

Unconventional secretion of *Magnaporthe oryzae* effectors in rice cells is regulated by tRNA modification and codon usage control

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Microbial pathogens deploy effector proteins to manipulate host cell innate immunity, often using poorly understood unconventional secretion routes. Transfer RNA (tRNA) anticodon modifications are universal, but few biological functions are known. Here, in the rice blast fungus *Magnaporthe oryzae*, we show how unconventional effector secretion depends on tRNA modification and codon usage. We characterized the *M. oryzae* Uba4–Urm1 sulfur relay system mediating tRNA anticodon wobble uridine 2-thiolation (s^2U_{34}), a conserved modification required for efficient decoding of AA-ending cognate codons. Loss of s^2U_{34} abolished the translation of AA-ending codon-rich messenger RNAs encoding unconventionally secreted cytoplasmic effectors, but mRNAs encoding endoplasmic reticulum–Golgi-secreted apoplastic effectors were unaffected. Increasing near-cognate tRNA acceptance, or synonymous AA- to AG-ending codon changes in *PWL2*, remediated cytoplasmic effector production in $\Delta uba4$. In *UBA4*⁺, expressing recoded *PWL2* caused Pwl2 super-secretion that destabilized the host–fungus interface. Thus, U_{34} thiolation and codon usage tune pathogen unconventional effector secretion in host rice cells.

Transfer RNA nucleoside modifications are abundant and occur across all domains of life^{1–5}, but biological relevance for many is poorly understood^{3,4,6–9}. The genome of the devastating rice blast fungus *Magnaporthe oryzae*^{10–14} carries a *UBA4* orthologue (MGG_05569), encoding the evolutionary conserved E1-like Urm1-activating enzyme, and MGG_03978, encoding the Uba4 substrate ubiquitin related modifier 1 (Urm1). In eukaryotes, Uba4 and Urm1 act together to modify the tRNA wobble uridine-34 anticodon base with sulfur to tune messenger RNA translation¹⁵ (Fig. 1a). tRNA thiolation is a universal modification, but it is not required for general translation and is dispensable in yeast⁴, although its loss affects proteome integrity, metabolic homeostasis and stress responses^{3,7,9}. We seek to understand *M. oryzae* metabolism and growth during rice infection and hypothesized that the *M. oryzae* Uba4–Urm1 sulfur relay system contributed to fungal colonization of the host rice cell.

The Uba4–Urm1 sulfur relay system is required for 2-thiolation of position 34 wobble uridines (s^2U_{34}) in the anticodon loop of cytoplasmic (but not mitochondrial) tRNAs^{6,15,16} (Fig. 1a). s^2U_{34} is a universally conserved modification on tRNAs decoding AA-ending cognate codons (i.e., codons carrying adenine in the second and wobble third positions)^{1–3,7,17} (Fig. 1b). A methoxycarbonylmethyl group (mcm)⁵ also decorates U_{34} , and the loss of both modifications is lethal¹. U_{34} thiolation involves the transfer of sulfur from the ubiquitin-like Urm1 following its activation by Uba4^{7,15,16,18} (Fig. 1a). The loss of U_{34} thiolation⁷ slows cognate codon decoding at AA-ending codons, causing ribosome pausing and resulting in reduced translation speeds and increased protein aggregation^{3,6–8,17}. Despite its ubiquity, however, translation defects in s^2U_{34} mutants are minor^{3,9}, and few physiological roles for the tRNA anticodon wobble U_{34} thiolation modification are known^{4,6,7,9,17}.

To test our hypothesis that the Uba4–Urm1-mediated 2-thiolation modification of wobble uridines in *M. oryzae* tRNA anticodons

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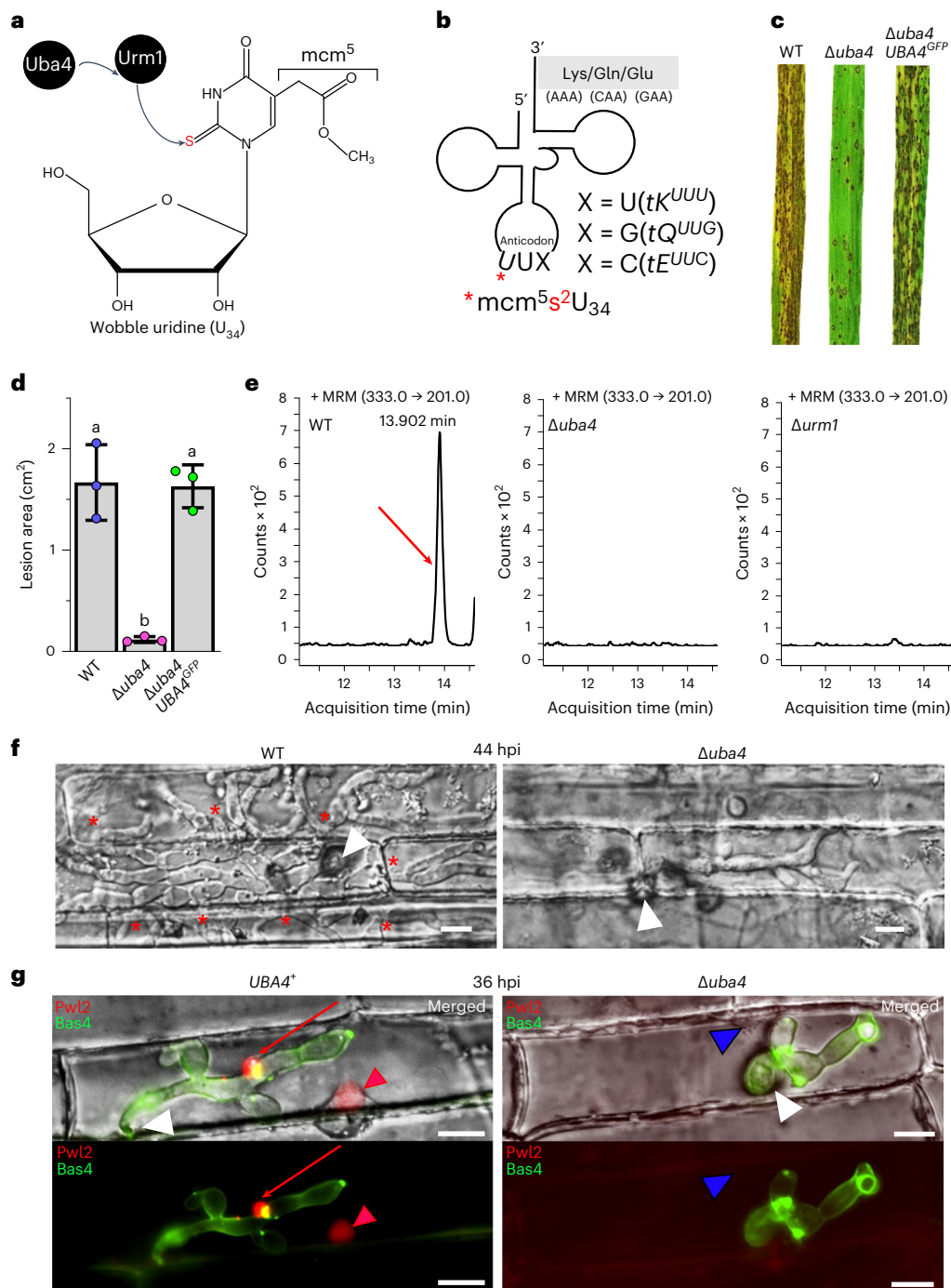


Fig. 1 | tRNA anticodon wobble uridine 2-thiolation is required for fungal pathogenicity. a, b, 2-Thiolation of tRNA anticodon wobble U₃₄ is mediated by the conserved Uba4–Urm1 sulfur relay system⁷ (**a**) and is a universal modification at tRNA anticodons corresponding to AA-ending codons (**b**). **c,** The *M. oryzae* UBA4 gene is required for pathogenicity on leaves of the susceptible rice cultivar CO-39. Spores of the indicated strains were sprayed on 3-week-old seedlings at a rate of 1×10^5 spores per ml. Images were taken at 5 dpi and are representative of at least 5 leaves from each of 3 independent biological replicates. **d,** Quantification of rice leaf lesions after 5 dpi following infection with the indicated strains. Disease lesion areas were determined from 10 cm leaf sections using ImageJ and analysed according to one-way ANOVA followed by Tukey's HSD test for multiple comparison ($P < 0.05$). Values are means $\pm$ s.d. calculated from three biological replicates. Different letters above bars indicate significant differences. $P = 0.0006$ between WT and $\Delta uba4$, $P = 0.9808$ between WT and $\Delta uba4$ UBA4^{GFP}, $P = 0.0007$ between $\Delta uba4$ and $\Delta uba4$ UBA4^{GFP}. **e,** Mass chromatograms of the mcm⁵s²U tRNA modification in vegetative mycelia of

the indicated strains. The y axes are ion counts; the x axes are acquisition times. The mcm⁵s²U modification peak position is indicated by a red arrow. **f,** Live-cell imaging at 44 hpi of detached rice leaf sheath epidermal cells colonized with the indicated strains. White arrowheads indicate penetration sites; red asterisks indicate where IH have spread into neighbouring cells. Scale bars, 10 μ m. **g,** Live-cell imaging at 36 hpi of detached rice leaf sheath epidermal cells colonized with the indicated strains carrying genes encoding the fluorescent cytoplasmic effector Pwl2-mCherry:NLS and the fluorescent apoplastic effector Bas4-GFP. At this time point, 100% of WT BICs carry Pwl2-mCherry:NLS, but Pwl2-mCherry:NLS fluorescence was not observed in any $\Delta uba4$ -infected cells. Calculations and representative images are based on the observations of 50 infected rice cells per leaf sheath per treatment; $n = 3$ biological replicates. White arrowheads indicate penetration sites; red arrows indicate Pwl2 in BICs; red arrowheads indicate Pwl2 in host nuclei; blue arrowheads indicate host nuclei without Pwl2. Scale bars, 10 μ m.

contributes to host plant infection, we functionally characterized the *M. oryzae* Uba4–Urm1 sulfur relay system. In doing so, we discovered a previously unknown connection between tRNA wobble U₃₄ thiolation-dependent decoding of AA-ending cognate codons, codon usage frequency and the unconventional secretion of *M. oryzae* cytoplasmic effector proteins required for virulence.

Results

Rice infection and U₃₄ thiolation requires *UBA4* and *URM1*

To determine whether *M. oryzae* tRNA thiolation was required for rice infection, we first disrupted *UBA4* in the genome of our wild-type (WT) isolate Guy11 by homologous recombination using *ILV1* as a selectable marker conferring sulphonyl urea resistance¹⁹. Three independent *Δuba4*-carrying mutant strains were recovered, and each had identical phenotypes (Extended Data Fig. 1a–e). One deletant was used to generate the *Δuba4* *UBA4*-GFP complementation strain. On media plates, *Δuba4* radial growth and sporulation rates were significantly ($P \leq 0.05$) reduced compared to WT (Extended Data Fig. 1a,b). Reduced radial growth of *M. oryzae* *Δuba4* mutant strains is consistent with observations in yeast, where tRNA thiolation is required for optimal nutrient sensing and metabolic homeostasis in a biochemical manner independent of protein translation rates⁹. When equal numbers of *M. oryzae* spores were applied to 3-week-old rice seedlings of the susceptible cultivar CO-39, infection with WT and the complementation strain resulted in typical expanded necrotic lesions on leaves by 5 days post inoculation (dpi), but the *Δuba4* mutant strain produced only pinpoint lesions²⁰ and was thus non-pathogenic (Fig. 1c, quantified in Fig. 1d). Liquid chromatography–mass spectrometry (LC–MS) analysis of tRNAs from vegetative mycelia showed that, consistent with yeast mutants¹⁶, both the *M. oryzae* *Δuba4* mutant strain and an *Δurm1* deletant that we also generated in Guy11, and which was physiologically indistinguishable from *Δuba4* (Extended Data Fig. 1f–k), were abolished for the mcm⁵S²U tRNA modification (Fig. 1e; raw values with replicates in Supplementary Table 1) but not for the mcm⁵U tRNA modification (Supplementary Table 1), compared to WT. Like in yeast, *M. oryzae* Uba4-GFP localized to the fungal cytoplasm (Extended Data Fig. 2). Taken together, we conclude that *M. oryzae* *UBA4* and *URM1* are required for optimal axenic growth and sporulation, rice plant infection and cytoplasmic tRNA anticodon U₃₄ thiolation.

Following adhesion to host leaf surfaces, *M. oryzae* spores germinate, and each form specialized dome-shaped infection structures (appressoria); appressoria penetrate the host cuticle under enormous turgor at 24 h post inoculation (hpi)^{21,22} and elaborate bulbous, branching invasive hyphae (IH) that fill the first infected living rice cell by 44–48 hpi before migrating to neighbouring cells²³. Live-cell imaging of optically clear detached rice leaf sheath epidermal cells showed that *UBA4* was not required for leaf cuticle penetration by appressoria (Extended Data Fig. 1c,d) or for initial IH elaboration after penetration (Fig. 1f) but was essential for filling the first infected rice epidermal cell with IH and for extensive IH spread to neighbouring cells (Fig. 1f and Extended Data Fig. 1e). Similar results were obtained for *Δurm1* (Extended Data Fig. 1i–k). Thus, *UBA4* and *URM1* are required for extended *M. oryzae* biotrophic growth in living host rice cells.

