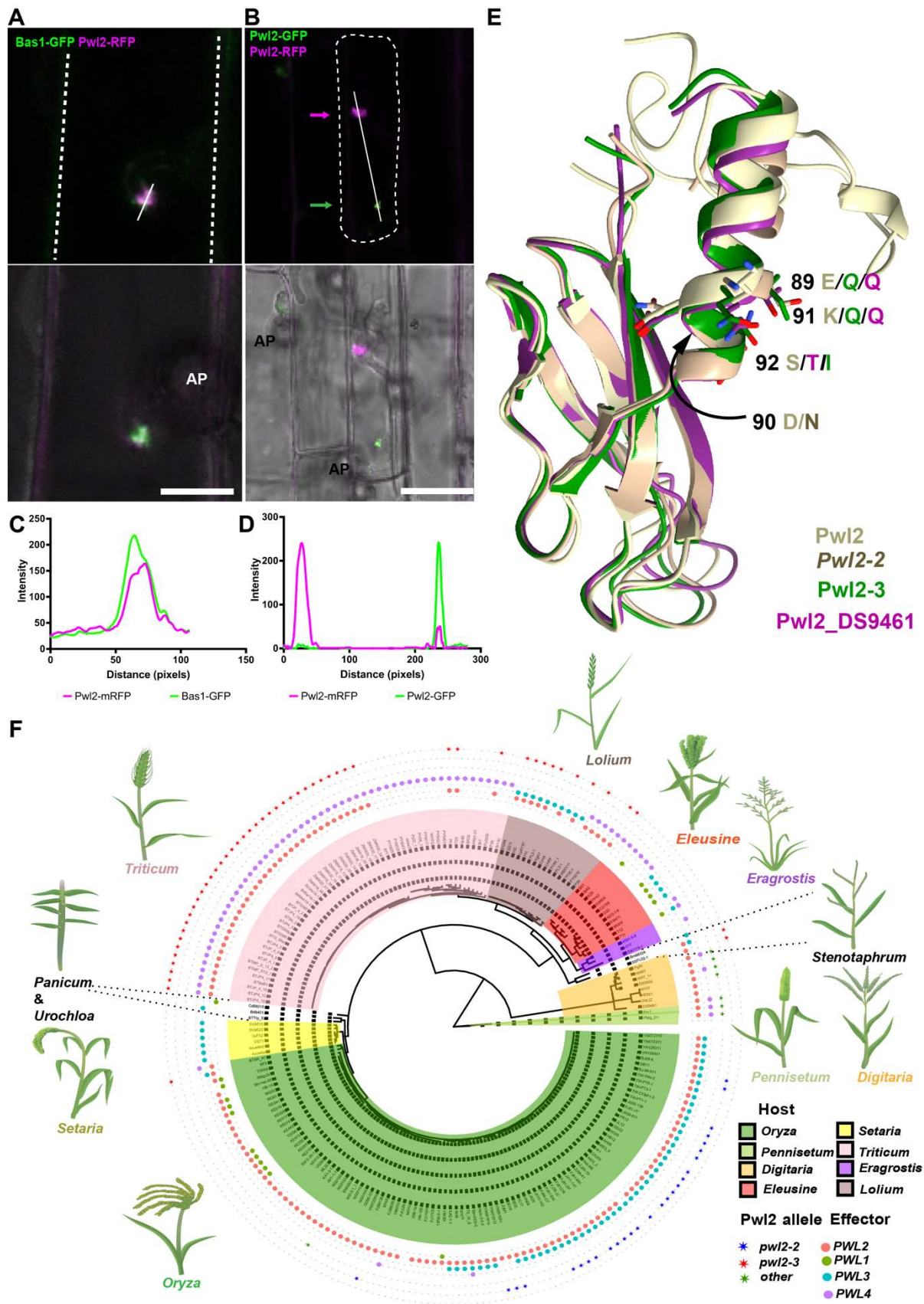


Fig. 1. Pwl2 is a BIC localized effector that is ubiquitous in isolates of *M. oryzae* and displays conserved structural features.

(A+C) Micrographs showing Pwl2 secreted through the Biotrophic Interfacial Complex (BIC) in rice cells during early infection. Conidial suspension at $1 \times 10^5 \text{ mL}^{-1}$ of *M. oryzae* strain co-expressing Bas1-GFP and Pwl2-mRFP were inoculated onto a susceptible cultivar Moukoto rice leaf sheath and images captured at 26 hpi. Fluorescence of the two effectors was observed as small punctate signals in the same BIC. (C+D) Co-infection assay of rice leaf sheath with *M. oryzae* strains expressing Pwl2-mRFP and Pwl2-GFP at 30 hours post-infection (hpi). Micrographs and line scan graph shows there is an absence of mixed fluorescence confirming the BIC does not contain Pwl2 transferred from rice cells. BICs indicated by magenta arrows for mRFP and green arrows for GFP. (E) Superimposition of different Pwl2 variants predicted using AlphaFold2 onto the resolved Pwl2 structure (companion paper to this study by Zdrzalek *et al* (15)) indicating region of polymorphism. (F) Phylogenetic analysis and *PWL* gene family distribution in *M. oryzae*. A maximum parsimony tree including *M. oryzae* strains and isolates from related species *Magnaporthe grisea* and *Pyricularia pennisetigena*. The heatmap indicates the presence/absence of genes in the *PWL* family. *PWL1* is found in *Eleusine* and *Oryza* lineages. *PWL2* is conserved across various *M. oryzae* lineages and isolates infecting crabgrass and pearl millet. *PWL3* is specific to *Oryza*, *Lolium*, *Setaria*, and *Eleusine* lineages. *PWL4* is predominantly found in *Eleusine*, *Eragrostis*, *Lolium*, and *Triticum* lineages. Maximum parsimony tree of *M. oryzae* isolates was generated using a standalone, whole genome multiple alignment/SNP calling program, kSNP3. We used *Pwl1*, *Pwl2*, *Pwl3* and *Pwl4* protein sequence to query for presence/absence polymorphism of the gene families using tblastn.

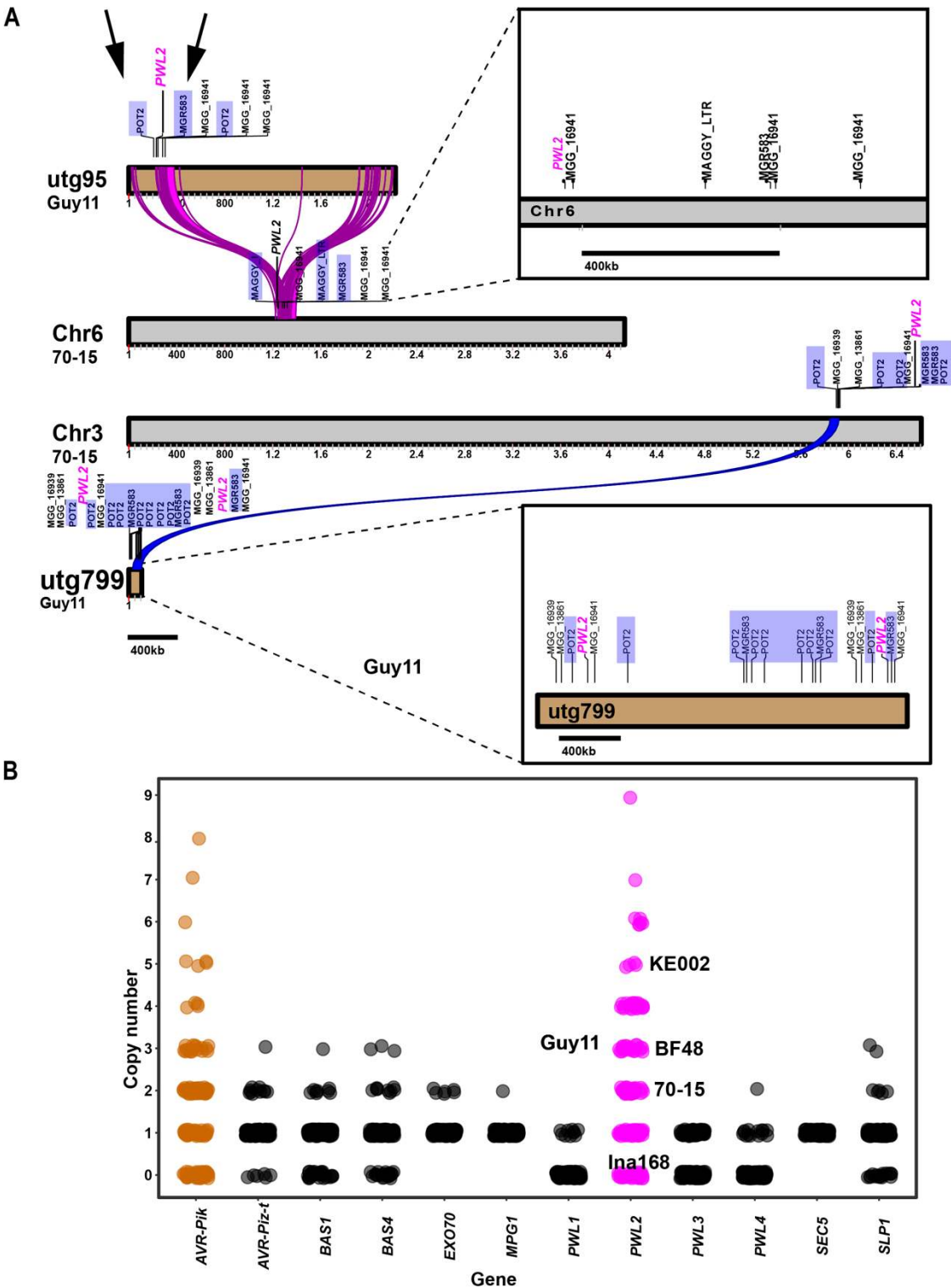


Fig. 2. *PWL2* occurs in multiple loci and shows high copy number variations in field isolates.

(A) Schematic showing the estimated chromosomal location of *PWL2* on multiple loci (Chr3 and 6) of the reference genome 70-15 and on equivalent region of assembled contigs of laboratory strain Guy11 genome. *PWL2* is flanked by *POT2* and *MGR583* repeated sequences suggesting a possible involvement in translocation of events into different loci in the genome. Arrows indicate location of *PWL2*. (B) A k-mer analysis on sequenced raw reads was used to

determine copy number variation of *PWL* family genes in different *M. oryzae* isolates. Plot shows high copy number of *PWL2* in analyzed genomes (n = 286) compared to the other *PWL* gene family members, another effector *SLP1*, and selected control genes *MPG1* and *SEC5*. Similarly, *AVR-Pik* shows multiple copies in different isolates. Copy number of *PWL2* in selected isolates Guy11, KE002, BF48, 70-15 is indicated with Ina168 that lack *PWL2* used as a negative control.

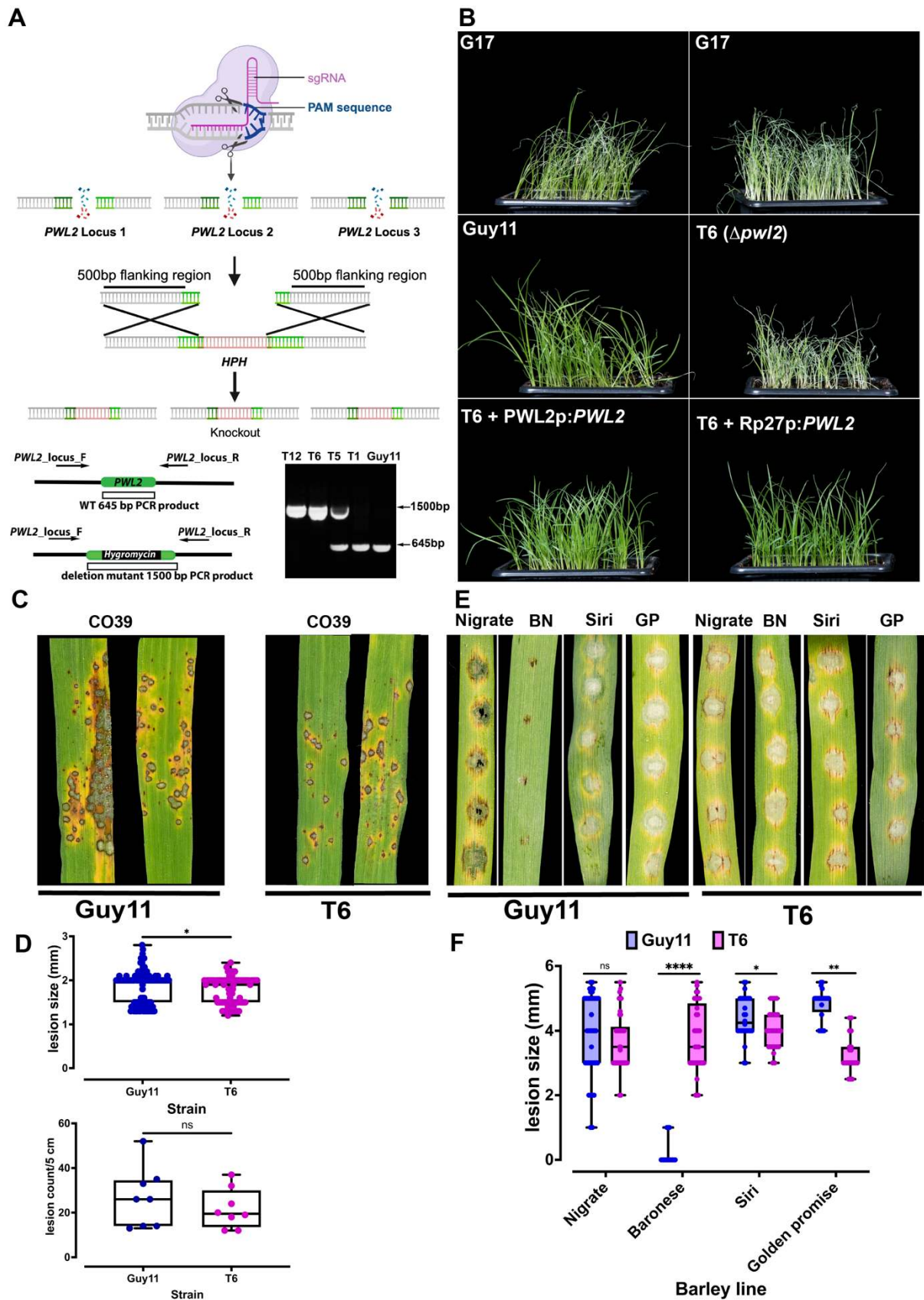


Fig. 3. CRISPR-Cas9 *Δpwl2* mutants demonstrate gain of virulence on weeping lovegrass and barley carrying *Mla3*.

(A) Schematic illustration of the CRISPR-Cas9 mediated gene editing process for inserting the Hygromycin Phosphotransferase (*HPH*) gene cassette at the *PWL2* locus. The guided sequence (sgPWL2) directs Cas9 to introduce a double stranded break at the *PWL2* locus, with the DNA repair template containing the Hygromycin resistance gene cassette and flanking regions of the *PWL2* gene. This leads to mutations in the form of indels or gene replacements. Figure created with BioRender <https://biorender.com/>. Positive transformants were identified by amplifying the *PWL2* coding sequence from the wild type Guy11 and the hygromycin cassette from transformants with the three copies deleted. (B) Comparison of disease symptoms in weeping lovegrass (*Eragrostis curvula*) infected with different *M. oryzae* isolates. In the top panel, typical disease lesions produced by *E. curvula*-infecting isolate G17 are shown. In the middle panel, CRISPR-Cas9 deletion mutants exhibit gain of virulence on weeping lovegrass compared to Guy11. In the bottom panel, complemented strains with both native and constitutive RP27 promoters showed a loss of virulence on weeping lovegrass. (C) *Δpwl2* mutants display reduced pathogenicity on rice cultivar CO39. Conidial suspensions at 1×10^5 mL⁻¹ spores/mL from Guy11 and *Δpwl2* T6 were used to inoculate 21-d-old seedlings of the blast-susceptible cultivar CO39, and disease symptoms recorded after 5 dpi. (D) The box plots show the lesion size (top) and lesion density (bottom) of seedlings infected with Guy11 and *Δpwl2* T6. The lower horizontal line shows the minimum value, and the upper horizontal line shows the maximum value. The lower border and upper border of the box show the lower quartile and upper quartile, respectively. The line in the box shows the median. (E) The *Δpwl2* mutant exhibits enhanced pathogenicity on barley cultivar Baroness (*Mla3*). Conidial suspensions at 1×10^5 mL⁻¹ spores/mL from Guy11 and *Δpwl2* T6 were used to inoculate 10-day old seedlings of barley lines Golden Promise, Siri, Nigrata and Baroness, and disease symptoms recorded after 5 dpi. (F) The box plot shows lesion size of barley seedlings infected with Guy11 and *Δpwl2* T6. The lower horizontal line shows the minimum value, and the upper horizontal line shows the maximum value. The lower border and upper border of the box shows the lower quartile and upper quartile, respectively. The line in the box shows the median. Significance between groups of samples was performed using Unpaired Student's t-test. **** $P < 0.0001$, ** $P < 0.01$, $P < 0.05$, NS indicates no significant difference.