Loss of *UBA4* abolishes Pwl2 effector accumulation in the biotrophic interfacial complex

During the biotrophic growth phase of rice cell infection, *M. oryzae* IH are separated from host cytoplasm by an extended interfacial zone comprising host-derived membranes^{24,25}. To better understand the role of *UBA4* in biotrophy, we hypothesized that the biotrophic interfacial compartments separating IH from plant host cytoplasm would be perturbed in *Δuba4* compared to WT, as was observed previously for another biotrophic growth mutant²⁵. WT IH are wrapped in the plant host-derived extra-invasive hyphal membrane^{14,23}, forming a matrix into which apoplastic effectors like Bas4 are secreted via the

conventional ER–Golgi pathway. A focal, plant lipid-rich membrane structure, the biotrophic interfacial complex (BIC), forms an additional compartment, outside IH, in the first infected rice cell and at the tips of IH spreading to adjacent cells^{14,26}. Cytoplasmic effectors like Pwl2 (which confers host species specificity) and AVR-Pita (which confers avirulence on rice lines carrying the corresponding *Pita* R gene) are secreted into the BIC by an unconventional protein secretion pathway before being translocated into the host cell²⁴. Effector genes are only expressed in planta, and therefore fluorescently labelled effectors are cellular probes to monitor both effector deployment and biotrophic interfacial membrane integrity²⁵. Using the pBV591 vector²⁶, we generated a *Δuba4* mutant strain producing, under native promoters, Pwl2 fused to mCherry (which also carries an added C-terminal nuclear localization signal (NLS) to concentrate Pwl2 in the rice nucleus and thus confirm secretion into the host cell²⁴) and Bas4 fused to green fluorescent protein (GFP). Like our previously generated *PWL2*-mCherry:NLS and *BAS4*-GFP-expressing control strain²⁷, we found that at 36 hpi, Bas4-GFP correctly outlined *Δuba4* IH (Fig. 1g) indicating, contrary to our expectations, that the extra-invasive hyphal membrane was not eroded in *Δuba4* and the apoplast was intact. However, whereas Pwl2-mCherry:NLS accumulated in *UBA4*⁺ control BICs and in the nuclei of *UBA4*⁺-infected rice cells by 36 hpi (Fig. 1g), in contrast, Pwl2-mCherry:NLS was not visible in *Δuba4* IH or associated rice nuclei (Fig. 1g). Together, these initial results suggested that *UBA4* was required for Pwl2 production and/or secretion and accumulation in the BIC, and also possibly for BIC integrity, but was not required for Bas4 production and secretion or for apoplastic space integrity.

PWL2 mRNA translation requires U₃₄ thiolation

We initially considered that the loss of Pwl2 secretion and accumulation in *Δuba4* BICs indicated BIC integrity (but not apoplastic integrity) was disrupted in *Δuba4*. However, closer inspection revealed *Δuba4* BICs by bright-field microscopy and, like for *UBA4*⁺ (and as reported by others), Bas4-GFP visibly accumulated under *Δuba4* BICs (Fig. 2a). Thus, *UBA4* is not required for BIC formation but is required for Pwl2-mCherry:NLS accumulation in BICs. Similarly, a strain expressing Pwl2-GFP and Bas4-mCherry:NLS, constructed to ensure that the defect in Pwl2 BIC accumulation in *Δuba4* was not due to the fluorescent tag, showed Bas4-mCherry:NLS was secreted into the *Δuba4* apoplast and accumulated under the BIC, but Pwl2-GFP was not detected in any *Δuba4*-infected cells (Extended Data Fig. 3). To understand why Pwl2 did not accumulate in *Δuba4* BICs, we considered that in yeast, tRNA anticodon wobble U₃₄ thiolation by Uba4 prevents ribosome pausing and protein aggregation by improving the efficiency of AA-ending cognate codon decoding^{7,17}. We thus hypothesized that the loss of Pwl2 secretion and accumulation in *Δuba4* BICs indicated *PWL2*-mCherry:NLS mRNA (but not *BAS4*-GFP mRNA) was translationally impaired in planta in the *M. oryzae* *Δuba4* mutant strain due to ribosome pausing at AA-ending codons. In yeast, paromomycin treatment mitigates ribosomal pausing at AA-ending codons in U₃₄ thiolation deficient mutant strains by increasing near-cognate tRNA acceptance⁷. We infused leaf sheaths with 1 mg ml^{−1} paromomycin in 0.2% gelatin at 36 hpi and observed the effects at 44 hpi. In the control *UBA4*⁺ strain, *PWL2*-mCherry:NLS mRNA translation and *BAS4*-GFP mRNA translation were not affected by paromomycin treatment, and both Pwl2-mCherry:NLS and Bas4-GFP were correctly deployed in IH, although, consistent with its role as a protein synthesis inhibitor, *UBA4*⁺ IH growth was severely attenuated compared to the untreated control (Extended Data Fig. 4). In *Δuba4* IH, paromomycin treatment restored Pwl2-mCherry:NLS secretion and accumulation in BICs (Fig. 2b). Furthermore, in the *Δurm1* mutant strain (which closely resembled *Δuba4*; Extended Data Fig. 1f–k), we also observed loss of Pwl2-mCherry:NLS accumulation in the *Δurm1* BIC, and this was similarly (but weakly) remediated by paromomycin treatment (Fig. 2b). Concordant with yeast findings⁷, remediation of Pwl2-mCherry:NLS production by paromomycin suggests that

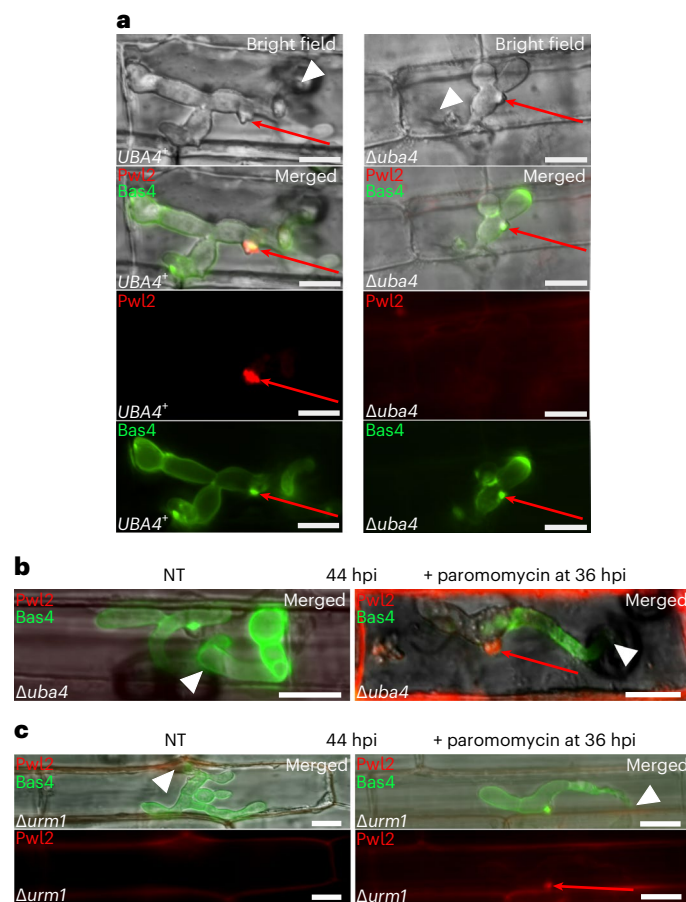


Fig. 2 | The tRNA thiolation modification is required for *PWL2* (but not *BAS4*) mRNA translation. **a**, The *Δuba4* mutant strain is never observed accumulating PwL2 in BICs. Live-cell images of detached rice leaf sheath epidermal cells were taken at 36 hpi. White arrowheads indicate penetration sites; red arrows indicate visible BICs. Scale bars, 10 μ m. **b, c**, Paromomycin treatment of detached rice leaf sheath epidermal cells colonized with *Δuba4* (**b**) or *Δurm1* (**c**) mutant strains at 36 hpi restored PwL2 translation and secretion into the BIC by 44 hpi in 100% of treated cells. PwL2-mCherry fluorescence was weak following paromomycin treatment, and the required increase in signal intensity revealed autofluorescence in host rice cell walls. White arrowheads indicate penetration sites; red arrows indicate visible BICs. Scale bars, 10 μ m. NT, no treatment. In **a–c**, calculations and representative images are based on the observations of 50 infected rice cells per leaf sheath per treatment, repeated in triplicate.

ribosomal pausing is occurring in both the *Δuba4* and *Δurm1* mutant strains, likely due to inefficient decoding of AA-ending cognate codons, leading to the loss of *PWL2-mCherry:NLS* mRNA translation. We conclude that the *M. oryzae* Uba4–Urm1 sulfur relay system is required for s^2U_{34} tRNA modification-dependent efficient codon decoding of *PWL2-mCherry:NLS* mRNA (but not *BAS4-GFP* mRNA) in planta.

AVR-Pita secretion requires U_{34} thiolation

We next asked whether tRNA thiolation was only required for *PWL2* mRNA translation or whether it was required for other unconventionally secreted cytoplasmic effectors that accumulate in the BIC, of which few are experimentally determined, including AVR-Pita²⁶. To address this, we generated *Δuba4* and *UBA4*⁺ strains expressing *BAS4-GFP* along with *AVR-Pita-mCherry:NLS* expressed under its native promoter. Figure 3 shows that AVR-Pita-mCherry:NLS weakly accumulated in *UBA4*⁺ BICs compared to PwL2-mCherry:NLS, as previously described²⁶. However, no AVR-Pita-mCherry:NLS protein accumulation was observed in *Δuba4* BICs unless *Δuba4*-infected rice cells were treated with paromomycin

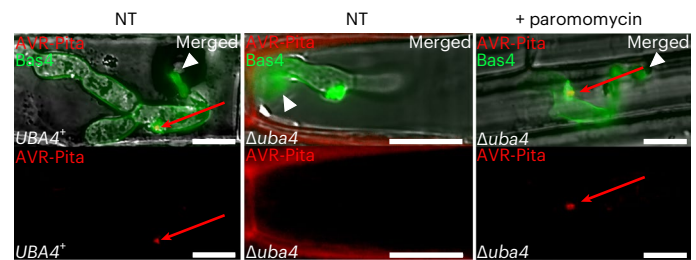


Fig. 3 | Translation of the unconventionally secreted AVR-Pita effector requires tRNA thiolation. Live cell imaging at 44 hpi of detached rice leaf sheath epidermal cells shows that in 100% of observed cells, AVR-Pita was secreted into the *UBA4*⁺ BIC, but in the *Δuba4* strain, the accumulation of AVR-Pita in BICs was abolished unless cells were treated with paromomycin at 36 hpi. Calculations and representative images are based on the observations of 50 infected rice cells per leaf sheath per treatment, repeated in triplicate. White arrowheads indicate penetration sites; red arrows indicate AVR-Pita in BICs. Scale bars, 10 μ m. For the *Δuba4* NT panel, the red channel signal intensity was increased to confirm that AVR-Pita was not accumulating in the BIC, revealing autofluorescence in the host rice cell wall.

(Fig. 3). In contrast, the experimentally determined apoplastic effector Slp1²⁸ was, like Bas4, successfully produced and secreted into the apoplast by a *Δuba4* strain expressing *SLP-GFP* under its native promoter (Extended Data Fig. 5). Therefore, Uba4–Urm1-dependent tRNA wobble U_{34} thiolation is required for the translation of mRNAs encoding at least two unconventionally secreted cytoplasmic effectors but not for the translation of mRNAs encoding at least two ER–Golgi secreted apoplastic effectors.

Synonymous codon recoding remedies *PWL2* mRNA translation

To confirm that the loss of *PWL2* and *AVR-Pita* (but not *BAS4* or *SLP1*) mRNA translation was due to inefficient decoding of AA-ending cognate codons following the loss of tRNA thiolation, we reasoned that recoding AA-ending codons to synonymous AG-ending codons carrying guanine in the third position (which do not require the s^2U_{34} modification for decoding^{3,17}) would remediate cytoplasmic effector mRNA translation and unconventional secretion in the absence of tRNA wobble U_{34} thiolation. To test this, we generated a *Δuba4* strain expressing *BAS4-GFP* along with a recoded form of *PWL2-mCherry:NLS* (hereafter termed *PWL2* (AA $\rightarrow$ AG)) where all AA-ending codons were recoded to synonymous AG-ending codons (Fig. 4a and Extended Data Fig. 6). Expressing *PWL2* (AA $\rightarrow$ AG) in *Δuba4* fully remediated PwL2 production and its secretion and accumulation in the *Δuba4* BIC (Fig. 4b). In some cases, expressing *PWL2* (AA $\rightarrow$ AG) resulted in large and/or unstable *Δuba4* BICs (Extended Data Fig. 7). We conclude that during host infection, because *PWL2* (AA $\rightarrow$ AG) mRNA is under the same promoter and encodes the same amino acid sequence as *PWL2-mCherry:NLS*, then the translation of native *PWL2* mRNA (encoding an unconventionally secreted effector) must require tRNA wobble U_{34} thiolation-dependent efficient decoding of AA-ending cognate codons.