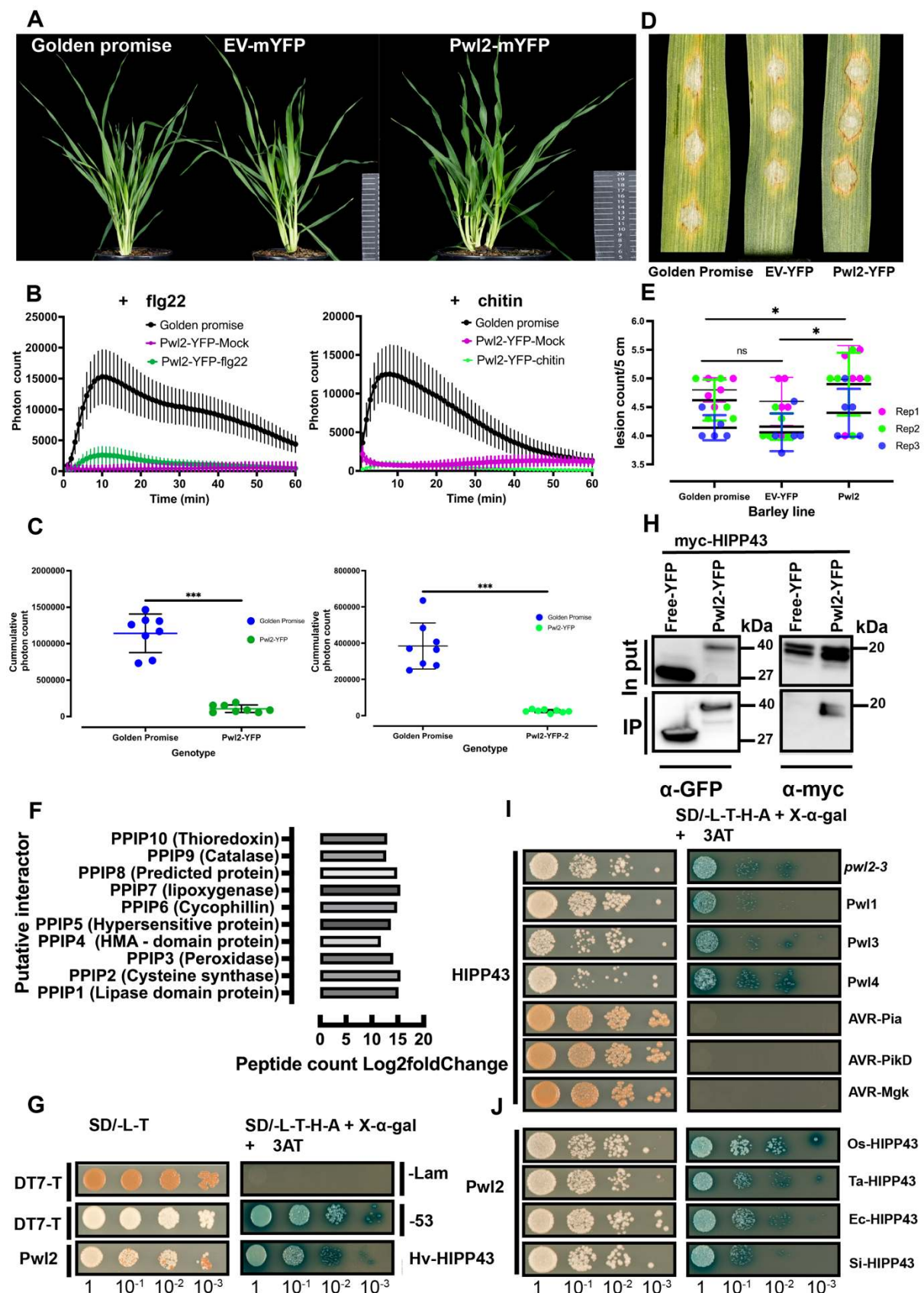
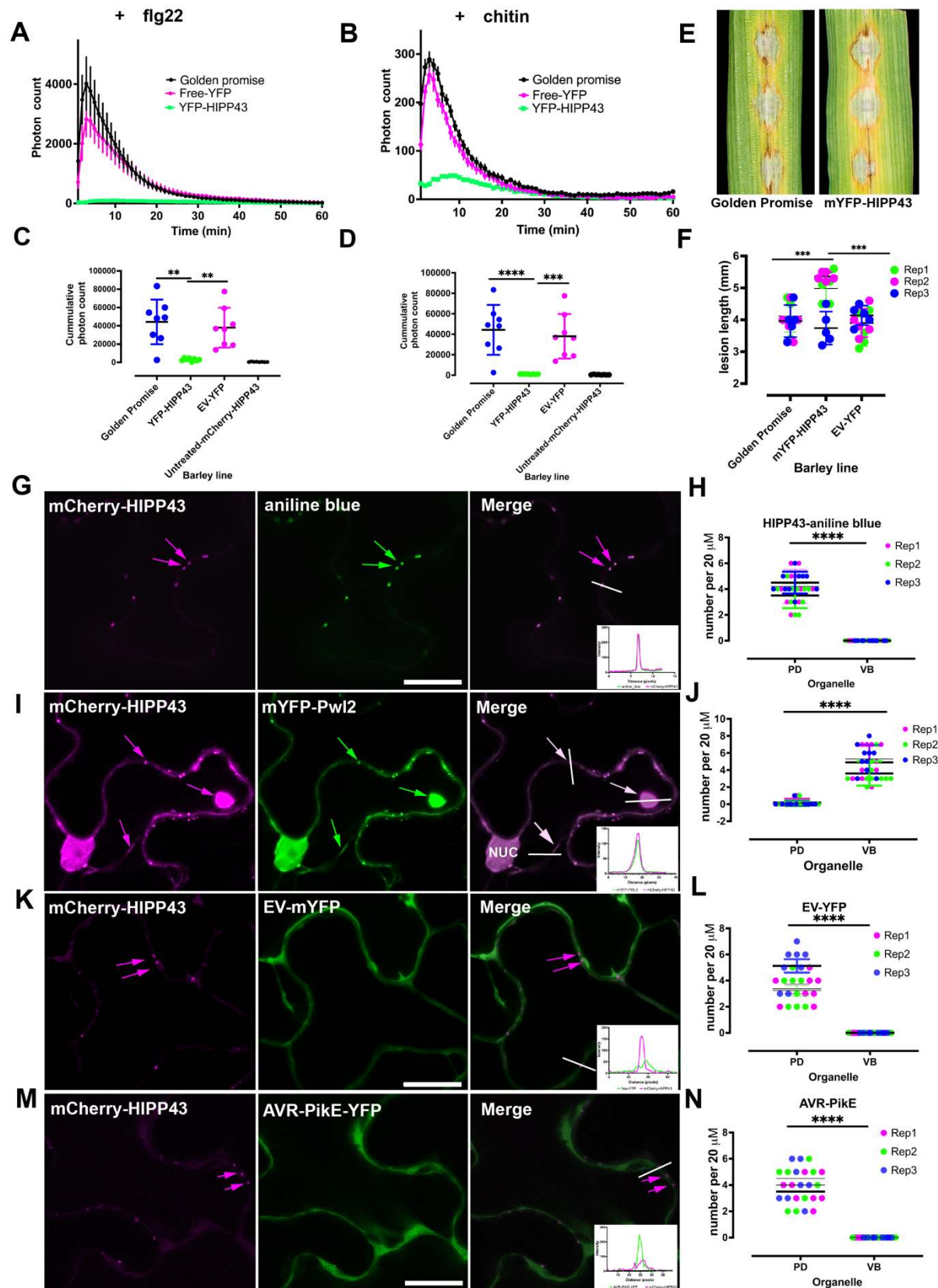


Fig. 4. Pwl2 suppresses PAMP-induced ROS in transgenic barley lines and interacts with a host HIP43.

(A) Transgenic lines expressing Pwl2-YFP, control (free YFP) did not show growth defect compared to the background line, Golden Promise. (B) ROS production in leaf discs collected from 4-week-old stable transgenic lines expressing Pwl2-YFP, control (free YFP) and Golden Promise induced by 1 μ M chitin or flg22 ($n > 8$). Points represent mean; error bars represent SEM. (C) Dot plot shows cumulative ROS production over 60 min for Golden Promise control and Pwl2-YFP. The lower horizontal line shows the minimum value, and the upper horizontal line shows the maximum value, the middle line shows the mean value. Significance between groups of samples was performed using Unpaired Student's t-test. (D) Leaf drop infection on barley transgenic lines expressing free-YFP, Pwl2-YFP compared to infection on wild type Golden Promise 3-4 dpi. (E) The dot plot represents the number of rice blast disease lesions per 5 cm on Golden Promise, EV-YFP and Pwl2-YFP transgenic lines in three independent repetitions of the experiment. Unpaired Student's t-test was performed to determine significant differences. (F) Putative Pwl2 interacting peptides were immunoprecipitated from protein extracted from 3-week-old stable Golden Promise transgenic lines expressing Pwl2-YFP or free cytoplasmic YFP using anti-GFP antibodies, and LC-MS/MS was performed to identify unique putatively interacting peptides. The scale 0-20 represents the Log2fold change in peptides when compared to the control. (G) Pwl2 interacts with *HvHIP43* in a yeast-two-hybrid assay. Simultaneous co-transformation of pGAD-Pwl2 (prey vector) and pGBK-HIP43 (bait vector) and PGBKT7-53 and pGADT7-T (positive control) pGADTT7-T and pGBKT7-Lam (negative control) into Y2H gold strain resulted in the activation of four reporter genes and growth on high-stringency medium (-Ade, -Leu, -Trp, -His +X- α -gal and 3-amino-1,2,4-triazole). Co-transformation also activates the expression of *MEL1*, which results in the secretion of α -galactosidase and the hydrolysis of X- α -gal in the medium, turning the yeast colonies blue (H) Co-immunoprecipitation (co-IP) of Pwl2-YFP and Myc-HIP43 in *N. benthamiana* leaves. C-terminal GFP tagged Pwl2 and C-terminal Myc-HIP43 was cloned into the vector pGW514 and transformed into *Agrobacterium* strain *GV301* and co-infiltrated into *N. benthamiana* leaves and left to incubate for 48 h. Immunoprecipitates were obtained with anti-GFP or anti-Myc affinity matrix beads and probed with anti-GFP-peroxidase and anti-Myc-peroxidase (HRP-conjugated) antibodies. Total protein extracts were also probed with appropriate (HRP-conjugated) antibodies. (I) Yeast-two-hybrid analysis shows; upper panel, *HvHIP43* interacts with *PWL* gene family products Pwl1, Pwl3, Pwl4 and variant *pwl2-3*. (J) Pwl2 binds to HIP43 homologs from rice (*OsHIP43*), wheat (*TaesHIP43*), weeping love grass (*EcHIP43*), and foxtail millet (*SiHIP43*).



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Fig. 5. HvHIPP43 suppresses PAMP-induced ROS in transgenic barley.

(A-D) ROS production measured from leaf disks collected from 4-week-old stable transgenic line expressing YFP-HIPP43 and Golden promise (control) in the absence and presence of 1 μ M flg22 or 1 mg/ml Chitin ($n > 8$). For ROS assay, line graphs (upper panel) points represent mean per time point and as cumulative ROS (C+D) production over 60 min, error bars represent SEM. For dot plot graphs show the average cumulative photon count. The lower horizontal line shows the minimum value, the upper horizontal line shows the maximum value, the middle line shows the mean value. Significance between groups of samples was performed using unpaired Student's t-test. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$. (E) Leaf drop infection on barley transgenic lines expressing YFP-HIPP43 compared to wild type Golden Promise dpi. Conidial suspensions at 1×10^5 mL⁻¹ spores/mL from Guy11 were used to inoculate 21-d-old seedlings of the blast-susceptible Golden Promise control and Golden Promise expressing YFP-HIPP43. Disease symptoms were recorded after 5 dpi. (F) Dot plot showing the average lesion size on barley lines infected with Guy11. The lower horizontal line shows the minimum value, the upper horizontal line shows the maximum value, the middle line shows the mean value. Significance between groups of samples was performed using unpaired Student's t-test. *** $P < 0.001$. (G+H) Micrographs and line scan graph showing mCherry-HIPP43 localizing as small puncta on the plasma membrane when expressed in *N. benthamiana*. Staining of callose using aniline blue overlaps with mCherry-HIPP43, confirming mCherry-HIPP43 localises exclusively at the plasmodesmata (PD) localization and is absent from vesicular bodies (VB). (I+J) Micrographs and line scan graph showing the presence of YFP-Pw12, alters m-Cherry-HIPP43 localisation, where it was observed to translocate to the cytoplasm, cytoplasmic and vesicular structures (K+L) Micrographs and line scan graph showing free-YFP fluorescence is observed in the cytoplasm and does not overlap with mCherry-HIPP43 PD localization. (M+N) Micrographs and line scan graph showing mCherry-HIPP43 remains in the PD in the presence of cytoplasmically localised AVR-PikE. Scale bars represent 20 μ m. Significance between groups of samples was performed using unpaired Student's t-test. **** $P < 0.0001$.