BAS4 translation is unaffected by AA-ending codon recoding

To better understand why *BAS4* but not *PWL2* mRNAs are translated in *Δuba4*, we next expressed in *Δuba4* a version of *BAS4-GFP* where all AG-ending codons were recoded to synonymous AA-ending codons (Extended Data Fig. 8a), hereafter *BAS4* (AG $\rightarrow$ AA), along with *PWL2* (AA $\rightarrow$ AG) as a positive control for protein translation, with the expectation that *BAS4* (AG $\rightarrow$ AA) mRNAs would not be translated in *Δuba4*. However, in contrast to native *PWL2* mRNA, which was not translated in *Δuba4* unless all AA-ending codons were recoded to synonymous AG-ending codons, the recoding of all AG-ending codons in *BAS4-GFP*

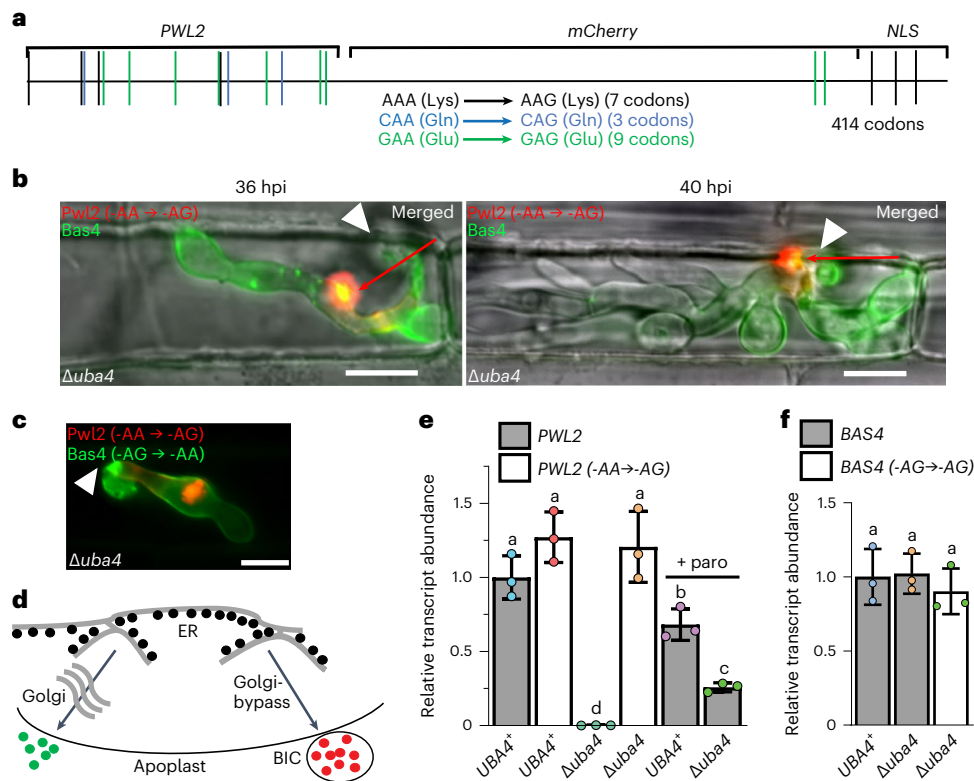


Fig. 4 | Unconventional protein secretion but not conventional ER–Golgi secretion in host cells is AA-ending codon decoding-dependent. **a**, Synonymous codon changes in *PWL2-mCherry:NLS* to yield *PWL2(-AA→-AG)*. **b**, Live-cell imaging at 36 hpi and 40 hpi of detached rice leaf sheath epidermal cells infected with *Duba4* shows *PWL2* mRNA translation was remediated in 100% of infected cells by synonymous codon recoding. **c**, In 100% of observed cells, synonymous AG-ending to AA-ending codon changes in *BAS4-GFP* did not affect *Bas4-GFP* secretion in *Duba4*. Images were taken at 36 hpi. In **b** and **c**, white arrowheads indicate penetration sites; red arrows indicate BICs. Scale bars, 10 μ m. Calculations and representative images are based on the observations of 50 infected rice cells per leaf sheath per treatment, repeated in triplicate. **d**, Unconventionally secreted effectors requiring tRNA wobble U_{34} thiolation carry signal peptides and are likely secreted through the Golgi-bypass pathway²⁹. **e**, Using *mCherry*-specific primers, qPCR analysis of cDNAs obtained at 42 hpi from detached rice leaf sheaths infected with the indicated strains shows that *PWL2*

mRNA abundance is codon decoding-dependent. Paro is treatment with 1 mg ml⁻¹ paromomycin in 0.2% gelatin. Values are the means $\pm$ s.d.; $n = 3$ biological replicates. Different letters above bars indicate significant differences at $P \leq 0.05$ (Student–Newman–Keuls test). **f**, Using *GFP*-specific primers, qPCR analysis of cDNAs obtained at 42 hpi from detached rice leaf sheaths infected with the indicated strains shows that *BAS4* mRNA abundance is not codon decoding-dependent. Values are the means $\pm$ s.d.; $n = 3$ biological replicates. Same letters above bars indicate no significant differences at $P \leq 0.05$ (Tukey’s HSD of one-way ANOVA). In **e** and **f**, values were calculated for each strain by normalizing the expression of *PWL2-mCherry:NLS*, *PWL2(-AA→-AG)*, *BAS4-GFP* and *BAS4(-AG→-AA)* against the *M. oryzae* actin-encoding gene *MoACT*, then normalizing these values against either the relative transcript abundance of *PWL2-mCherry:NLS* in untreated *UBA4*⁺ for *PWL2-mCherry:NLS* and *PWL2(-AA→-AG)* or against *BAS4-GFP* in *UBA4*⁺ for *BAS4-GFP* and *BAS4(-AG→-AA)*. Exact *P* values are given in Source Data Extended Data Fig. 4.

to synonymous AA-ending codons did not, as hypothesized, abolish *Bas4* secretion into the $\Delta uba4$ apoplast (Fig. 4c; more examples shown in Extended Data Fig. 8b). Thus, the s^2U_{34} tRNA modification is not required for translating conventional ER–Golgi-secreted *BAS4(-AG→-AA)* mRNAs, although we cannot rule out that some changes to translation speed may occur. In *M. oryzae*, both apoplastic and cytoplasmic effectors carry signal peptide sequences targeting them for synthesis on rough ER, but cytoplasmic effectors are then likely secreted via the type IV unconventional secretion pathway, which bypasses the Golgi apparatus²⁹ (Fig. 4d). Our results suggest that the s^2U_{34} tRNA modification and efficient AA-ending codon decoding is required for the folding of newly synthesized peptides in the type IV Golgi-bypass pathway but not in the ER–Golgi network.

***PWL2* mRNA levels are AA-ending codon decoding-dependent**

In addition to translational differences between *PWL2* and *BAS4* mRNAs, differences in transcript stability were gleaned from quantitative real-time PCR (qPCR). Using *mCherry*-specific primers, we found that, compared to *PWL2-mCherry:NLS* expression in *UBA4*⁺ IH, *PWL2-mCherry:NLS* transcripts were barely detected in $\Delta uba4$ IH.

However, recoded *PWL2(-AA→-AG)* expression levels in both *UBA4*⁺ and $\Delta uba4$ IH were indistinguishable from native *PWL2* expression levels in *UBA4*⁺, while paromomycin treatment partially remediated *PWL2-mCherry:NLS* expression levels in $\Delta uba4$ (Fig. 4e). In contrast, using *GFP*-specific primers, we found that *BAS4-GFP* expression levels, including of the recoded *BAS4(-AG→-AA)* version, were not affected in $\Delta uba4$ IH compared to *UBA4*⁺ (Fig. 4f). Thus, *PWL2* mRNA stability but not *BAS4* mRNA stability in *M. oryzae* IH is dependent on the efficient decoding of AA-ending cognate codons by s^2U_{34} -modified tRNAs.

Cytoplasmic effector mRNAs are enriched in AA-ending codons

To better understand the dependency of *PWL2* and *AVR-Pita* mRNA translation (but not *BAS4* or *SLP1* mRNA translation) on *Uba4*-dependent tRNA wobble U_{34} thiolation, we next asked whether AA-ending codon usage was biased or unbiased in cytoplasmic effector mRNAs compared to apoplastic effector mRNAs. To address this, we analysed AA- and synonymous AG-ending codon usage in all *M. oryzae* protein coding sequences as well as in *PWL2*, *AVR-Pita*, *BAS4* and *SLP1* mRNAs (Extended Data Fig. 9a). We also examined mRNAs encoding proteins

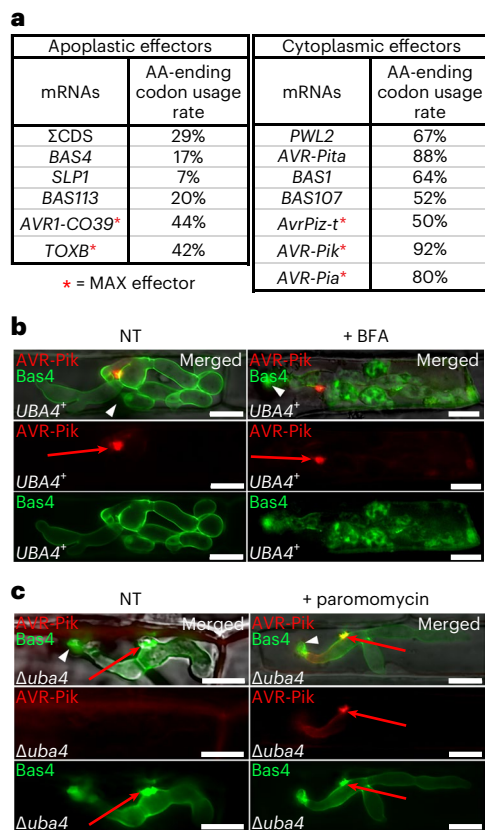


Fig. 5 | Unconventionally secreted cytoplasmic effector mRNAs are AA-ending codon-rich. **a**, Cytoplasmic effector mRNAs are enriched for AA-ending codons relative to synonymous AG-ending codons, whereas apoplastic effector mRNA codon usage, and codon usage from across all 12,936 protein coding sequences in the *M. oryzae* genome (ΣCDS), is biased towards AG-ending codons. mRNA AA-ending codon usage rates are calculated using the codon counts in Extended Data Fig. 9a–c and are given as the fraction of AA-ending codons as a percentage of the total number of AA- and synonymous AG-ending codons. **b**, Live-cell imaging of a *UBA4*⁺ strain expressing *AVR-PIK-mCherry:NLS* and *Bas4-GFP* treated with BFA, which inhibits conventional ER–Golgi secretion, shows that AVR-Pik is secreted into the BIC via the unconventional protein secretion pathway. Infected detached rice leaf sheath epidermal cells were treated at 36 hpi with 50 μg ml^{−1} BFA for 4 h before observation. Blocked ER–Golgi secretion in BFA-treated samples resulted in Bas4 retention in IH, but AVR-Pik secretion into BICs was unaffected. **c**, Live-cell imaging of detached rice leaf sheath epidermal cells infected with a *Δuba4* strain expressing *AVR-PIK-mCherry:NLS* and *Bas4-GFP* shows that AVR-Pik is produced and secreted into the BIC in a Uba4-mediated, codon decoding-dependent manner. AVR-Pik was not detected in any untreated *Δuba4*-infected rice cells. Infected cells treated with 1 mg ml^{−1} paromomycin at 36 hpi and viewed at 44 hpi showed remediated AVR-Pik translation and secretion into the *Δuba4* BIC in all observed cells. In **b** and **c**, white arrowheads indicate penetration sites; red arrows indicate BICs. Scale bars, 10 μm. Calculations and representative images are based on the observations of 50 infected rice cells per leaf sheath per treatment, repeated in triplicate.

with stringent evidence for BIC accumulation (AvrPiz-t, Bas1 and Bas107)^{24,26,30}, confirmed brefeldin A (BFA)-insensitive unconventional secretion (Bas1 and Bas107)²⁴ and confirmed BFA-sensitive conventional ER–Golgi secretion (Bas113)²⁴ (Extended Data Fig. 9b). By calculating the AA-ending codon usage rate (that is, the fraction of AA-ending codons as a percentage of the total number of AA- and synonymous AG-ending codons), we found that cytoplasmic effector mRNAs were enriched for AA-ending codons relative to synonymous AG-ending codons whereas in contrast, apoplastic effector mRNA codon usage,

like codon usage across all *M. oryzae* protein coding sequences, was biased against AA-ending codons in favour of AG-ending codons (Fig. 5a; underlying codon data in Extended Data Fig. 9a–c). This suggests cytoplasmic effector mRNA codon usage is under selection for AA-ending codon enrichment while apoplastic effector mRNA codon usage, like genome-wide codon usage, is not.

AvrPiz-t is a MAX (*Magnaporthe oryzae* avirulence and ToxB-like) effector, a class of sequence-unrelated but structurally conserved fungal effectors that also includes AVR-Pik, Avr-Pia and Avr1-CO39 from *M. oryzae* and ToxB from the wheat pathogen *Pyrenophora tritici-repentis*^{31–33} (Extended Data Fig. 9c). Transient expression studies in rice protoplasts³⁴ suggest AVR-Pik and AVR-Pia are cytoplasmic MAX effectors³², while *M. oryzae* AVR1-CO39 and *P. tritici-repentis* ToxB are apoplastic MAX effectors^{32,35}. Figure 5a shows that despite protein structural conservation, AA-ending codon usage rates are higher for AVR-Pik and AVR1-Pia mRNAs than for AVR1-CO39 mRNAs and *P. tritici-repentis* TOXB mRNAs. Thus, cytoplasmic MAX effector mRNAs are enriched for AA-ending codons, whereas apoplastic MAX effector mRNAs across species are depleted for AA-ending codons relative to synonymous AG-ending codons (Fig. 5a and Extended Data Fig. 9c).

Based on the 92% AA-ending codon usage rate for AVR-Pik mRNAs (Fig. 5a), we predicted that AVR-Pik was secreted into the BIC via the unconventional protein secretion pathway in a tRNA wobble U₃₄ thiolation-dependent manner. By expressing AVR-Pik-mCherry:NLS and BAS4-GFP, Fig. 5b shows how, in *UBA4*⁺, BFA treatment, which inhibits conventional ER-to-Golgi secretion, prevented Bas4 secretion (which is retained in IH) but did not affect AVR-Pik accumulation in the BIC, confirming AVR-Pik is unconventionally secreted. Figure 5c shows that in *Δuba4* mutant strains, AVR-Pik was not produced and secreted into the BIC unless infected cells were treated with paromomycin. Thus, as predicted from AA-ending codon usage rates, AVR-Pik is produced and secreted into the BIC via the unconventional protein secretion pathway in an AA-ending codon decoding-dependent manner.

Codon usage tunes Pwl2 secretion to foster BIC stability

We next asked, why would *M. oryzae* evolve AA-ending codons in *PWL2* mRNA—whose efficient decoding requires tRNA thiolation by the multi-step Uba4–Urm1 sulfur relay system—when a strain lacking *UBA4* can translate *PWL2* mRNAs carrying all AG-ending synonymous codons instead? To address this, we expressed *PWL2* (AA→AG), along with *BAS4-GFP*, in the *UBA4*⁺ WT strain. Figure 6a shows that, relative to the *PWL2-mCherry:NLS*-expressing control strain, the expression of *PWL2* (AA→AG) resulted in the formation of giant, brightly fluorescing BICs that were unstable and often either split or elongated rather than spherical. Note that Pwl2 (AA→AG) accumulated in rice nuclei, indicating that the protein was correctly folded and translocated into the host cell. BICs were also visualized by brightfield and were obviously increased in size in the *UBA4*⁺ strain expressing recoded *PWL2* (AA→AG) compared to the *UBA4*⁺ strain expressing native *PWL2* (Fig. 6a). Pwl2 (AA→AG) accumulated in expanded and fragmented BICs following BFA treatment, which blocks the secretion of apoplastic (but not cytoplasmic) effectors through the conventional ER–Golgi pathway²⁴, indicating recoded Pwl2 continued to be trafficked via the unconventional protein secretion pathway and did not spill into the ER–Golgi network (Extended Data Fig. 10). Considering *PWL2* (AA→AG) transcript abundance in *UBA4*⁺ was not significantly different to native *PWL2* mRNA levels (Fig. 4e), these data together suggest recoding AA-ending codons to synonymous AG-ending codons in the *UBA4*⁺ background leads to unregulated *PWL2* (AA→AG) mRNA translation resulting in Pwl2 (AA→AG) protein overproduction and super-secretion into the BIC. Inoculating susceptible rice plants with spores of the *UBA4*⁺ strain expressing *PWL2* (AA→AG) resulted in fewer and smaller disease lesions compared to leaves inoculated with the *UBA4*⁺ strain expressing native *PWL2* (Fig. 6b,c), indicating that giant, unstable BICs resulting from recoded Pwl2 super-secretion are

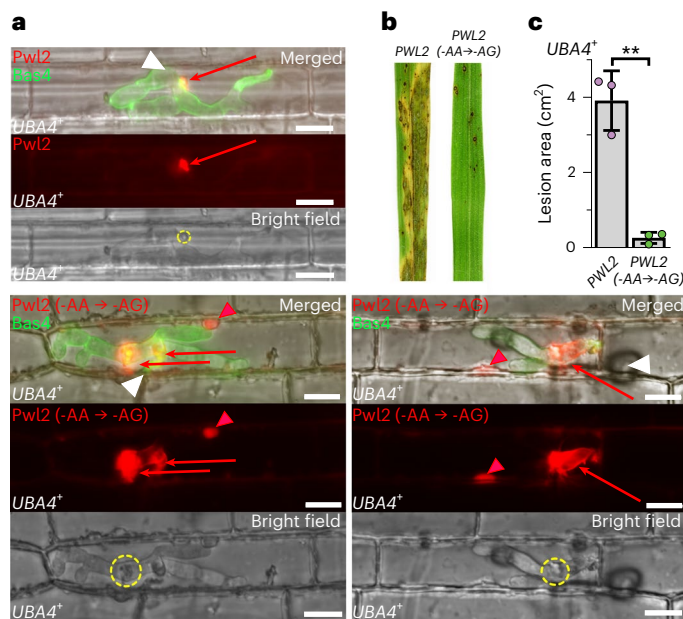


Fig. 6 | AA-ending codon usage tunes unconventional protein secretion in living host cells. a, Live-cell imaging at 38 hpi of detached rice leaf sheath epidermal cells infected with the indicated strains shows that, compared to the *PwL2-mCherry:NLS*-expressing *UBA4*⁺ control strain (top left), expressing *PwL2(-AA→-AG)* in *UBA4*⁺ resulted in recoded *PwL2* super-secretion into BICs, which are destabilized (two examples shown at bottom left and right). Hatched yellow circles indicate BIC sizes, which are expanded in IH expressing recoded *PwL2* compared to those expressing native *PwL2*. White arrowheads indicate penetration sites; red arrows indicate *PwL2* or recoded *PwL2* in BICs; red arrowheads indicate recoded *PwL2* in host nuclei. Scale bars, 10 μm. One hundred per cent of infected rice cells displayed the phenotypes shown. Representative images are based on the observations of 50 infected rice cells per leaf sheath per treatment, repeated in triplicate. **b**, Spores of *UBA4*⁺ expressing *BAS4-GFP* along with native *PwL2* (fused to *mCherry:NLS*) or recoded *PwL2(-AA→-AG)* were applied to 3-week-old seedlings of the susceptible rice cultivar CO-39 at a rate of 1×10^5 spores per ml. Leaves were imaged at 5 dpi. Images are representative of at least five leaves from three biological replicates. **c**, Sections of 10 cm length of infected leaves at 5 dpi were imaged, and the disease lesion areas were quantified using ImageJ. Values are the means $\pm$ s.d. of three biological replicates. Areas were analysed by the two-tailed unpaired *t*-test. **Statistically significant differences ($P < 0.05$). $P = 0.013$ between strains *UBA4*⁺ *PwL2-mCherry:NLS* *BAS4-GFP* and *UBA4*⁺ *PwL2(-AA→-AG)* *BAS4-GFP*.

detrimental to infection; thus, cytoplasmic effector codon usage may influence *M. oryzae* fitness in the field. We conclude that AA-ending codon usage in *PwL2* mRNA modulates translation to precisely tune *PwL2* production and optimize rates of unconventional protein secretion into the BIC. In doing so, tRNA wobble U_{34} thiolation and codon usage bias directly contribute to *M. oryzae* BIC integrity and facilitate biotrophic growth in living rice cells.

Discussion

To subvert host cell innate immunity, animal and plant microbial pathogens secrete effector proteins and virulence factors³⁶, often via unconventional pathways^{24,29,37,38} whose molecular underpinnings are only starting to emerge²⁹. tRNA wobble U_{34} thiolation is required for efficient decoding of AA-ending cognate codons to prevent transient ribosomal pausing, which affects protein folding⁷ and protein homeostasis¹⁷. However, U_{34} thiolation mutants have minor translational defects⁹, and specific biological roles for this universal modification are poorly understood. Here we describe a physiological role for tRNA U_{34} thiolation in tuning unconventional but not conventional

protein secretion and show how such tuning is essential for fungal virulence.

In our study, the loss of tRNA thiolation eliminated unconventional protein secretion due to ribosomal pausing at AA-ending codons, which prevented protein translation and secretion, whereas expressing recoded *PwL2* mRNA carrying all AG-ending codons caused unregulated *PwL2* mRNA translation and *PwL2* super-secretion that gave rise to giant, unstable BICs and the loss of fungal virulence. Thus, efficient AA-ending codon decoding by tRNA wobble s^2U_{34} anticodons tunes the translation of cytoplasmic effector mRNAs, which carry more AA-ending codons than apoplastic mRNAs, in order to control the rate of unconventional cytoplasmic effector secretion and maintain BIC stability. In contrast, ribosomal pausing on the relatively small number of AA-ending codons carried by native *BAS4* and *SLP1* mRNAs (2 and 1, respectively) did not prevent peptide synthesis, nor, counterintuitively, did changing all AG-ending codons to AA-ending codons in recoded *BAS4* (to give a total of 12 AA-ending codons in *BAS4(-AG→-AA)*, comparable to the 14 AA-ending codons in native *PwL2*). Why is efficient AA-ending codon decoding necessary for cytoplasmic but not apoplastic effector mRNA translation and protein secretion? Although counterintuitive, our results are consistent with findings in yeast, where the integrity of ER–Golgi proteins was less affected by changes in decoding speeds arising from the loss of the s^2U_{34} modification than cytosolic proteins due to different co-translational mechanisms and folding constraints across the ER membrane compared to the cytoplasm⁷. We suggest that similarly, our results showing the different outcomes of *PwL2* and *BAS4(-AG→-AA)* mRNA translation in $\Delta uba4$ may be consistent with the notion that proteins secreted unconventionally through the type IV Golgi-bypass pathway are subjected to different co-translational mechanisms and protein folding constraints compared to conventional ER–Golgi secreted proteins. Consequently, the unconventional protein secretion pathway (but not the conventional ER–Golgi network) is intolerant of ribosome pausing and changes to mRNA translation speeds resulting from inefficient AA-ending codon decoding, which may cause misfolding and elimination of newly synthesized peptides⁷. When considering that local translation elongation rates are tightly controlled by individual codons³⁹, and that intragenic codon frequency bias leads to variable ribosome decoding speeds along mRNAs to optimize co-translational protein folding and protein translocation across membranes⁴⁰, it is conceivable that unconventionally secreted effector protein mRNAs (but not apoplastic effector mRNAs) may evolve AA-ending codon-enriched mRNAs precisely in order for nascent peptides to be translated at speeds that optimize protein folding and secretion through the type IV Golgi-bypass pathway, which is the only known means of targeting effector proteins to BICs for entry into host rice cells. For sequence-unrelated but structurally conserved MAX effectors, this suggests cytoplasmic MAX effectors may be under dual selection for both sequence-unrelated structural conservation and AA-ending codon usage frequency. Indeed, AA-ending codon usage rates successfully predicted an unconventionally secreted cytoplasmic MAX effector, AVR-Pik, from among MAX effectors that shared sequence-unrelated structural folds. AA-ending codon usage rates could thus be used to complement other efforts to identify new effectors, such as those involving proteomics or structural approaches⁴¹, although functional confirmation will still be necessary.

The codon-decoding dependency of *PwL2* but not *BAS4* mRNA stability provides additional evidence for the notion that ribosomal pausing affects the translation of cytoplasmic but not apoplastic effector proteins due to differences in co-translational and protein folding microenvironments. To maintain protein homeostasis, protein quality control pathways that detect stalled or slowed and colliding ribosomes during elongation trigger mRNA decay as well as the degradation of nascent peptides^{42–44}. Following ribosome collision detection by the E3 ubiquitin ligase Znf598 (Hel2 in yeast⁴²), and in coordination with aberrant protein degradation, non-functional ribosome-associated mRNAs

are eliminated via a quality control pathway called the No-Go Decay (NGD) mRNA surveillance pathway. During NGD, aberrant mRNAs undergo endonucleolytic cleavage and degradation to release stalled ribosomes^{42,45–47}. *PWL2* mRNA decay and undetectable *Pwl2* protein production (and their reversal by codon recoding or paromomycin treatment) suggest a similar response to ribosomal pausing at AA-ending codons in *M. oryzae*. Tightly linking mRNA stability with protein quality control may, along with translational speed control, provide an additional layer of cytoplasmic effector production fine-tuning. Indeed, one could speculate that NGD may underpin the in planta-specific expression of fungal effector genes. Thus, future studies directed at understanding the coupling of ribosome pausing at AA-ending codons with nascent protein degradation and NGD, and their connections to secretion, are warranted.

Uncovering an effector mRNA codon usage code⁴⁸ that may adapt translation elongation speeds to co-translational protein folding constraints in the type IV Golgi-bypass pathway could help identify new effectors secreted by unconventional routes not just in *M. oryzae* but also in other plant pathogenic fungi and oomycetes that use this alternative effector secretion route^{37,41}, thereby facilitating the search for novel sources of durable plant host resistance. In addition, parasitic protists including *Leishmania* and the malaria parasite *Plasmodium* traffic virulence factors by unconventional secretion pathways³⁸. Our results may aid in deciphering the poorly understood rules of cargo selection, sorting and translocation³⁸ in these harrowing agents of human affliction. Together, we have shed new light on a microbial process central to many challenging plant and human diseases.

Methods

Strains, culture conditions and physiological analyses

All fungal strains used in this study are listed in Supplementary Table 2. The *M. oryzae* isolate Guy11¹¹ was used as the parental WT strain. Strains were grown on complete media (CM) plates²² and were measured and imaged after 10 days growth using a Sony Cyber-shot digital camera (14.1 megapixels). Spores were collected from 10-day-old CM plates and counted on a haemocytometer, repeated in triplicate. Spores for downstream applications were collected from 12- to 14-day-old oatmeal agar plates (1.5% *w/v* prepared using Oatmeal Agar, Bioworld). For whole-plant inoculations, spores were applied in 0.2% gelatin at a rate of 1×10^5 spores per ml to 3-week-old seedlings of the susceptible rice cultivar CO-39. Infected leaves were collected, dried and imaged at 5 dpi. Images are representative of at least five leaves from each of three independently inoculated plants per strain. For disease area calculations, 10 cm sections of infected leaves were used in triplicate and quantified using ImageJ. Appressorial formation and penetration rates, and cell-to-cell movement rates, were determined by inoculating three detached rice leaf sheaths (from three different plants) per strain with 0.5×10^5 spores per ml. Leaf sheaths were prepared for microscopy as described previously²².

Strain construction

UBA4 and *URM1* were deleted in the Guy11 WT strain by the PCR-based split-marker method¹⁹ using the primers in Supplementary Table 3. Briefly, the coding region of *UBA4* and *URM1* was replaced by the *ILV1* gene conferring sulphonyl urea resistance. At least three independent deletants per gene were confirmed by PCR and subsequently characterized. One deletant of each gene was used to receive the respective add-back complementation vector carrying *UBA4* or *URM1* fused to *GFP*, vectors carrying *PWL2-mCherry:NLS* and *BAS4-GFP*, and, for $\Delta uba4$, codon recoded versions of these genes or the other effector genes described in the text.

LC-MS-based tRNA modification detection

LC-MS-based tRNA modification detection^{49,50} was performed by Arraystar on tRNAs of the indicated strains that were separated from total

small RNAs extracted from vegetative hyphae cultured in liquid CM culture for 42 h. Three biological replicates of each strain were used for the following tRNA modification analysis. After extensive washing with distilled H₂O, mycelia were homogenized in liquid nitrogen, and total RNAs were extracted using the TRIzol reagent (Invitrogen), according to the manufacturer's standard procedure. The prepared total RNAs were then subjected to small RNA isolation using the PureLink microRNA Isolation Kit (Invitrogen) by following the manufacturer's instruction manual. Small RNA quality was estimated by running on a denatured agarose gel, and the RNA quantity was measured using a Nanodrop Spectrophotometer. More than 10 µg of small RNAs per biological replicate were provided to Arraystar for further tRNA isolation and modification analysis. There, tRNAs were isolated from small RNAs by 7.5% PAGE (29:1 acrylamide:bisacrylamide) containing 7 M urea. The 60–90 nt band of tRNA was excised from the gel, extracted with 0.3 M NH₄Ac and precipitated with glycogen and ethanol, then quantified using the Qubit RNA HS Assay kit (ThermoFisher). Purified tRNA was hydrolysed to single, dephosphorylated nucleosides in a 50 µL reaction volume containing 10 U benzonase (Sigma), 0.1 U phosphodiesterase I (US Biological) and 1 U alkaline phosphatase (NEB) following incubation at 37 °C for 3 h. The hydrolysed nucleoside solution was deproteinized using a Satorius 10,000 Da molecular weight cut-off spin filter. The mononucleoside analytes were then separated on an Agilent Zobax SB-Aq liquid chromatography column with flow rate determined by a solvent gradient generated from mixing solution A (0.1% (vol/vol) final concentration of formic acid in HPLC-grade water) and solution B (0.1% (vol/vol) final concentration of formic acid in 100% acetonitrile). tRNA modifications including mcm⁵-²U and mcm⁵U were identified by an Agilent 6460 Triple Quadrupole mass analyser running in multiple reaction monitoring (MRM) mode at the parent to daughter ion transition of (*m/z* 333.0 → 201.0). LC-MS data were acquired using Agilent Qualitative Analysis software (B.07.00). MRM peaks of each modified nucleoside were extracted and normalized to the quantity of purified tRNA for each sample.