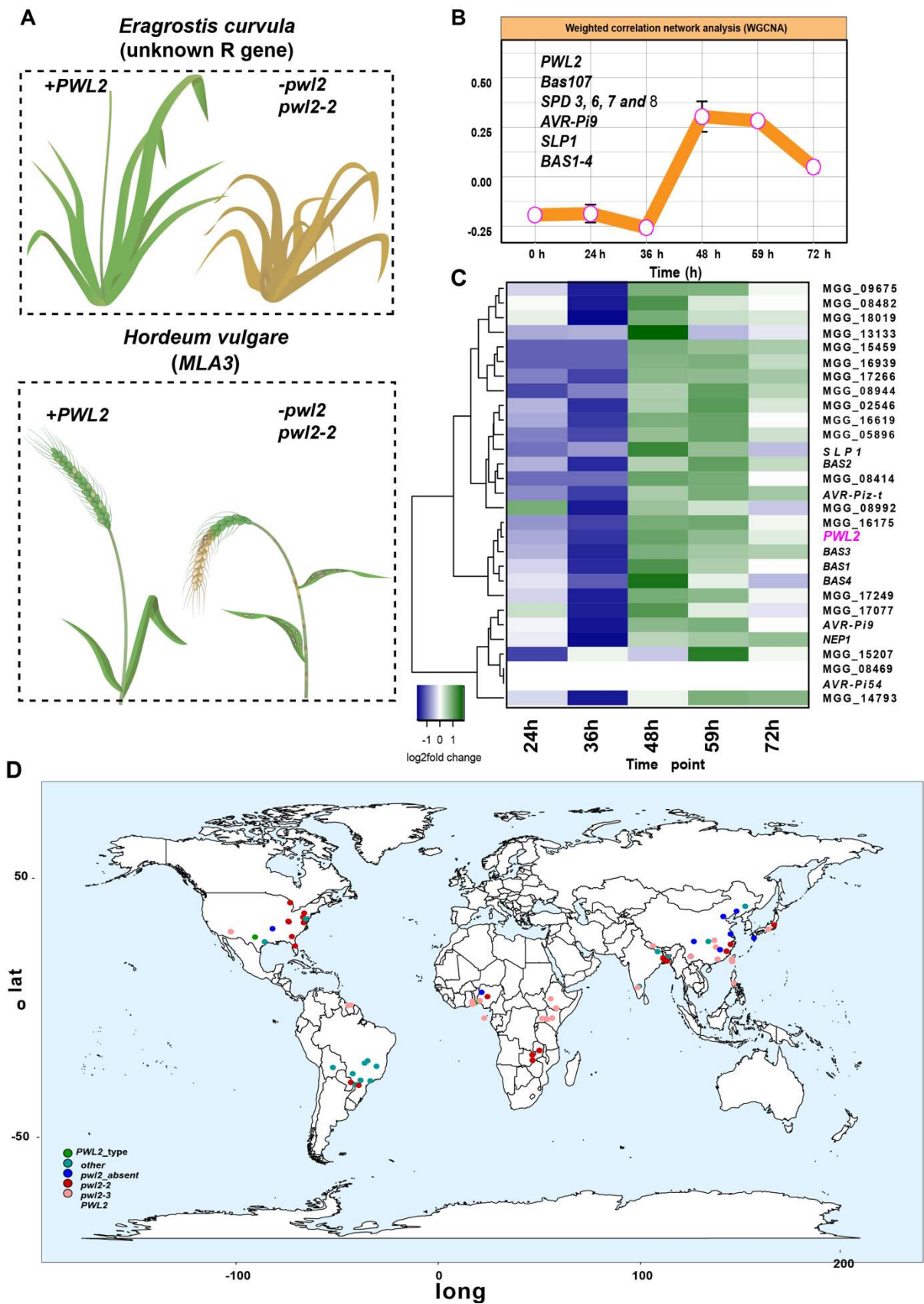
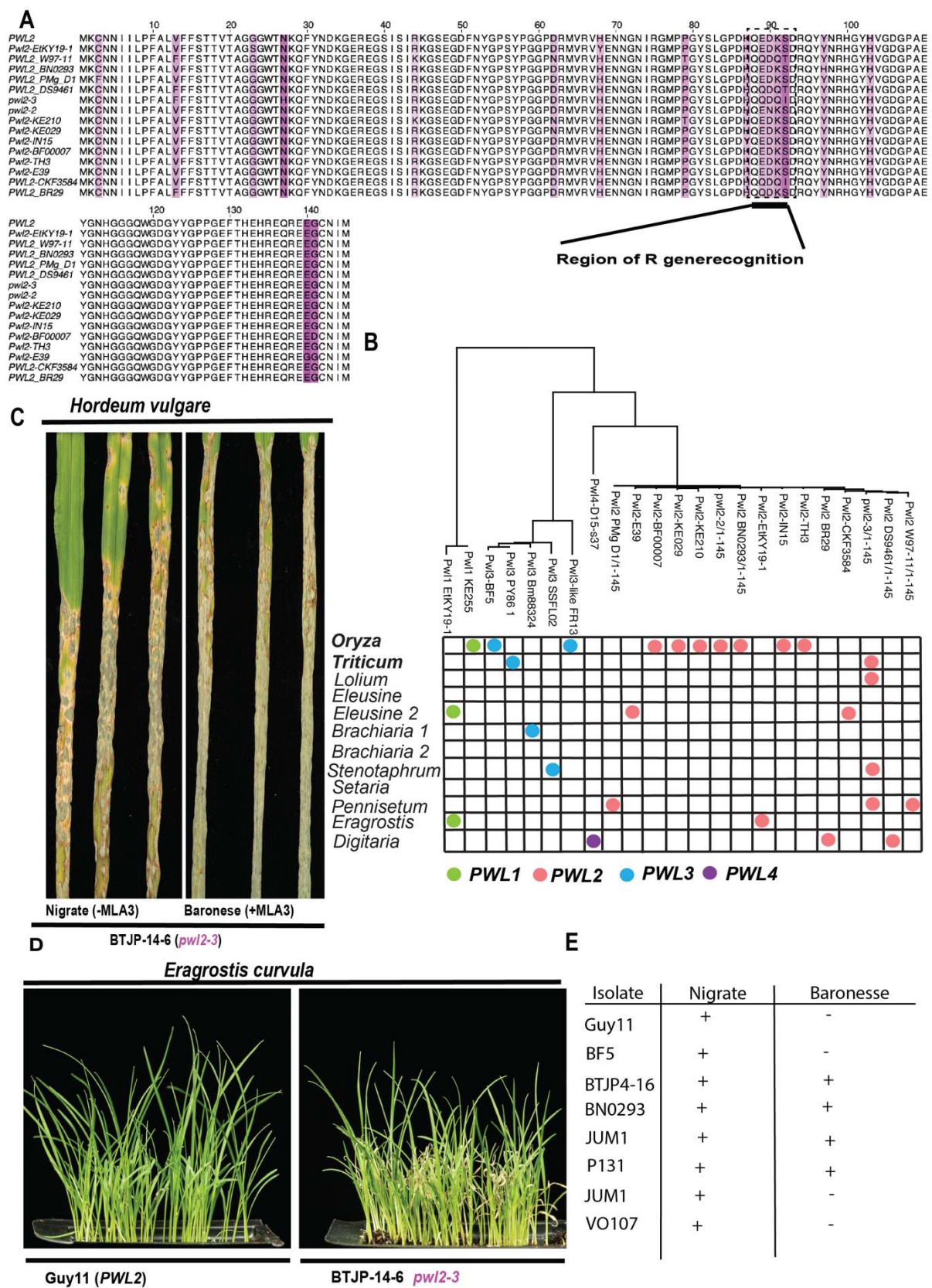


Fig. S1. Pwl2 is co-expressed *in-planta* with other known effectors and is ubiquitous in isolates of *M. oryzae* showing global distribution.

(A) Illustration of how *PWL2* has been described as a host range determinant gene in weeping lovegrass and barley. *M. oryzae* strain carrying *PWL2* produces incompatible reaction on weeping lovegrass and cv. Baronesse (*Mla3*). (B) A susceptible rice line Moukoto, was infected by a *M. oryzae* strain KE002 using leaf drop infection method and samples collected at 24, 36, 48, 59 and 72 hpi. RNA isolated from infected rice leaves were sequenced and the reads normalized against *M. oryzae* mycelia KE002 RNA to determine differentially expressed genes. A weighted gene co-expression network analysis grouped gene expression profile into 10 co-expression modules. The line graph (WGCNA) showing the representative eigengene for module 4 in *PWL2* is co-expressed with other known effector protein encoding, *AVR-Pi9*, *SLPI*, *BASI-4* and a necrosis inducing effector *NEPI*. (C) Heatmap showing relative expression levels (log2fold change) of *PWL2* and other known effectors during infection. (D) Global distribution of Pwl2 alleles in *M. oryzae* isolates with publicly available genomes. Tblastn was used to determine presence of Pwl2 in *M. oryzae* genomes and R package *rworldmap* v.1.3.4 used to create the map and occurrence of different alleles according to isolate point of collection. *pwl2-2* mostly present in isolates from Asia while *pwl2-3* present in wheat blast isolates from South America.



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Fig. S2. A variant of Pwl2, pwl2-3 is not recognized by Mla3 and cognate resistance against Pwl2 in weeping lovegrass.