Live-cell imaging

For live-cell imaging of fluorescent fusion proteins, 3- to 4-week-old detached rice leaf sheaths of the susceptible cultivar CO-39 were inoculated with 1×10^5 spores per ml of the indicated strains, unless otherwise stated. For each strain, time points and treatments were conducted using leaf sheaths from at least three different plants. Appressoria formation rates were calculated at 24 hpi from 50 germinating spores per strain, repeated in triplicate. Appressorial penetration rates were calculated at 30 hpi by determining how many of 50 appressoria per biological replicate, repeated in triplicate, had formed IH in rice cells by 30 hpi. Rates of spread of IH of the indicated strains to neighbouring cells by 48 hpi was calculated from 50 primary infected rice leaf sheath epidermal cells per biological replicate, repeated in triplicate. Infected detached rice leaf sheaths were imaged using a Nikon Eclipse Ni-E upright microscope and NIS Elements software. Excitation/emission was 488 nm/505–531 nm for GFP and 543 nm/590–632 nm for mCherry. For treatments, 1 mg ml⁻¹ paromomycin (Sigma-Aldrich) in 0.2% gelatin was added to cells at 36 hpi and its effects observed at 44 hpi. For the BFA assay, 4-week-old rice sheaths were inoculated with 3×10^4 conidia per ml of each strain and incubated at 25 °C in the dark. At 36 hpi, the infected leaf sheath samples were infused with mock (0.1% DMSO in H₂O) or freshly prepared BFA (50 µg ml⁻¹ in 0.1% DMSO in H₂O) at room temperature (23 °C), left for 4 h, then trimmed into ultrathin sections for imaging using Nikon Eclipse Ni-E Upright Microscope or, for best results, were further immersed in BFA and incubated at 23 °C for an additional 3 h.

Vector construction

The primers used for constructing the *UBA4-GFP* and *URM1-GFP* complementation vectors are in Supplementary Table 3. Briefly, *UBA4* and

URM1 were amplified with the indicated primers to produce PCR products with 15–20 bases homologous to the pGTN vector. Purified PCR product was fused with linearized pGTN using the In-Fusion HD Cloning Kit (Clontech). The ligation mix was transferred into the *Escherichia coli* DH5a strain with ampicillin antibiotic screening, and all colonies were identified by PCR. Plasmids were sequenced to confirm they were error-free, then transformed into *M. oryzae* strains. *M. oryzae* transformants were screened by geneticin resistance and confirmed by PCR.

All other new vectors used in this study were constructed at GenScript USA, using the vector pBV591 as template. For the domain swap studies, *BAS4* and its native promoter replaced *PWL2* on pBV591 to give *BAS4-mCherry:NLS*; *PWL2* and its native promoter replaced *BAS4* to give *PWL2-GFP*. The *PWL2* sequence and its native promoter were replaced in pBV591 by *AVR-Pita* or *AVR-PIK* and their native promoters to generate *AVR-Pita-mCherry:NLS* and *AVR-Pik-mCherry:NLS* (along with *BAS4-GFP*). The *BAS4* sequence and native promoter were replaced in pBV591 with the *SLP1* gene and its native promoter²⁸ to generate *SLP1-GFP*. For vectors carrying codon recoded genes, first, the recoded *PWL2* (-AA→-AG) sequence replaced *PWL2* in pBV591. Then, *BAS4* in this *PWL2* (-AA→-AG)-carrying vector was replaced with recoded *Bas4* (-AG→-AA). The *PWL2*, *BAS4*, *PWL2* (-AA→-AG), *Bas4* (-AG→-AA), *AVR-Pita*, *AVR-Pik* and *SLP1* genes were synthesized at GenScript USA, subcloned onto the respective vectors using their Clone EZ method and confirmed by sequencing.

qPCR

Leaf sheath samples were collected at 42 hpi, and total RNA was extracted from each sample using the RNeasy mini kit (Qiagen). RNA was treated with DNase I (Invitrogen) and converted to cDNA using qScript (Quantas). qPCR was performed on a QuantStudio 3 real-time qPCR system (ThermoFisher Scientific) using the recommended reagents with primers listed in Supplementary Table 3. Thermocycler conditions were: 5 min at 95 °C, followed by 40 cycles of 95 °C for 30 s, 63 °C for 30 s and 68 °C for 1 min. qPCR data were analysed using the QuantStudio Design and Analysis software package (version 1.5.2), and fold changes were calculated using the $\Delta\Delta C_t$ method⁵¹.

Codon usage analysis

The complete coding sequence database of *M. oryzae*, which contains 12,836 protein coding sequences, was obtained from the Joint Genome Institute Genome portal (<https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=Magor1>, project name: *Pyricularia oryzae* 70-15 v3.0). The codon usage pattern was analysed with CUSP, a command line program from EMBOS (The European Molecular Biology Open Software Suite; <http://bioinfo.pbi.nrc.ca:8090/EMBOSS/>) (version 6.6), utilizing the high-performance computing resources provided by the Holland Computing Center at the University of Nebraska-Lincoln.

Statistics and reproducibility

Values represent the mean $\pm$ s.d. of three independent biological replicates. Sample sizes were chosen based on previous studies in the field^{22,25,27}. Microsoft Excel 2016 was used to calculate the means $\pm$ s.d. for the fluorescent protein distribution patterns during live-cell imaging. For comparing the mean values of the colony diameters, appressorium formation rates, penetration rates, cell-to-cell movement rates and lesion area rates, the one-way analysis of variance (ANOVA) function in PASW Statistics 18.0 (PASW Statistics) with the Tukey's honestly significant difference (HSD) multiple comparison test was used. For comparing the mean values of sporulation rates, the Welch ANOVA with Games–Howell post hoc test was used. For comparing the mean values of lesion areas, statistical analysis was performed with two-tailed unpaired *t*-test or one-way ANOVA with Tukey's multiple comparisons test, as indicated. For comparing target gene expression levels between different strains, the quantified transcript abundance

was calculated as the fold change as described in the figure legends, and statistical analysis was carried out using one-way ANOVA with the Student–Newman–Keuls test or the Tukey HSD multiple comparison tests in PASW Statistics. The R package agricolae was utilized to acquire precise *P* values for the Student–Newman–Keuls test, with modifications made to the source code of the SNK test function. A resulting *P* value of <0.05 was considered significant between group means.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The *M. oryzae* *UBA4* and *URM1* gene sequences are available at the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov>) under the accession numbers MGG_05569 and MGG_03978, respectively. Other gene sequences used in the course of this study can be found at the National Center for Biotechnology Information under the following accession numbers: MGG_13863 for *PWL2*, MGG_10914 for *BAS4*, MGG_15370 for *AVR-Pita*, MGG_10097 for *SLP1*, FJ807764 for *BAS1*, MGG_18041 for *AvrPiz-t*, MGG_10020 for *BAS107*, MGG_05785 for *BAS113*, JN035619.1 for *AVR-Pik*, AB434708 for *Avr-Pia*, AF463528.1 for *AVR1-CO39*, and AAK31287.1 for *TOXB*. Strains generated during the course of this study are available from the corresponding author upon request and with an appropriate Animal and Plant Health Inspection Service permit. Source data are provided with this paper.

Code availability

Underlying source code associated with the *P* value calculations in Fig. 4e is available in Source Data Fig. 4.

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Author contributions

R.A.W. conceived the project and obtained funding. R.A.W. designed the experiments. R.A.W. and G.L. interpreted the data. G.L., N.D. and Z.G. generated the strains and performed the experiments. G.L. performed the bioinformatic analyses. R.A.W. wrote the paper, with contributions from all authors.

Competing interests

The authors declare no competing interests.

Additional information

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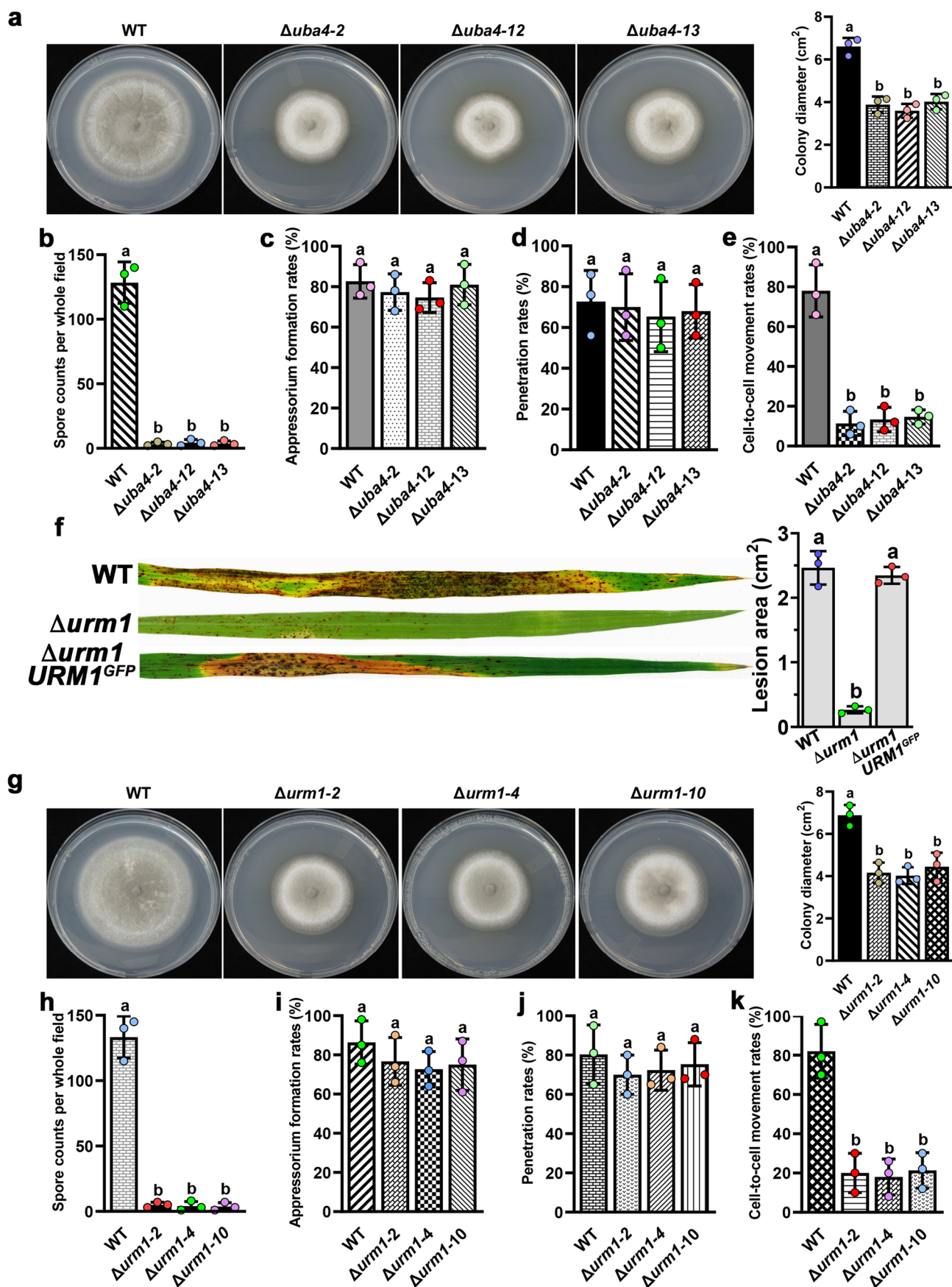
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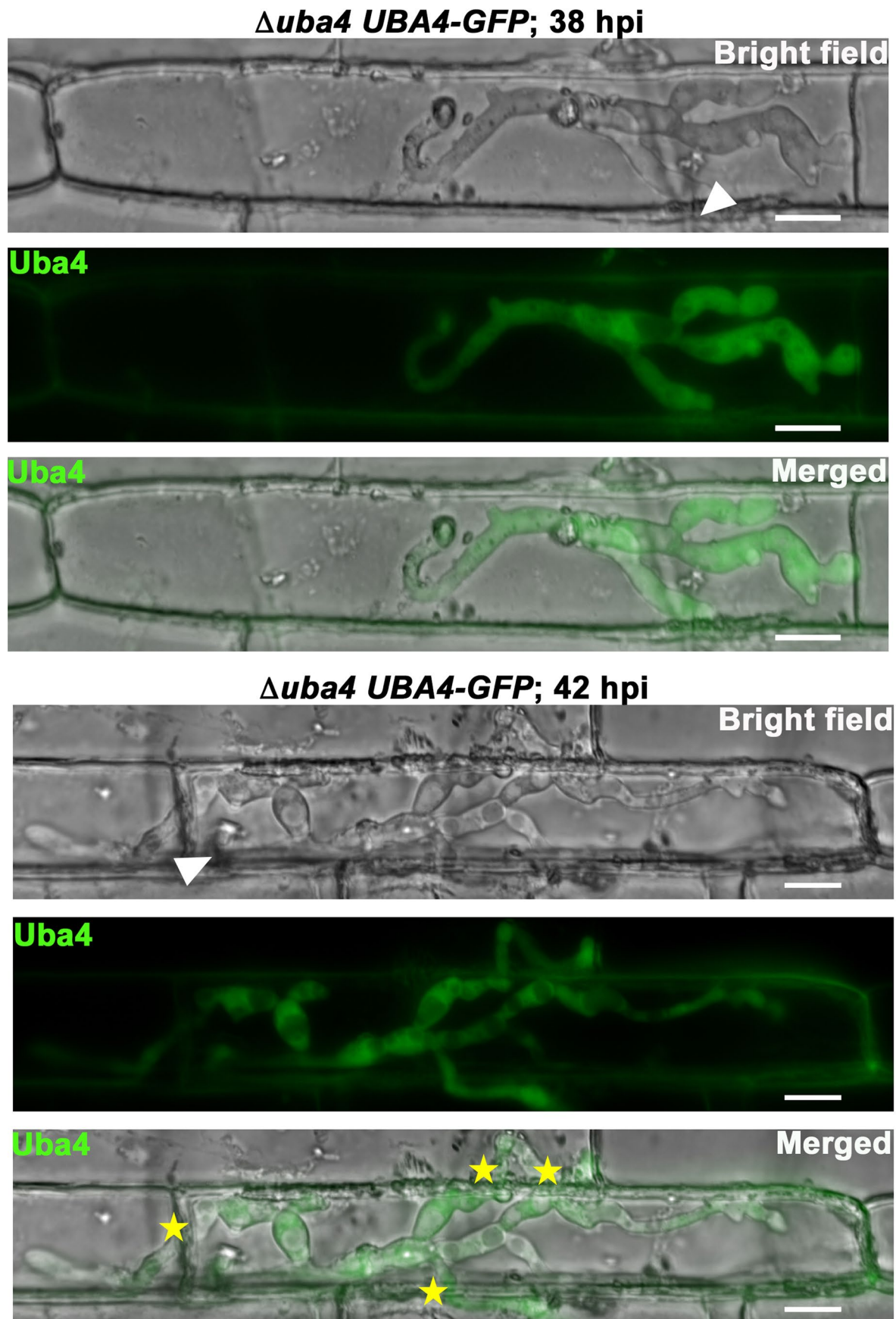
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Extended Data Fig. 1 | See next page for caption.

Extended Data Fig. 1 | Loss of *UBA4* or *URM1* in *M. oryzae* impairs biotrophic growth in host rice cells. a–e, Three independent transformants carrying deletions in *UBA4* had identical phenotypes. Compared to WT, $\Delta uba4$ strains were reduced in radial growth (a) and sporulation (b) on complete media (CM) by 10 days post inoculation (dpi), but appressorium formation rates on detached rice leaf sheath surfaces at 24 hpi (c), and penetration rates of appressoria on host rice leaf sheath surfaces at 30 hpi (d), were not affected by the loss of *UBA4*; however, cell-to-cell movement of $\Delta uba4$ invasive hyphae (IH) from the first infected rice cell into neighbouring cells at 48 hpi was reduced (e). f, Compared to WT and the $\Delta urm1$ *URM1-GFP* complementation strain, loss of *URM1* abolished *M. oryzae* pathogenicity on rice leaves. Left, Leaves were imaged at 5 dpi. Images are representative of at least 5 leaves from three biological replicates. Right, 10 cm lengths of infected rice leaves were imaged at 5 dpi. Disease lesion areas were quantified using ImageJ and analyzed according to one-way ANOVA

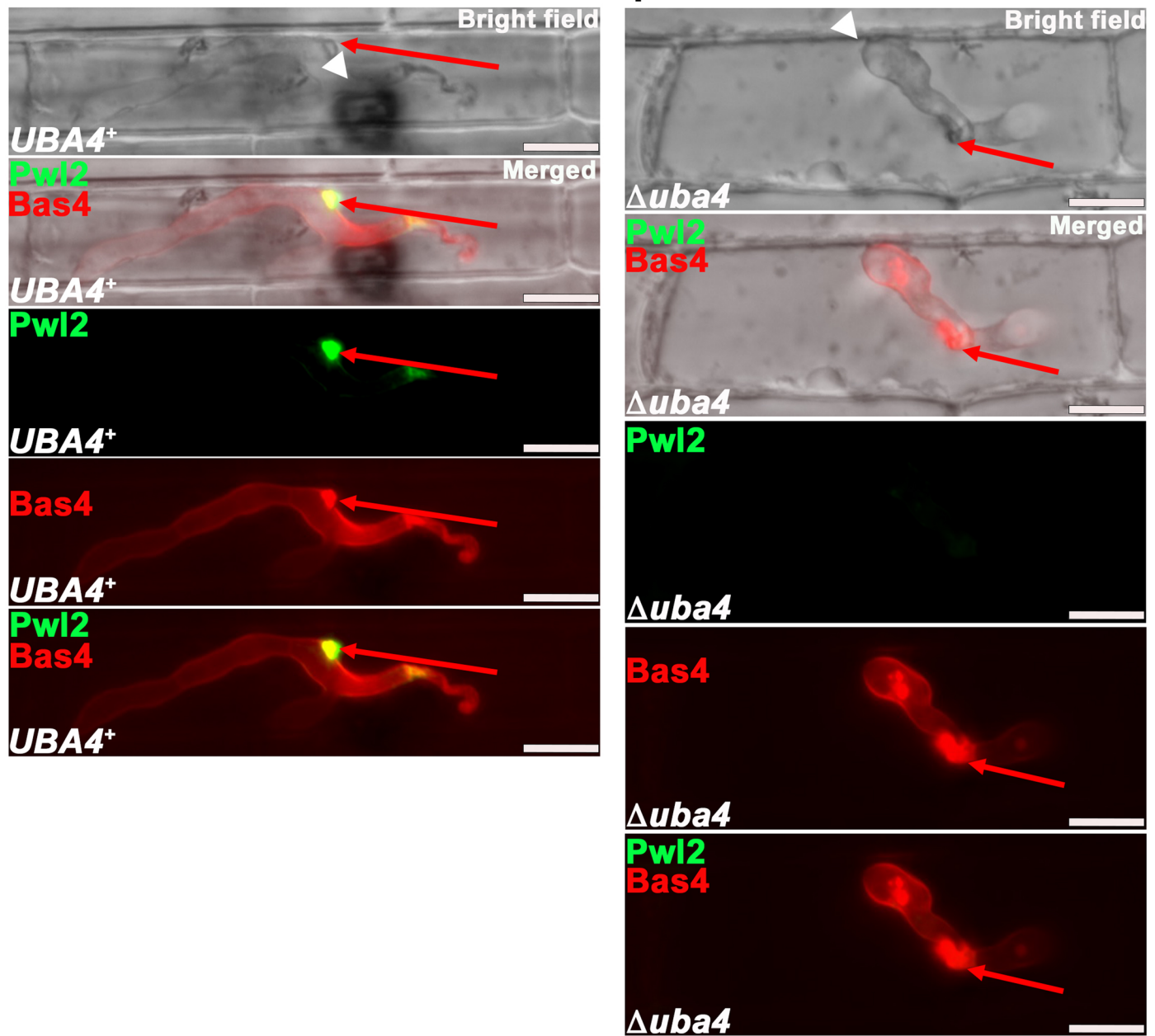
followed by a Tukey HSD test for multiple comparisons. Values are the means $\pm$ s.d. from three biological replicates. Different letters indicate significant differences ($p < 0.05$). g–k, Three independent transformants carrying deletions in *URM1* had identical phenotypes, and also strongly resembled the $\Delta uba4$ strains in growth (g), sporulation rates (h), appressorium formation (i) and function (j), and cell-to-cell movement rates (k), compared to WT. a, g, Colonies of each strain were imaged and measured after 10 days growth on CM. Images are representative of three independent experiments. a–e, g–k, Values are means $\pm$ s.d. from three biological replicates. Bars with different letters indicate significant differences at $P \leq 0.05$. *P*-values were determined by one-way ANOVA with Tukey's multiple comparisons test (a, c–g, i–k) or by Welch ANOVA with Games-Howell post hoc test (b, h). Exact *P* values are given in **Source Data** **Extended Data Fig. 1. c, i**, Spores of the indicated strains were inoculated onto detached rice leaf sheaths at a rate of 0.5×10^5 spores ml⁻¹.



Extended Data Fig. 2 | Uba4 is localized to the cytoplasm in invasive hyphae. Live-cell imaging of the $\Delta uba4$ UBA4-GFP complementation strain in detached rice leaf sheath epidermal cells at 38 hpi and 42 hpi shows that the Uba4-GFP fusion protein localizes to the cytoplasm of fungal invasive hyphae. White

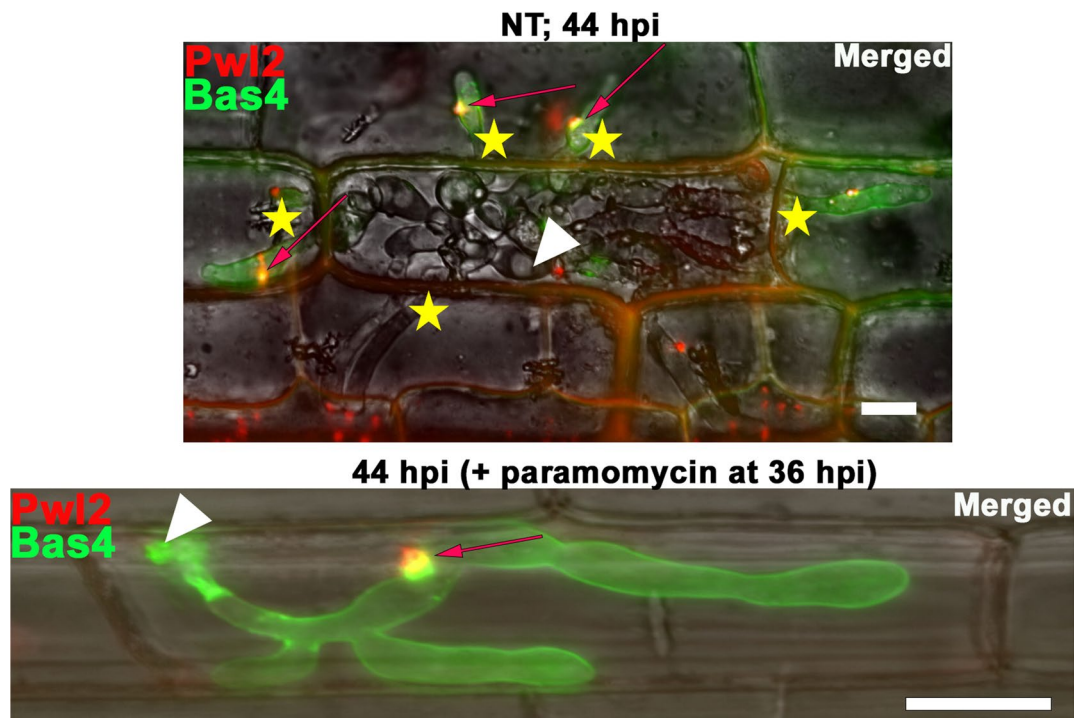
arrowheads indicate penetration sites, stars indicate movement of invasive hyphae into neighbouring cells. Scale bars, 10 μ m. Representative images are based on the observations of 50 infected rice cells per leaf sheath per treatment, repeated in triplicate.

36 hpi



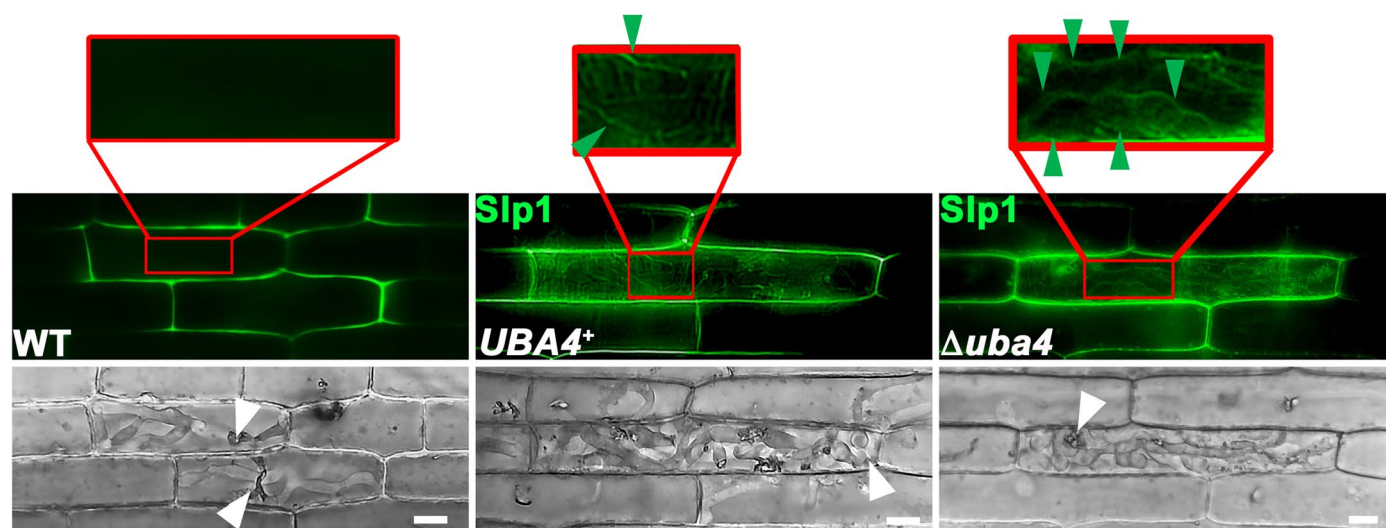
Extended Data Fig. 3 | Bas4-mCherry:NLS but not Pwl2-GFP is secreted by Δ *uba4*. Live-cell imaging at 36 hpi of the indicated strains expressing *PWL2*-GFP and *BAS4*-mCherry:NLS in detached rice leaf sheath epidermal cells shows how in 100 % of observed infected cells, Pwl2-GFP accumulated in BICs of the *UBA4*⁺ strain but not in Δ *uba4* BICs, while Bas4-mCherry:NLS accumulated in the

apoplast of both strains. Calculations and representative images are based on the observations of 50 infected rice cells per leaf sheath per treatment, repeated in triplicate. White arrowheads indicate penetration sites; red arrows indicate Pwl2 in BICs. Scale bars, 10 μ m.