(A) Multiple amino acid sequence alignment of Pwl2 protein and naturally occurring variants. Alignment was carried using ClustalW (Jalview). (B) Neighbor-joining tree based on amino acid sequence of variants encoded by *PWL* gene family. Tree IDs contains isolate name from which the variant was identified, and each dot represent presence in each host-limited forms. (C and D) Wheat blast isolate BTJP-14-6 carrying a *PWL2* variant, *pwl2-3* with Glu-89-Gln, Lys-91-Gln and Ser-92-Ile substitutions was used for spray inoculation on barley accessions cv. Golden promise (-*Mla3*) and cv. Baroness (+*Mla3*) or (D) weeping lovegrass seedlings. Disease symptoms on barley and weeping lovegrass were scored 5 and 7 days respectively, post-infection. Observations were consistent in three independent infection replicates. (E) Summary of disease reaction of isolates carrying variants of Pwl2 on Nigrate (-*Mla3*) and Baroness (+*Mla3*). Lesions on barley were scored 5 days post-infection.

A

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      10      20      30      40      50      60      70      80
PWL1      MKFNKT IPLY I LAFFSTAV IAGGRKWTNKV IYNDKGEREGS I S IRKGAEGDFNCGPGYPGGPDRMVRVHEDNGN IRGMPPGYRLGPDDE
PWL1_KE255 MKFNN I IPLY I LAFFSTAV IAGGRKWTNKV IYNDKGEREGS I S IRKGAEGDFNCGPGYPGGPDRMVRVHEDNGN IRGMPPGYRLGPDDE
PWL1_EIKY19-1 MKFNKT IPLY I LAFFSTAV IAGGRKWTNKV IYNDKGEREGS I S IRKGAEGDFNCGPGYPGGPDRMVRVHEDNGN IRGMPPGYRLGPDDE

      100     110     120     130     140
PWL1      DKRDNQYYSRNGYHVGDGPAEYQNHGGGQWGDGYGPPGE I TNQHGKRQGDQGCHIM
PWL1_KE255 DKRDNQYYSRNGYHVGDGPAEYQNHGGGQWGDGYGPPGE I TNQHGKRQGDQACHIM
PWL1_EIKY19-1 DKRDNQYYSRNGYHVGDGPAEYQNHGGGQWGDGYGPPGE I TNQHGKRQGDQGCHIM
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B

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      10      20      30      40      50      60      70      80
PWL3_PY86_1 MKINTS I LALT LALLPGMATAGRKWLNKKLWDAN GQSAGSVS I VKGGQGS I NTD TGP I TAEGSYD I YERNGK I EGPPGYK YTD RYED
PWL3      MKINTS I LALT LALLPGMATAGRKWLNKKLWDAN GQSAGSVS I VKGGQGS I NTD TGP I TAEGSYD I YERNGK I EGPPGYK YTD RYED
PWL3_Bm88324 MKINTS I LALT LALLPGMATAGRKWLNKKLWDAN GQSAGSVS I VKGGQGS I NTD TGP I TAEGSYD I YERNGK I EGPPGYK YTD RYED
PWL3_SSFL02 MKINTS I LALT LALLPGMATAGRKWLNKKLWDAN GQSAGSVS I VKGGQGS I NTD TGP I TAEGSYD I YERNGK I EGPPGYK YTD RYED
PWL3-BF5      MKINTS I LALT LALLPGMATAGRKWLNKKLWDAN GQSAGSVS I VKGGQGS I NTD TGP I TAEGSYD I YERNGK I EGPPGYK YTD RYED

      90     100     110     120     130
PWL3_PY86_1 RKDDRYYNTHGYHVGDGPAEYQNYGGGHWGDGYGPPGEFVKTSEYE -
PWL3      RKDDRYYNTHGYHVGDGPAEYQNYGGGHWGDGYGPPGEFVKTSEYD -
PWL3_Bm88324 RKDDRYYNTHGYHVGDGPAEYQNYGGGHWGDGYGPPGEFVKTSEYD -
PWL3_SSFL02 RKDDRYYNTHGYHVGDGPAEYQNYGGGHWGDGYGPPGEFVKTSEYD -
PWL3-like FR13 RKDDRYYNTHGYHVGDGPAEYQNYGGGHWGDGYGPPGEFVKTSEYD -
PWL3-BF5      RKDDRYYNTHGYHVGDGPAEYQNYGGGHWGDGYGPPGEFVKTSEYE -
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C

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      10      20      30      40      50      60      70      80      90     100
PWL4_CD156 MKINTS I LALALALLPGMATAGRKWFNKK IYDENGESAGSLSVVKGGSY IN IGPSAPGQRDLVEFRESGGK I QGGPPGYRYTSDHEEDQRDNRYNTHGY
D15-s37      MKINTS I LALALALLPGMATAGRKWFNKK IYDENGESAGSLSVVKGGSY IN IGPSAPGQRDLVEFRESGGK I QGGPPGYRYTSDHEEDQRDNRYNTHGY
PwH      MKINTS I LALALALLPGMATAGRKWFNKK IYDENGESAGSLSVVKGGSY IN IGPSAPGQRDLVEFRESGGK I QGGPPGYRYTSDHEEDQRDNRYNTHGY

      110     120     130
PWL4_CD156 HVGDGPAEYGDHGGGHWGDGYGPPGEFVKTSEYED
D15-s37      HVGDGPAEYGDHGGGHWGDGYGPPGEFVKTSEYED
PwH      HVGDGPAEYGDHGGGHWGDGYGPPGEFVKTSEYED
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Fig. S3. *M. oryzae* isolates carry variants of Pwl1, Pwl3 and Pwl4 and copy number variation occurs in all rice blast genetic lineage groups. Multiple amino acid sequence alignment of Pwl1 (A), Pwl3 (B) and Pwl4 (C) protein and naturally occurring variants. Alignment was carried using ClustalW (Jalview) regions or low conservation are shown in magenta.

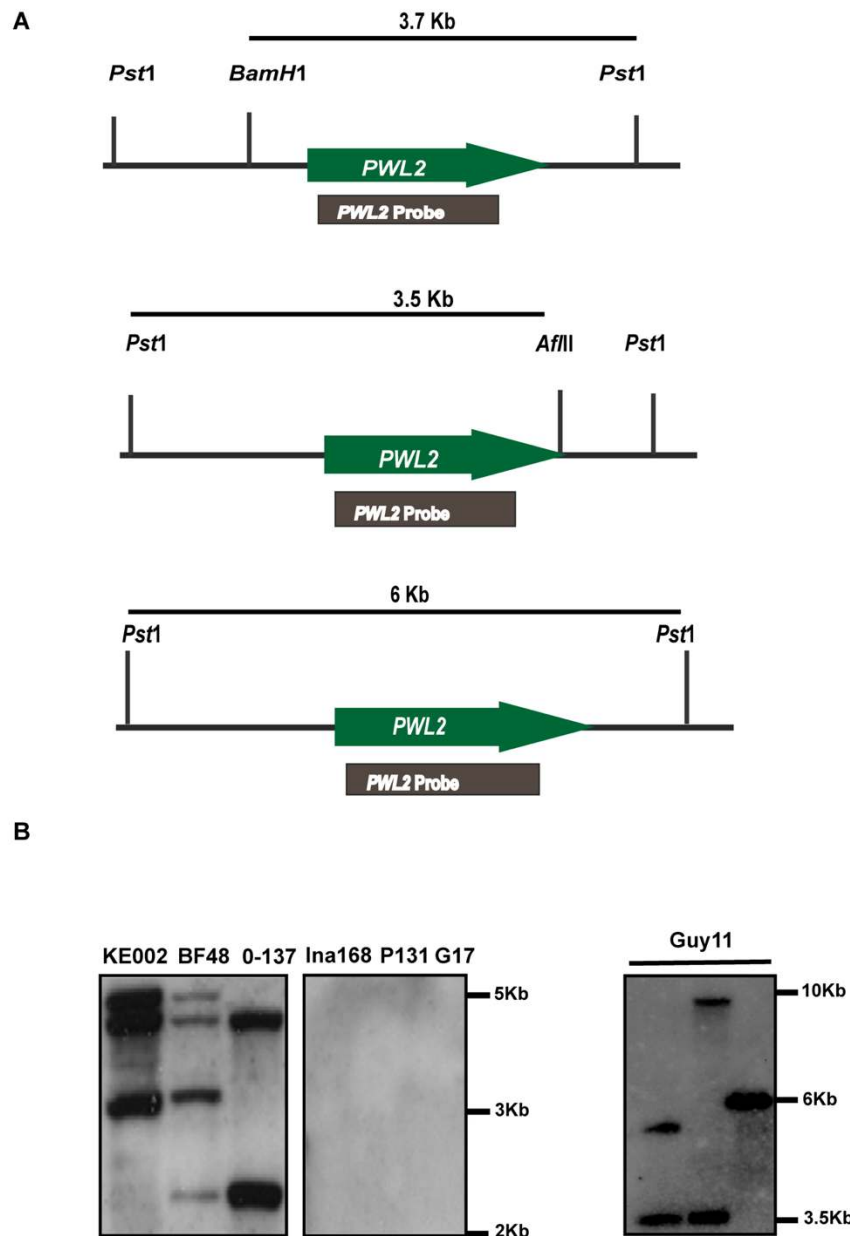


Fig. S4. Southern blot to determine copy number variation of *PWL2* in selected *M. oryzae* isolates.

(A) Genomic DNA was digested using different enzyme combination *Bam*H1/*Pst*1 and probed using the *PWL2* 438 bp probe. (B) The order of digested genomic DNA from different isolates is indicated on the lanes. Size estimates are from the *PWL2* loci annotated *M. oryzae* 70-15 genome. When Guy11 genomic DNA was digested using *Pst*1 and *Afl*III or single digested using *Afl*III, two bands were detected, a single digest using *Pst*1 only produce a single band.