Extended Data Fig. 4 | Paromomycin treatment impairs biotrophic growth in WT but does not prevent effector translation or secretion. Live-cell imaging at 44 hpi of detached rice leaf sheath epidermal cells colonized with the wild type Guy11 strain expressing *PWL2-mCherry:NLS* and *BAS4-GFP*. Compared to the non-treated (NT) control (*Top*), treatment with paromomycin at 36 hpi (*Bottom*) impaired biotrophic growth and prevented cell-to-cell spread of invasive hyphae

by 44 hpi, but effectors were nonetheless translated and secreted, and the BIC and apoplastic space were intact. White arrowheads indicate penetration sites; red arrows indicate Pwl2 in BICs; stars indicate movement of invasive hyphae into neighbouring cells. Scale bars, 10 μm. Representative images are based on the observations of 50 infected rice cells per leaf sheath per treatment, repeated in triplicate.



Extended Data Fig. 5 | Slp1 is secreted into the $\Delta uba4$ apoplast. Live-cell imaging at 44 hpi of detached rice leaf sheath epidermal cells infected with the indicated strains expressing *SLP1*-GFP shows how in 100 % of observed infected cells, compared to WT lacking *SLP1*-GFP, Slp1 accumulated in the apoplast of both *UBA4*⁺ and $\Delta uba4$ strains. Slp1-GFP fluorescence was weak in both strains and

can be best seen outlining IH in the blow-up boxes, indicated with green arrow heads. Calculations and representative images are based on the observations of 50 infected rice cells per leaf sheath per treatment, repeated in triplicate. White arrowheads indicate penetration sites. Scale bars, 10 μ m.

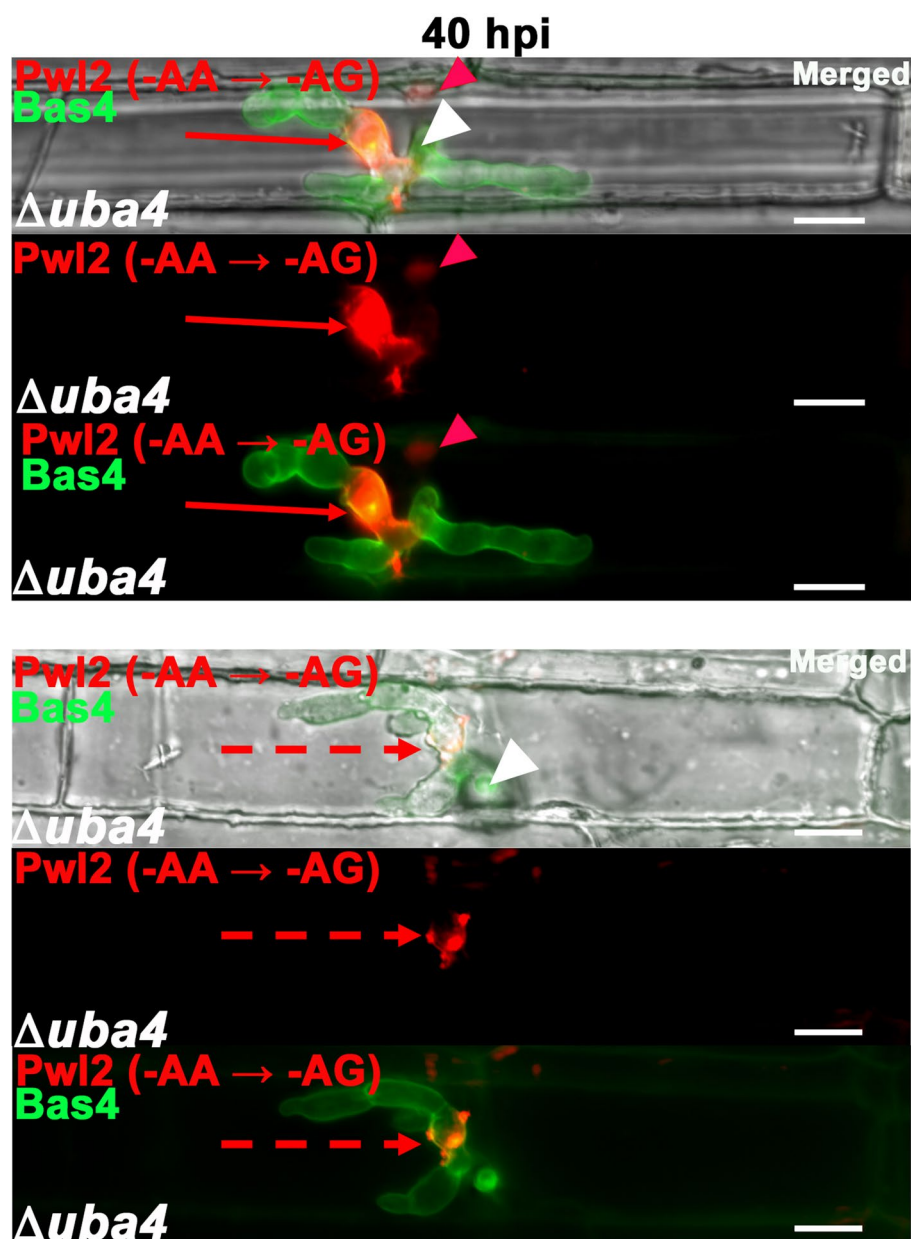
Pwl2-mCherry:NLS**AAA** (Lys) to AAG (Lys) (7)**CAA** (Gln) to CAG (Gln) (3)**GAA** (Glu) to GAG (Glu) (9)

```

atg aaa tgc aac aac atc atc ctc cct ttt gct ttg gtc ttt ttt tcg acc act gta acc
M K C N N I I L P F A L V F F S T T V T
gcc ggt ggc ggg tgg act aac aaa caa ttt tac aac gac aaa ggc gaa aga gag ggc tca
A G G G W T N K Q F Y N D K G E R E G S
att tca att agg aag ggc tcg gaa ggc gat ttt aac tat ggc ccc agt tat cct gga ggg
I S I R K G S E G D F N Y G P S Y P G G
ccc gat agg atg gta cgg gtt cat gaa aac aac ggc aac atc cgc ggg atg ccc ccg gga
P D R M V R V H E N N G N I R G M P P G
tat tct cta ggc cct gat cat cag gaa gat aaa agc gat cgt caa tat tat aac agg cac
Y S L G P D H Q E D K S D R Q Y Y N R H
gga tat cat gtt ggt gat gga ccc gcc gaa tac gga aat cac gga ggc ggg caa tgg ggc
G Y H V G D G P A E Y G N H G G G Q W G
gac gga tat tat gga ccg cca ggc gag ttt aca cat gag cac cgt gaa cag cga gaa gag
D G Y Y G P P G E F T H E H R E Q R E E
ggc tgc aat att atg gga tcc atg gtg agc aag ggc gag gag gat aac atg gcc atc atc
G C N I M G S M V S K G E E D N M A I I
aag gag ttc atg cgc ttc aag gtg cac atg gag ggc tcc gtg aac ggc cac gag ttc gag
K E F M R F K V H M E G S V N G H E F E
atc gag ggc gag ggc gag ggc cgc ccc tac gag ggc acc cag acc gcc aag ctg aag gtg
I E G E G E G R P Y E G T Q T A K L K V
acc aag ggt ggc ccc ctg ccc ttc gcc tgg gac atc ctg tcc cct cag ttc atg tac ggc
T K G G P L P F A W D I L S P Q F M Y G
tcc aag gcc tac gtg aag cac ccc gcc gac atc ccc gac tac ttg aag ctg tcc ttc ccc
S K A Y V K H P A D I P D Y L K L S F P
gag ggc ttc aag tgg gag cgc gtg atg aac ttc gag gac ggc ggc gtg gtg acc gtg acc
E G F K W E R V M N F E D G G V V T V T
cag gac tcc tcc ctg cag gac ggc gag ttc atc tac aag gtg aag ctg cgc ggc acc aac
Q D S S L Q D G E F I Y K V K L R G T N
ttc ccc tcc gac ggc ccc gta atg cag aag aag acc atg ggc tgg gag gcc tcc tcc gag
F P S D G P V M Q K K T M G W E A S S E
cgg atg tac ccc gag gac ggc gcc ctg aag ggc gag atc aag cag agg ctg aag ctg aag
R M Y P E D G A L K G E I K Q R L K L K
gac ggc ggc cac tac gac gct gag gtc aag acc acc tac aag gcc aag aag ccc gtg cag
D G G H Y D A E V K T T Y K A K K P V Q
ctg ccc ggc gcc tac aac gtc aac atc aag ttg gac atc acc tcc cac aac gag gac tac
L P G A Y N V N I K L D I T S H N E D Y
acc atc gtg gaa cag tac gaa cgc gcc gag ggc cgc cac tcc acc ggc ggc atg gag gag
T I V E Q Y E R A E G R H S T G G M E E
ctg tac aag tcc gga ctc aga tct cga gct gat cca aaa aag aag aga aag gta gat cca
L Y K S G L R S R A D P K K K R K V G S -
aaa aag aag aga aag gta gat cca aaa aag aag aga aag gta gga tct taa
K K K R K V D P K K K R K V G S -

```

Extended Data Fig. 6 | Location of AA-ending codons in *PWL2-mCherry:NLS*. AA-ending codons in the *PWL2-mCherry:NLS* coding sequence²⁶ that were recoded to synonymous AG-ending codons in *PWL2* (–AA → –AG) are shown in red. The first *mCherry* codon is highlighted in yellow. The start of the *NLS* sequence is highlighted in green. The linker sequence is highlighted in grey.



Extended Data Fig. 7 | Expressing *PWL2* (-AA → -AG) in $\Delta uba4$ affects BIC integrity. Live-cell imaging at 40 hpi of detached rice leaf sheath epidermal cells infected with $\Delta uba4$ expressing *PWL2* (-AA → -AG) and *BAS4*-GFP. White arrowheads indicate penetration sites; red arrowheads indicate recoded PwI2 in

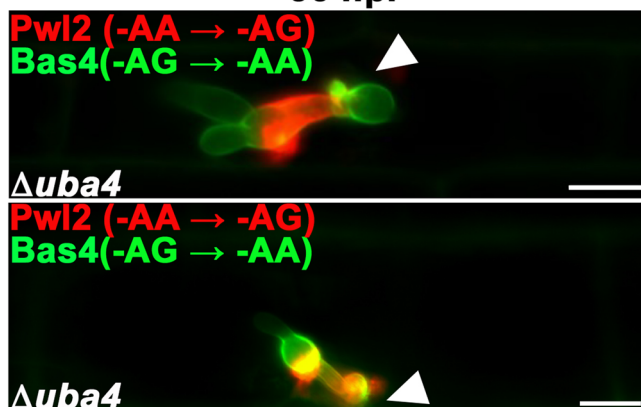
host nuclei; solid red arrows indicate recoded PwI2 in enlarged BICs; dashed red arrows indicate recoded PwI2 in fractured BICs. Scale bars, 10 μ m. Representative images are based on the observations of 50 infected rice cells per leaf sheath per treatment, repeated in triplicate.

a**Bas4-GFP****AAG** (Lys) to **AAA** (Lys) (22)**CAG** (Gln) to **CAA** (Gln) (10)**GAG** (Glu) to **GAA** (Glu) (20)

```

atg cag ctc tca ttc tca gca atc gcc atc ctt ttg gcc ttc gcc gtc aac cac gct acg
M  Q  L  S  F  S  A  I  A  I  L  L  A  F  A  V  N  H  A  T
acc gac tcg cac cag aat ctt gtt tgt gtc aga acc tac ccc gac aac aac agt gca gag
T  D  S  H  Q  N  L  V  C  V  R  T  Y  P  D  N  N  S  A  E
aac ctt gag gcc aca cga tgt gcc tgc gat tgg ctc aag aag aac ggc aaa tgc gac gac
N  L  E  A  T  R  C  A  C  D  W  L  K  K  N  G  K  C  D  D
tgc act atc tgg gag aac cga ctc tgc cat tcg gat gcc aag tct ctg gac gga aat gag
C  T  I  W  E  N  R  L  C  H  S  D  A  K  S  L  D  G  N  E
ttt gag gac gca tgc gtt agg aaa tgc cct aac ctt ggc gct act ggc tcg tct atc ccc
F  E  D  A  C  V  R  K  C  P  N  L  G  A  T  G  S  S  I  P
cct gct gga tcc atc gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg
P  A  G  S  G V S K G E E L F T G V V P I L
gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc gag ggc gag ggc
V  E  L  D  G  D  V  N  G  H  K  F  S  V  S  G  E  G  E  G
gat gcc acc tac ggc aag ctg acc ctg aag ttc atc tgc acc acc ggc aag ctg ccc gtg
D  A  T  Y  G  K  L  T  L  K  F  I  C  T  T  G  K  L  P  V
ccc tgg ccc acc ctc gtg acc acc ctg acc tac ggc gtg cag tgc ttc agc cgc tac ccc
P  W  P  T  L  V  T  T  L  T  Y  G  V  Q  C  F  S  R  Y  P
gac cac atg aag cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag
D  H  M  K  Q  H  D  F  F  K  S  A  M  P  E  G  Y  V  Q  E
cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag gtg aag ttc gag
R  T  I  F  F  K  D  D  G  N  Y  K  T  R  A  E  V  K  F  E
ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc atc gac ttc aag gag gac ggc aac
G  D  T  L  V  N  R  I  E  L  K  G  I  D  F  K  E  D  G  N
atc ctg ggg cac aag ctg gag tac aac tac aac agc cac aac gtc tat atc atg gcc gac
I  L  G  H  K  L  E  Y  N  Y  N  S  H  N  V  Y  I  M  A  D
aag cag aag aac ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc
K  Q  K  N  G  I  K  V  N  F  K  I  R  H  N  I  E  D  G  S
gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc ccc gtg ctg ctg
V  Q  L  A  D  H  Y  Q  Q  N  T  P  I  G  D  G  P  V  L  L
ccc gac aac cac tac ctg agc acc cag tcc gcc ctg agc aaa gac ccc aac gag aag cgc
P  D  N  H  Y  L  S  T  Q  S  A  L  S  K  D  P  N  E  K  R
gat cac atg gtc ctg ctg gag ttc gtg acc gcc gcc ggg atc act ctc ggc atg gac gag
D  H  M  V  L  L  E  F  V  T  A  A  G  I  T  L  G  M  D  E
ctg tac aag taa
L  Y  K  -

```

b**36 hpi**

Extended Data Fig. 8 | See next page for caption.