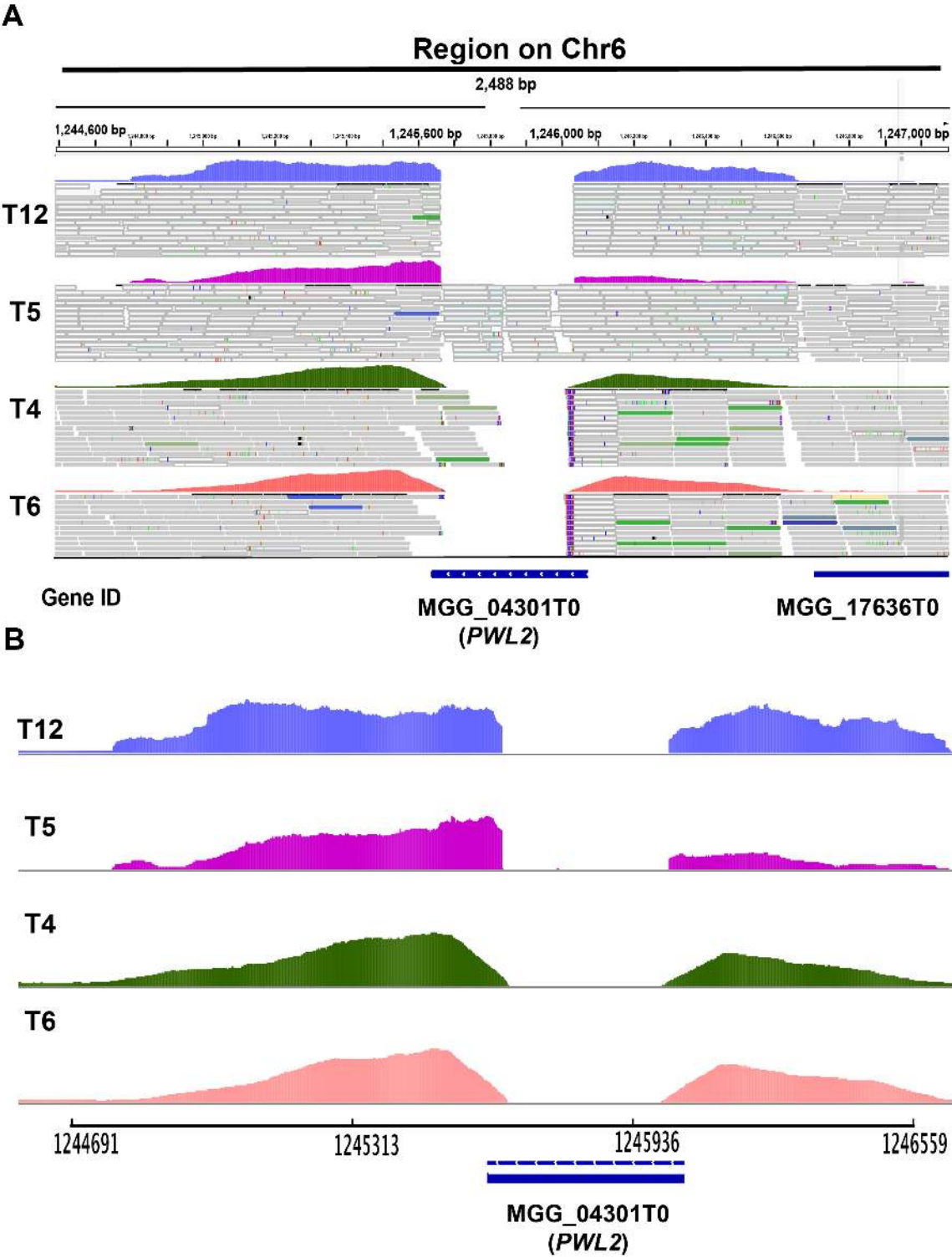


Fig. S5. Analysis of CRISPR-Cas9 mutants using Integrative Genomics Viewer (IGV).
(A) Reads alignment display of *PWL2* locus on Chromosome 6 from four sequenced *pwl2* mutant strains, T12, T5, T4 and T6 (lower panel). No reads aligned at *PWL2* (MGG_04301T0) locus for T6 and T12 and partial alignment for T4 and T5. Read depth around *PWL2* locus is higher compared to the neighbouring gene MGG_17636T0 suggesting the *PWL2* locus occurs multiple times. Read coverage and locus length are indicated. (B) Sashimi plot showing depth of reads from T12, T5, T4 and T6 that aligned to the region surrounding *PWL2* locus.

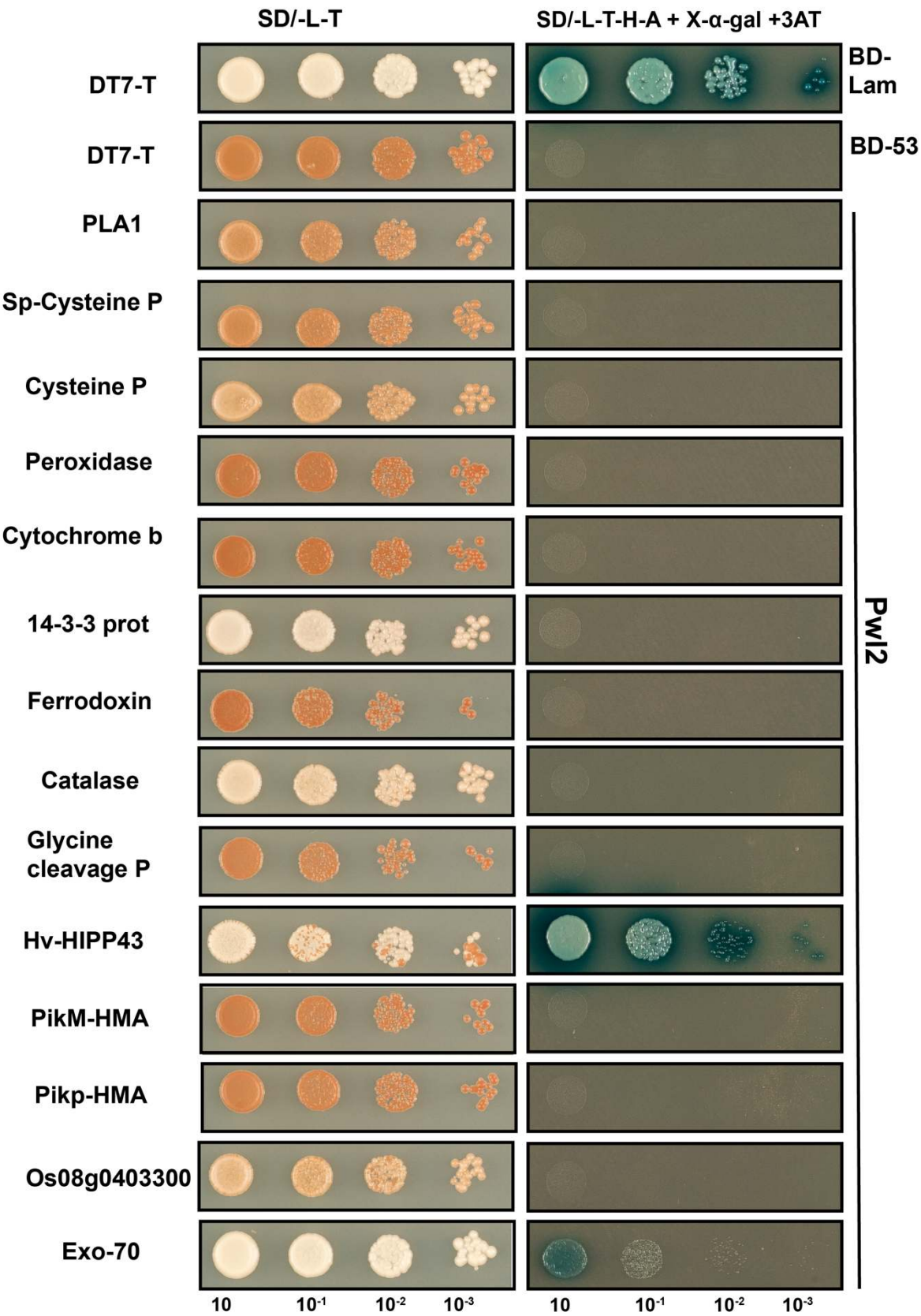


Fig. S6. Raw data results from Y2H assay with all other putative interactors from IP-MS.
 One-to-one yeast two-hybrid between Pwl2 and selected top candidates from IP-MS analysis, Pwl2 and Putative Pwl2 Interacting Proteins (PPIPs). HMA integrated rice NLR Pikm-1 and Pikp-1 were used as specificity controls. Simultaneous co-transformation of pGADT7-Pwl2 (prey vector) and pGBK-HIPP43 (bait vector) and PGBKT7-53 and pGADT7-T (positive control) pGADTT7-T and pGBKT7-Lam (negative control) into Y2H gold strain resulted in the activation of four reporter genes and growth on high-stringency medium (–Ade, –Leu, –Trp, –His +X-α-gal and 3-amino-1,2,4-triazole). Co-transformation also activates the expression of *MEL1*, which results in the secretion of α-galactosidase and the hydrolysis of X-α-gal in the medium, turning the yeast colonies blue. HIPP43 exclusively bind Pwl2 in SD/-L-T-H-A X-a-gal medium with 3AT added.

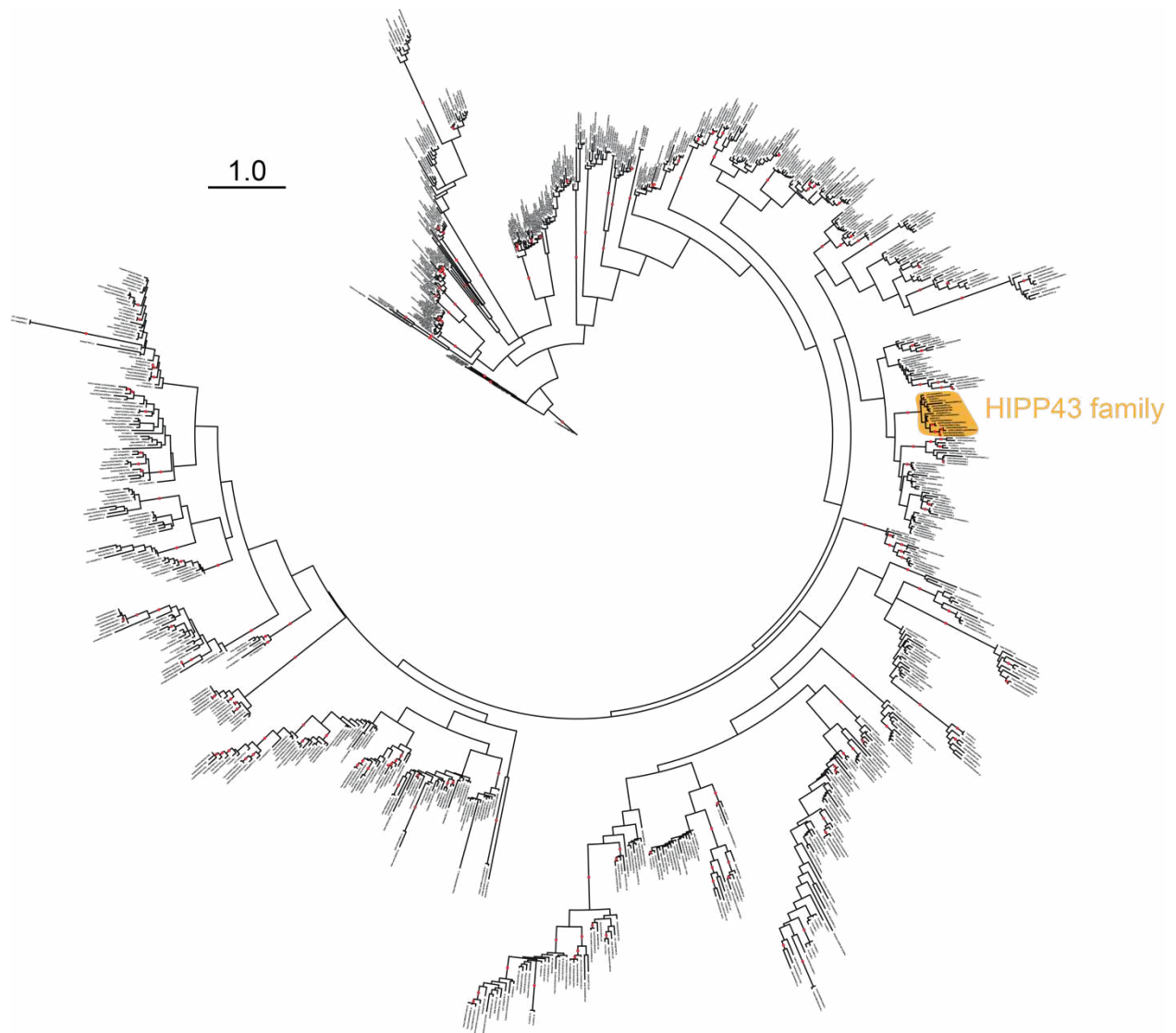


Fig. S7. Maximum likelihood phylogenetic tree of HMA domains from diverse grass species.

HMA domains were identified from proteins from the grass species *B. distachyon*, *H. vulgare*, *T. aestivum*, *O. sativa*, *Oropetium thomaeum*, *Sorghum bicolor*, *Setaria italica*, and *Zea mays*. Structure guided multiple sequence alignment was performed using MAFFT-DASH and phylogenetic analysis with RAXML. Scale bar indicates substitutions per site. Dots on branches indicate support of 80% or more based on 1,000 bootstraps. The HIP43 family formed a distinct clade with bootstrap support of 98%.

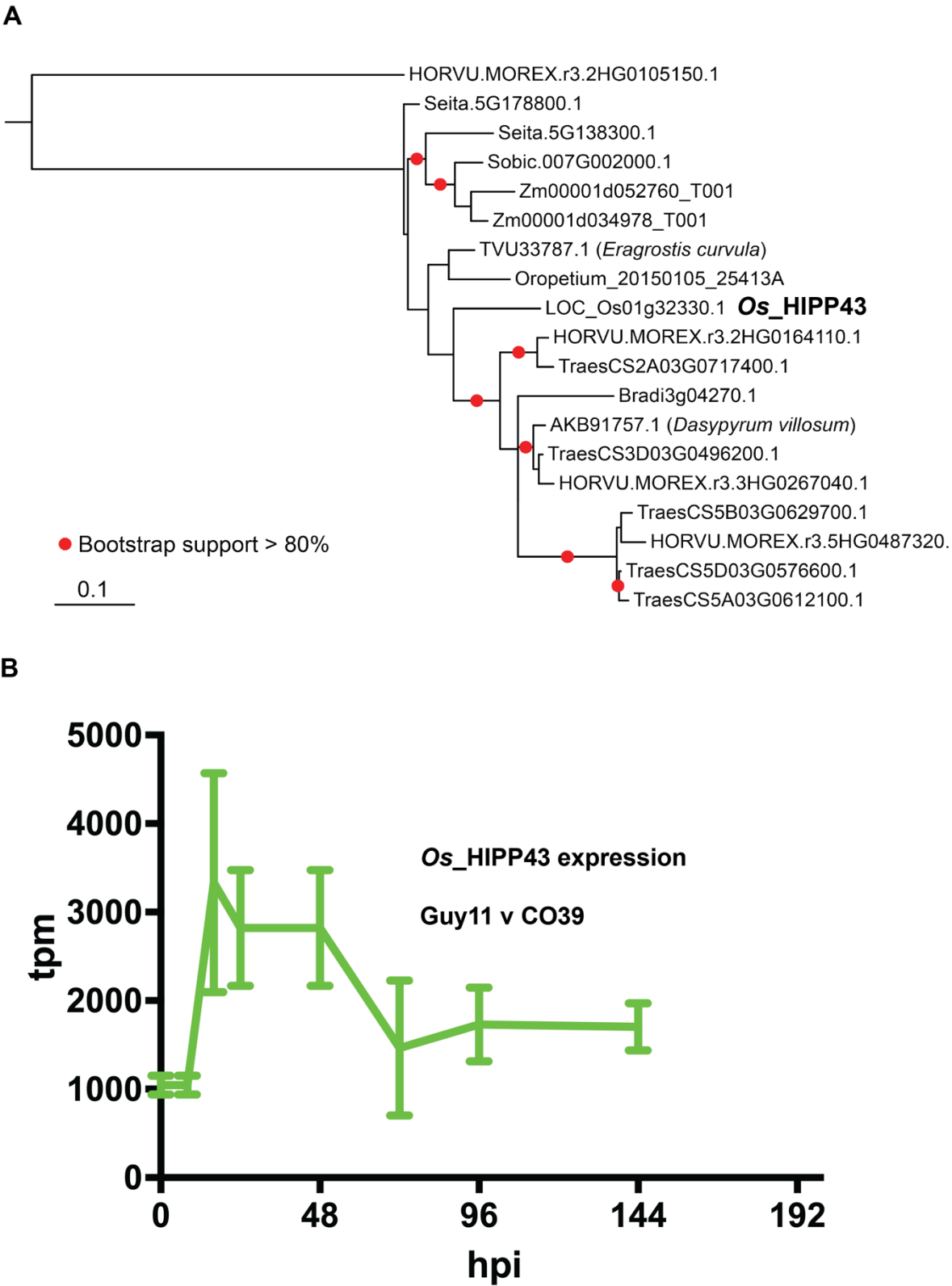


Fig. S8 Maximum likelihood phylogenetic tree for HIPP43 gene family.

(A) Full length coding sequence was identified for HIPP43 gene family members, aligned using MUSCLE translation alignment, and phylogenetic analysis with RAxML. Barley and wheat (i.e. *Triticeae* lineage) have three copies of HIPP43 per haploid genome. Dots on branches indicate support of 80% or more based on 1,000 bootstraps. (B) Expression at transcript level

937 of *OsHIPP43* during infection of a susceptible rice cv. CO39 by Guy11. Hour post inoculation
938 is shown on the X –axis.
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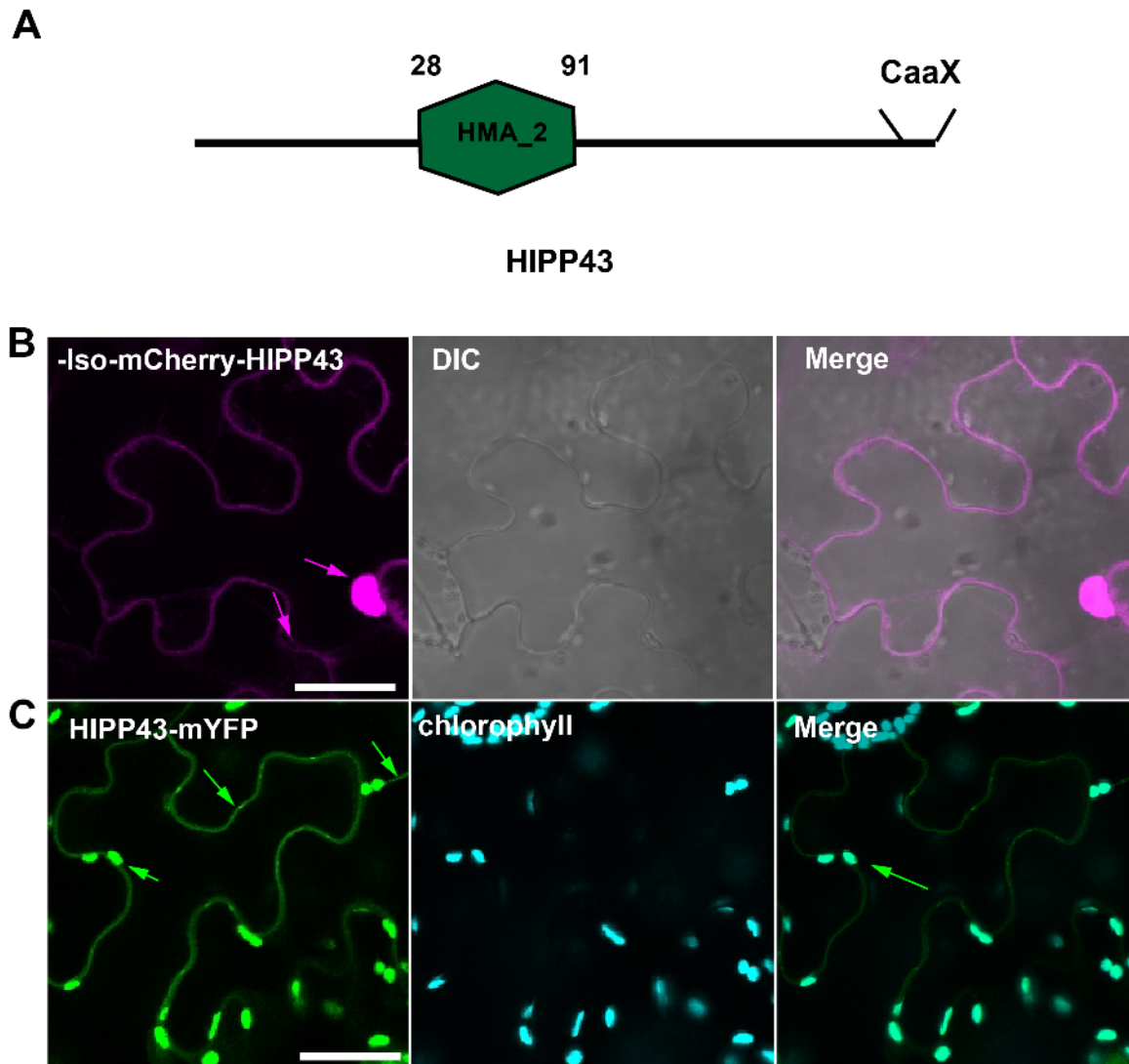


Fig. S9. HIPP43 isoprenylation motif is required for PD localisation.

(A) A schematic representation of domain structure of HIPP43 showing the HMA domain region and the isoprenylation motif at the C-terminus. (B+C) Micrographs showing localization of HIPP43 without the isoprenylation motif (-Iso-mCherry-HIPP43) and C-terminus tagged, HIPP43-YFP. (B) Deletion of the isoprenylation abolishes PD localisation but HIPP43 localises in the nucleus and partially in the plasma membrane and cytoplasm after 48h. (C) When HIPP43 was tagged at the C-terminus, localisation was observed in the plasma membrane, partially in the cytoplasm and PD, but predominantly in the chloroplast. Scale bars represent 20 μ m.

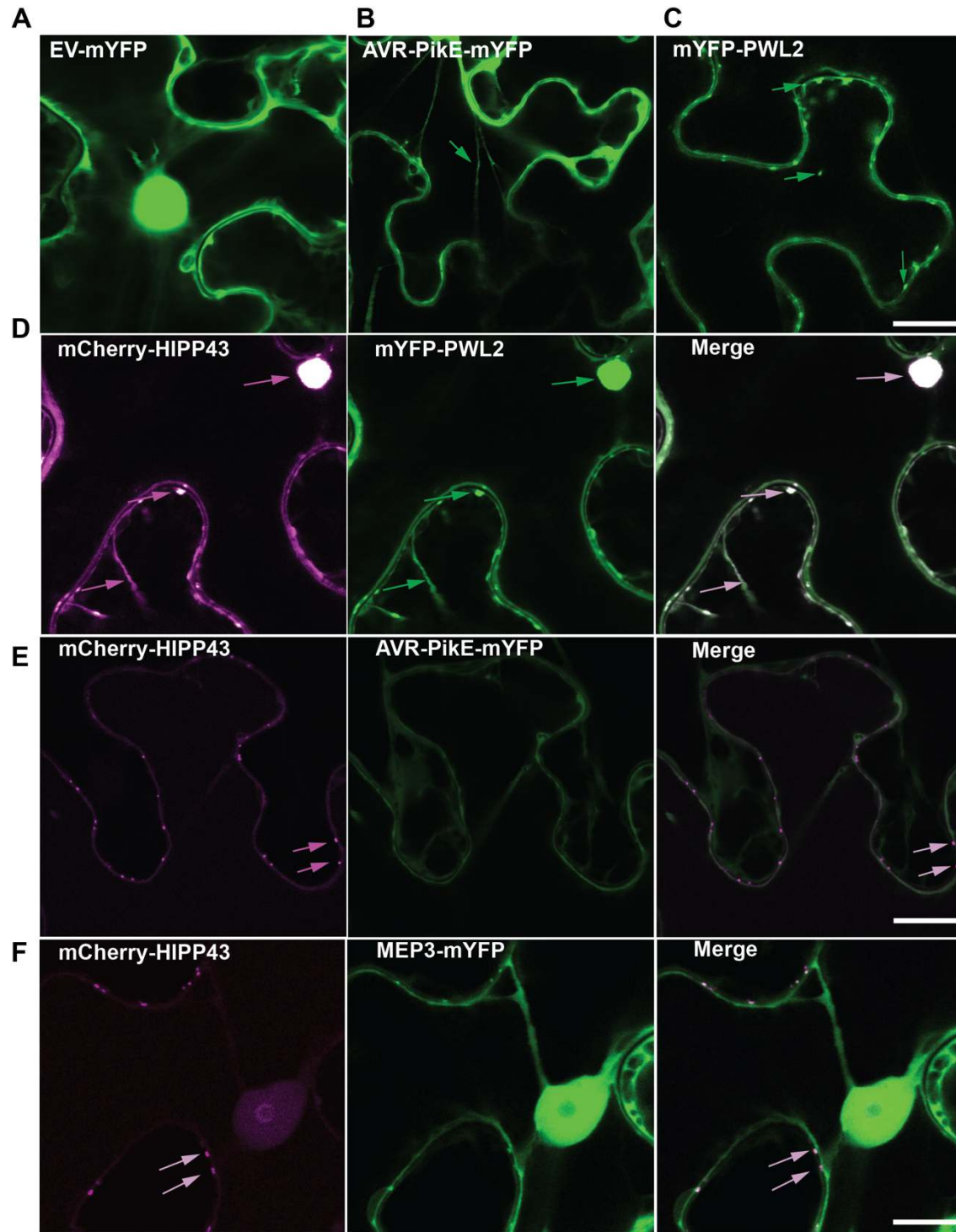


Fig. S10. Pwl2 promotes HIPP43 cytoplasmic localisation.

(A-C) Micrographs showing localization of free-YFP (A), AVR-PikeE-YFP (B) and YFP-Pwl2 (C). Both free-YFP and AVR-PikeE-YFP show localisation in the cytoplasm while YFP-Pwl2 showed localisation in cytoplasm and cytoplasmic mobile structures. (D-F) HIPP43 localisation in presence of AVR-PikeE and Mep3 is unaffected but altered in the presence of Pwl2. (D) Micrographs showing that the presence of YFP-Pwl2, alters m-Cherry-HIPP43 localization, where it was observed to translocate to the cytoplasm and cytoplasmic vesicular structures. Arrows indicated cytoplasmic localization and cytoplasmic mobile bodies (E+F)

967 Micrographs showing HIPP43 is co-localisation with two different MAX-fold effectors AVR-
 968 Pike and Mep3. mCherry-HIPP43 remains in the PD in the presence of cytoplasmically
 969 localized AVR-Pike and Mep3. Arrows indicated PD. Scale bars represent 20 μ m.
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Table S1 Primers used in this study.

Primer	Sequence
PWL2ORF	ATGAAATGCAACAACATCATCCTCCC
PWL2ORR	ACATAATATTGCAGCCCTCTTCTCGC
PWL2f	GGTTCTTATTATGGTCCCGGGTGA
PWL2r	GGTTCTTATTATGGTCCCGGGTGA
PWL2848F	CGCGGTGGCGGCCGCTCTAGAGACCGGGCACGAACCCGG CAGGC
PWL2-R	CATAATATTGCAGCCCTCTT
PWL2_GFPF	GGGCTGCAATATTATGATGGTGAGCAAGGGCGAGGAGCT G
KOLFF	ACAACGCGGTGTAAAGATTCGGGT
KOLFR	GTCGTGACTGGGAAAACCCTGGCGAGACCAAAGCAAAAG GGAGGATGA
KORFF	TCCTGTGTGAAATTGTTATCCGCTGCGAGAAGAGGGCTGC AATATTA
PG514-myc-F	AACACGGGGGACTCTAGAGAACAAAAGTTGATCTCTGA
YFPPWL2-F	GACGAGCTGTACAAGGGTGGCGGGTGGACTAAC
PWL2-N-term- YFPR	GTTAGTCCACCCGCCACCCTTGTACAGCTCGTC
PWL2-R-p514 HAPwl2F	GAACGATCGGGGAAATTCGTTACATAATATTGCAGCCCT AGAGAACACGGGGGACTCTAGAATGGGTGGCGGGTGGAC TAAC
PG514-myc-F	AACACGGGGGACTCTAGAGAACAAAAGTTGATCTCTGA
myc-HIPP43-R	TCCAAGACGCCCATAAGGTCCTCTTCAGAAA
myc-HIPP43F	ATTTCTGAAGAGGACCTTATGGGCGTCTTGGA
HIPP43-NOS-R	ACGATCGGGGAAATTCGTCACATGATGGTGCAG
PG514-mCherry-F	AACACGGGGGACTCTAGAATGGTGAGCAAGGGC
mCherry-HIPP43F	GGACGAGCTGTACAAGATGGGCGTCTTGGA
mCherry-HIPP43R	TCCAAGACGCCCATCTTGTACAGCTCGTCC
KT7_HIPP34F	CATGGAGGCCGAATTCATGGGCGTCTTGGA
KT7_HIPP34R	GCAGGTCGACGGATCCTCACATGATGGTGCAG

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