Extended Data Fig. 8 | Expressing *BAS4* ($-AG \rightarrow -AA$) in $\Delta uba4$ does not affect *Bas4* secretion. **a**, AG-ending codons in the *BAS4-GFP* coding sequence²⁶ that were recoded to synonymous AA-ending codons in *BAS4* ($-AG \rightarrow -AA$) are shown in red. The *GFP* start codon is highlighted in green. The linker sequence is highlighted in grey. **b**, Live-cell imaging at 36 hpi of detached rice leaf sheath

epidermal cells infected with $\Delta uba4$ expressing *PWL2* ($-AA \rightarrow -AG$) and *BAS4* ($-AG \rightarrow -AA$). Recoded *Bas4* and recoded *Pwl2* are correctly deployed, albeit sometimes resulting in enlarged or fractured BICs in the latter case. White arrowheads indicate penetration sites. Scale bars, 10 μ m. Images are based on the observations of 50 infected rice cells per leaf sheath, repeated in triplicate.

a

Σ CDS			BAS4		
Codon	Fraction	Number	Codon	Fraction	Number
GAA	0.296	98391	GAA	0	0
GAG	0.704	234297	GAG	1	5
AAA	0.249	66217	AAA	0.4	2
AAG	0.751	199963	AAG	0.6	3
CAA	0.338	77501	CAA	0	0
CAG	0.662	151801	CAG	1	2
PWL2			AVR-Pita		
Codon	Fraction	Number	Codon	Fraction	Number
GAA	0.64	7	GAA	0.86	12
GAG	0.36	4	GAG	0.14	2
AAA	0.8	4	AAA	0.92	12
AAG	0.2	1	AAG	0.08	1
CAA	0.6	3	CAA	0.83	5
CAG	0.4	2	CAG	0.17	1
SLP1					
Codon	Fraction	Number			
GAA	0	0			
GAG	0	0			
AAA	0	0			
AAG	1	5			
CAA	0.11	1			
CAG	0.89	8			

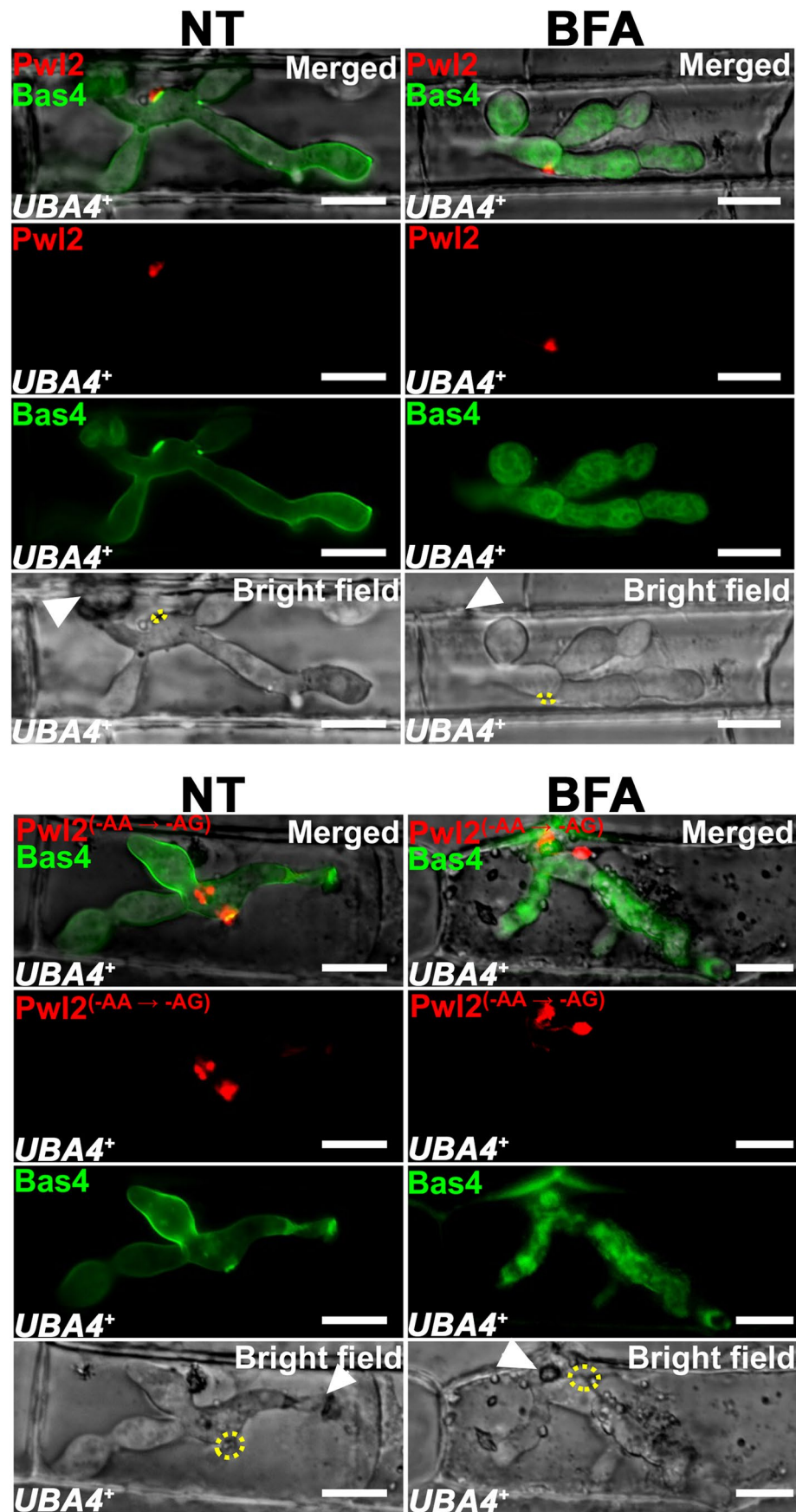
b

BAS1			AvrPiz-t		
Codon	Fraction	Number	Codon	Fraction	Number
GAA	0.44	4	GAA	1	2
GAG	0.56	5	GAG	0	0
AAA	0.6	3	AAA	0.5	3
AAG	0.4	2	AAG	0.5	3
CAA	0.88	7	CAA	0.33	2
CAG	0.13	1	CAG	0.67	4
BAS107			BAS113		
Codon	Fraction	Number	Codon	Fraction	Number
GAA	0.5	4	GAA	0.14	4
GAG	0.5	4	GAG	0.86	4
AAA	0.45	5	AAA	0.08	2
AAG	0.55	6	AAG	0.92	23
CAA	0.67	4	CAA	0.25	4
CAG	0.33	2	CAG	0.75	12

c

AVR-Pik			AVR-Pia			AVR1-CO39			TOXB		
Codon	Fraction	Number	Codon	Fraction	Number	Codon	Fraction	Number	Codon	Fraction	Number
GAA	0.75	3	GAA	0.75	3	GAA	0.33	2	GAA	0.33	1
GAG	0.25	1	GAG	0.25	1	GAG	0.67	4	GAG	0.67	2
AAA	1	8	AAA	1	4	AAA	0	0	AAA	0	0
AAG	0	0	AAG	0	0	AAG	4	1	AAG	1	2
CAA	1	1	CAA	0.5	1	CAA	1	2	CAA	1	2
CAG	0	0	CAG	0.5	1	CAG	0	0	CAG	0	0

Extended Data Fig. 9 | AA- and AG-ending codon counts for apoplastic and cytoplasmic effector mRNAs. Tables showing frequencies and numbers of synonymous AA-ending (red) and AG-ending (black) codons in all *M. oryzae* protein coding sequences (Σ CDS) (a) and in the indicated effector-encoding mRNAs (a-c). See text for details.



Extended Data Fig. 10 | See next page for caption.

Extended Data Fig. 10 | Brefeldin A treatment did not affect the secretion of recoded Pwl2 into the BIC. Live-cell imaging at 40 hpi of detached rice leaf sheath epidermal cells infected with *UBA4*⁺ strains expressing *BAS4-GFP* and either *PWL2* (*Top*) or recoded *PWL2* (*-AA* → *-AG*) (*Bottom*) shows how in both cases, and in 100 % of observed cells, Brefeldin A (BFA) treatment, which inhibits conventional ER-Golgi secretion, led to the retention of Bas4 in IH cytoplasm as expected, but Pwl2 secretion into the BIC was unaffected. This confirms that recoded Pwl2 (*-AA* → *-AG*) is, like Pwl2, secreted into the BIC via

the unconventional protein secretion pathway and that, moreover, no spillover secretion of Pwl2 (*-AA* → *-AG*) through the ER-Golgi pathway contributes to the enlarged, split-BIC phenotype observed for this strain. White arrowheads indicate penetration sites; hatched yellow circles indicate BICs, which are enlarged in the *UBA4*⁺ *PWL2* (*-AA* → *-AG*) strain compared to the *UBA4*⁺ *PWL2* strain. Scale bars, 10 μm. Calculations and representative images are based on the observations of 50 infected rice cells per leaf sheath per treatment, repeated in triplicate.

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- ☐ ☒ The statistical test(s) used AND whether they are one- or two-sided
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- ☐ ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☐ ☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☒ ☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection

Live-cell images were taken by a Nikon Eclipse Ni-E upright microscope. The mcm5s2U tRNA modification was measured by LC-MS using an Agilent 6460 Triple Quadrupole mass analyzer running in MRM mode. Quantitative gene expression data was measured by a QuantStudioTM 3 Real-Time qPCR system (ThermoFischer Scientific, USA). Plate images were taken by a Canon PowerShot ELPH 360 HS with 12x optical zoom. Images of *M. oryzae* infected whole leaves were imaged by scanning using the EPSON Perfection V550 Photo.

Data analysis

Values represent the mean $\pm$ SD of three independent biological replicates. Fluorescent protein distribution rates were analyzed using Microsoft Excel 2016. For comparing the mean values of the colony diameters, appressorium formation rates, penetration rates, and cell-to-cell movement rates, the one-way ANOVA function in PASW Statistics 18.0 (PASW Statistics Inc.) with the Tukey HSD multiple comparison test was used. For comparing the mean values of sporulation rates, the Welch ANOVA with Games-Howell post hoc test was employed. For comparing the mean values of lesion areas, statistical analysis was performed with two-tailed unpaired t-test or one-way ANOVA with Tukey's multiple comparisons test. For comparing target gene expression levels between different strains, the quantified transcript abundance was calculated as the fold change as described in the figure legends and statistical analysis was carried out using one-way ANOVA with the Student–Newman–Keuls test or the Tukey HSD multiple comparison tests in PASW Statistics. The R package agricolae was utilized to acquire precise P values for the Student–Newman–Keuls test, with modifications made to the source code of the SNK test function.

Live-cell images were processed by the software NIS-Elements (version 5.20.02). The mcm5s2U34 tRNA modification LC-MS data was analyzed using Agilent Qualitative Analysis software (B.07.00). The complete CDS database of Magnaporthe oryzae was obtained from the JGI Genome portal (<https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=Magor1>, Project name: Pyricularia oryzae 70-15 v3.0). The codon usage pattern was analyzed using the CUSP program of EMBOSS (The European Molecular Biology Open Software Suite, Cambridge, UK; <http://bioinfo.pbi.nrc.ca:8090/EMBOSS/>) in R utilizing the high performance computing resources in Holland Computing Center at the University of Nebraska-Lincoln. qPCR data was analyzed using the QuantStudioTM Design & Analysis software package (Version 1.5.2).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

The M. oryzae UBA4 and URM1 gene sequences are available at NCBI (<https://www.ncbi.nlm.nih.gov>) under the accession numbers MGG_05569 and MGG_03978, respectively. Other gene sequences used in the course of this study can be found at NCBI under the following accession numbers: MGG_13863 for PWL2, MGG_10914 for BAS4, MGG_15370 for AVR-Pita, MGG_10097 for SLP1, FJ807764 for BAS1, MGG_18041 for AvrPiz-t, MGG_10020 for BAS107, MGG_05785 for BAS113, JN035619.1 for AVR-Pik, AB434708 for Avr-Pia, AF463528.1 for AVR1-CO39, and AAK31287.1 for TOXB. Numerical and statistical source data that underlie the graphs in figures and extended data are provided with the paper. Strains generated during the course of this study are available from the corresponding author upon request and with an appropriate APHIS permit.

Field-specific reporting

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☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample size was chosen according to long-term experience in biological and biochemical experimentation, for example following Marroquin-Guzman et al. 2017, Nature Microbiology, and Li et al. 2020, New Phytologist. Pathogenicity-related assays were conducted according to published protocols. For inoculation assays, each independent experiment contains at least 3 randomly selected rice leaves. For analysis of the distribution of fluorescent proteins in growing fungal invasive hyphae, at least 50 invasive hyphae expressing fluorescent proteins were observed per replicate, n=3 biological replications.
Data exclusions	No data were excluded from analysis.
Replication	All experiments were performed in triplicate or with three biological replicates. All attempts at replicating the results were successful.
Randomization	Fungal strains were grown on complete media and randomly allocated for each experimental test. Rice seedlings were grown under the same conditions and randomly allocated for the inoculation assays. The distribution of fluorescent proteins in fungal invasive hyphae were observed in randomly selected infected rice cells. Biological samples used in LC/MS analyses were selected by random sampling.
Blinding	Investigators were not blinded to group allocation during the experiments, but data collection and assessment were repeated by multiple independent experiments to ensure the analysis is as objective as possible.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging