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CURRENT REVIEW

Paths of Least Resistance: Unconventional Effector Secretion by Fungal and Oomycete Plant Pathogens

Nawaraj Dulal and Richard A. Wilson†

Department of Plant Pathology, University of Nebraska–Lincoln, Lincoln, NE, U.S.A.

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Effector secretion by different routes mediates the molecular interplay between host plant and pathogen, but mechanistic details in eukaryotes are sparse. This may limit the discovery of new effectors that could be utilized for improving host plant disease resistance. In fungi and oomycetes, apoplastic effectors are secreted via the conventional endoplasmic reticulum (ER)-Golgi pathway, while cytoplasmic effectors are packaged into vesicles that bypass Golgi in an unconventional protein secretion (UPS) pathway. In *Magnaporthe oryzae*, the Golgi bypass UPS pathway incorporates components of the exocyst complex and a t-SNARE, presumably to fuse Golgi bypass vesicles to the fungal plasma membrane. Upstream, cytoplasmic effector mRNA translation in *M. oryzae* requires the efficient decoding of AA-ending codons. This involves the modification of wobble uridines in the anticodon loop of cognate tRNAs and fine-tunes cytoplasmic effector translation and secretion rates to maintain biotrophic interfacial complex integrity and permit host infection. Thus, plant-fungal interface integrity is intimately tied to effector codon usage, which is a surprising constraint on pathogenicity. Here, we discuss these findings within the context of fungal and oomycete effector discovery, delivery, and function in host cells. We show how cracking the codon code for unconventional cytoplasmic effector secretion in *M. oryzae* has revealed AA-ending codon usage bias in cytoplasmic effector mRNAs across kingdoms, including within the RxLR-dEER motif-encoding sequence of a bona fide *Phytophthora infestans* cytoplasmic effector, suggesting its subjection to translational speed control. By focusing on recent developments in understanding unconventional effector secretion, we draw attention to this important but understudied area of host-pathogen interactions.

Keywords: avirulence factors, fungal effectors, *Magnaporthe oryzae*, oomycete effectors, *Phytophthora infestans*, secretion

The normal state of plants is to be disease-free. This is due to the activation of robust plant innate immune responses following the detection of invading microorganisms and viruses. However, when plant defense responses can be skirted or suppressed, often via the deployment of effector proteins, pathogens destroy plant tissues, which may result in devastating crop losses. Effectors secreted by fungi and oomycete plant pathogens (Lovelace et al. 2023; S. Wang et al. 2023; Wilson and McDowell 2022) interfere with host innate immunity via roles, for example, in inhibiting plant defense signaling pathways or masking the invader from detection by the host plant (Petre and Kamoun 2014). Effectors are classified as apoplastic if they localize and function in the space formed between host cytoplasm and invasive hyphae (IH) or haustoria and are termed cytoplasmic if they are translocated into the host cell to target host cellular processes (Yi and Valent 2013). Detection of effectors by host intracellular nucleotide-binding and leucine-rich repeat (NLR) domain-containing immune receptors triggers immunity (Jones and Dangl 2006; Maidment et al. 2023).

Although widely separated taxonomically, both the rice blast fungus *Magnaporthe oryzae* and the Irish potato famine oomycete *Phytophthora infestans* secrete effectors by two routes: a Brefeldin A (BFA)-sensitive secretion route via the conventional ER-Golgi pathway for apoplastic effector secretion and a BFA-insensitive unconventional protein secretion (UPS) pathway for cytoplasmic effector delivery (Giraldo et al. 2013; Wang et al. 2017, 2018). Except for one leaderless effector protein in *M. oryzae* (MoNte1; Chen et al. 2023a), all experimentally described cytoplasmic effectors in *M. oryzae* and *P. infestans* carry, like apoplastic effectors, signal peptides (SPs) targeting them to the ER. However, due to their BFA insensitivity, cytoplasmic effectors must be secreted in a Golgi-independent manner (i.e., via the Golgi bypass pathway) (Fig. 1). In addition to SPs, *Phytophthora* cytoplasmic effectors carry RxLR (S. Wang et al. 2023) or LxLFLAK motifs (Fabro 2022) that may mediate translocation into host cells, either directly or by facilitating protein sorting or stability (Stuer et al. 2023; S. Wang et al. 2023). However, except for an RGD motif required for internalization of the *Pyrenophora tritici-repentis* host-selective protein toxin ToxA into host cells (Manning et al. 2008), similar conserved translocation sequences in fungi are absent or have not yet been discovered. Protein sequences specifying effector sorting into UPS pathways from the ER are unknown in either group.

The drive for novel sources of durable host plant resistance is facilitated by the identification and characterization of new effectors and their host targets, which is a key goal of molecular plant pathology. Understanding effector deployment may aid the discovery of new effectors or effector classes, inform on their evolutionary history, as well as suggest novel strategies for blocking effector secretion. The unconventional secretion of cytoplasmic effectors by the Golgi bypass pathway is a conserved means of delivering proteins into host cells via clathrin-mediated endocytosis (CME) in at least *M. oryzae* (Oliveira-Garcia et al. 2023) and *Phytophthora*

†Corresponding author: R. A. Wilson; rwilson10@unl.edu

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infestans (H. Wang et al. 2023). Although some aspects are known, however, a detailed molecular description of UPS covering all temporal events—from UPS regulation to cargo sorting to vesicle delivery at the plasma membrane—is lacking in any system. Here, we show how a recent breakthrough in our mechanistic understanding of UPS fine-tuning in *M. oryzae* led to the unanticipated uncovering, and subsequent cracking, of a codon code for effector secretion in *M. oryzae* that may be applicable to eukaryotic pathogens across kingdoms.

Effector Function and Discovery

Plant innate immunity

To suppress robust host defenses and cause disease, pathogens must avoid or overcome two tiers of plant innate immunity (Jones and Dangl 2006; Jones et al. 2024; Lu and Tsuda 2021; Ngou et al. 2022; Yuan et al. 2021). This need locks both partners in evolutionary warfare, resulting in elaborate molecular innovations for attack, defense, and counterattack. Following penetration of host cells by specialized cells such as pressurized and tightly adhered appressoria (*M. oryzae*), slicing appressoria-like structures (*P. infestans*), or hyphopodia (*V. dahlia*) (Bronkhorst et al. 2021; Rocha et al. 2020; Ryder et al. 2022; Zhao et al. 2016), the first line of host defense against filamentous eukaryotic pathogens is the recognition by cell-surface pattern recognition receptors (PRRs) of conserved pathogen-associated molecular patterns (PAMPs) (e.g., chitin) or damage-associated molecular patterns (DAMPs) (Ngou et al. 2022). PRR activation in response to PAMPs or DAMPs results in pattern-triggered immunity (PTI) stemming from reactive oxygen species (ROS) generation, calcium ion influx, mitogen-activated kinase (MAPK) induction, and transcriptional reprogramming leading to the upregulation of defense-related genes and the biosynthesis of antimicrobial compounds that collectively restrict pathogen invasion (Ngou et al. 2022). Although PTI is a powerful defense, pathogens can overcome it by secreting effector proteins either that mask detection (i.e., by preventing chitin-triggered immunity using chitin-binding LysM-domain-containing effectors to scavenge PAMP molecules [de Jonge et al. 2010; Mentlak et al. 2012]) or that otherwise inhibit the signal cascade emanating from PRR activation, such as through the targeting of the PRR-associated kinases BAK1 and BIK1 by the conserved fungal effector NIS1 (Irieda et al. 2019) or through the suppression of early transcriptional responses downstream of MAP kinase activation in the PTI pathway by the *P. infestans* WY-domain RxLR effector PiSFI3 (He et al. 2019). Pathogen virulence resulting from successful suppression of PTI is counteracted by direct or indirect recognition of effectors by host intracellular NLR-containing immune receptors (Jones and Dangl 2006; Ngou et al. 2022; Yuan et al. 2021). Effector recognition activates the second tier of defense, effector-triggered immunity (ETI), involving similar but stronger outputs to PTI including MAPK cascades, calcium flux, ROS bursts, transcriptional reprograming, and phytohormone signaling (Jones and Dangl 2006; Ngou et al. 2022; Yuan et al. 2021). Crosstalk between PTI and ETI is also apparent (Lu and Tsuda 2021; Yuan et al. 2021). In addition, although few examples are known, NLR immune receptors can be targets of effectors. For example, the *P. infestans* effector AVRcap1b suppresses the hypersensitive cell death response in *Nicotiana benthamiana* by interfering with the function of the NLR immune receptors NRC2 and NRC3 (Derevnina et al. 2021). A *P. capsici* effector, PcAvh103, suppresses immunity by disrupting the EDS1–PAD4 complex required, amongst other immune processes, for ETI (Li et al. 2020; Ngou et al. 2022). Thus, PTI and ETI operate to provide robust protection against most pathogens, but subversion of host defenses by

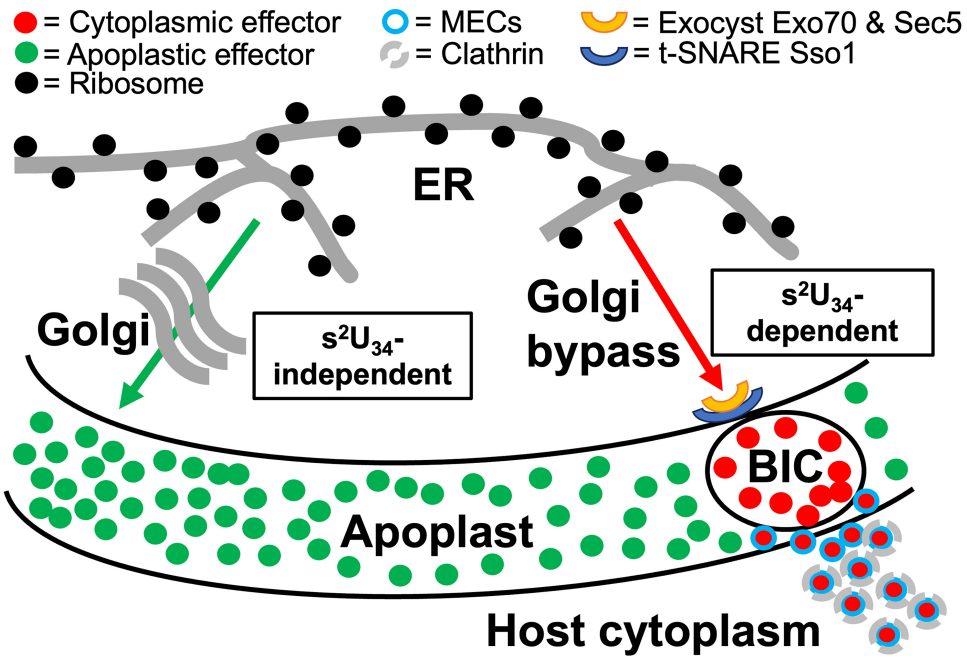

Fig. 1. Two effector secretion pathways operate in at least *Magnaporthe oryzae* and *Phytophthora infestans*. In both *M. oryzae* and *P. infestans* (shown here for *M. oryzae*), apoplastic effectors are secreted via the conventional endoplasmic reticulum (ER)-Golgi pathway, while cytoplasmic effectors, which carry signal peptides indicating they are targeted to the ER, bypass the Golgi to be secreted through the Golgi bypass unconventional protein secretion (UPS) pathway. Cytoplasmic effectors are delivered to the haustorium in *P. infestans* and secreted into the biotrophic interfacial complex (BIC) in *M. oryzae* before translocation into the host cell in a process, in both cases, involving plant clathrin-mediated endocytosis (CME). In *M. oryzae*, the translation of cytoplasmic effector mRNAs (but not apoplastic effector mRNAs) is dependent on a specific thiol modification on the wobble uridine (s^2U_{34}) of tRNAs decoding AA-ending codons. Delivery of Golgi bypass vesicles to the BIC requires the exocyst components Exo70 and Sec5 and the target soluble N-ethylmaleimide-sensitive factor attachment protein receptor (t-SNARE) Sso1. At the BIC, cytoplasmic effectors are packaged into dynamic vesicle-like membranous effector compartments (MECs), which associate with plant CLATHRIN LIGHT CHAIN 1 for uptake into the host cell. See text for details.

effectors targeting these pathways results in the catastrophic loss of plant health.

Effector classes and identification

Effector discovery is essential to the long-term goals of developing resistant crops. By knowing effector functions and host gene product targets, it could be possible to edit plant susceptibility genes when cognate NLRs are absent. Lines carrying designer NLR immune receptors with the potential to recognize any known effector are also being developed (Kourelis et al. 2023; Maidment et al. 2023). Furthermore, an understanding of effector evolution is important, as changes can lead to the emergence of new diseases (Le Naour-Vernet et al. 2023; Seong and Krasileva 2023). Computational approaches and machine learning tools aid effector discovery (Lovelace et al. 2023; Sperschneider and Dodds 2022; Wilson and McDowell 2022). RxLR and RxLR-dEER motifs downstream of the N-terminal SP have been used to find many putative cytoplasmic effector genes within the genomes of *Phytophthora* and downy mildew pathogens (S. Wang et al. 2023); oomycete cytoplasmic effectors can also carry WY domains and LxLFLAK motifs (Sperschneider and Dodds 2022). Fungal and oomycete apoplastic effectors are cysteine rich, whereas a hallmark of cytoplasmic effectors is enrichment for positively charged amino acids such as lysine (Sperschneider and Dodds 2022). Fungal effectors carry N-terminal SP sequences but otherwise lack common motifs that distinguish them as cytoplasmic effectors destined for the host cell. Fungal effectors may carry shared sequence-related motifs that define effector families, such as the cysteine-rich, GCD tripeptide, GRxV, and C-terminal FI/VGCA motifs encoded by the *HAG* class of effector genes in *M. oryzae* (Chen et al. 2022; Ebbole et al. 2021). However, due to rapid evolution, fungal effectors are diverse and often share little or no sequence similarity. Nonetheless, effectors can be grouped into related families using sequence-unrelated but structurally conserved protein folds. The *Magnaporthe* Avrs and ToxB-like (MAX) effector family, which includes apoplastic and cytoplasmic members (Li et al. 2023a), is sequence-unrelated but shares a common six-strand β-sandwich fold and is one of the few structural families to be functionally characterized and to have solved structures (de Guillen et al. 2015). Template-free modeling by TrRosetta in *M. oryzae* (Seong and Krasileva 2021) revealed new MAX family members, while AlphaFold and ChimeraX platforms grouped 863 putative effector proteins into 366 predicted structure clusters, including 38 predicted MAX effectors that are temporally co-expressed during biotrophy and necrotrophy (Yan et al. 2023). In a pangenome study of 120 *M. oryzae* isolates representing seven host-associated lineages, a combination of similarity searches and structure-guided alignments documented 58 to 78 MAX effector genes per genome (Le Naour-Vernet et al. 2023). Other characterized sequence-unrelated but conserved effector protein folds include the RNase fold found in RNase-like proteins expressed in haustoria (RALPH) effectors in *Blumeria graminis* f. sp. *hordei* (*Bgh*), which explain how conserved barley immune receptors recognize sequence-unrelated fungal *Bgh* effectors (Bauer et al. 2021), and the zinc finger protein fold that has functionally diversified in lineages of *M. oryzae* (De la Concepcion et al. 2024). In *Parastagonospora nodorum*, structural studies have revealed a novel fold in the SnTox3 effector protein (Outram et al. 2021).

Effector Delivery

Effector trafficking routes in fungi and oomycetes

Fungi and oomycetes deploy effectors into living host cells during the biotrophic stage of infection after forming IH (*M. oryzae*) or terminal haustoria (e.g., rust fungi and *Phytophthora infestans*) that are in close contact with host cytoplasm (Giraldo et al. 2013; Petre and Kamoun 2014; Wang et al. 2018; Yi and Valent 2013). Haustoria are wrapped in the extrahaustorial membrane (EHM), and *M. oryzae* IH are surrounded by plant-derived extra-invasive hyphal membranes (EIHM), with both forming extracellular spaces between the host cytoplasm and pathogen cell wall that receive apoplastic effectors. In *M. oryzae*, the EIHM matrix is sealed, and the leakage of effectors into the bulk apoplast is prevented (Yi and Valent 2013). Some examples of apoplastic effectors include Slp1 in *M. oryzae*, which masks fungal chitin detection by host surveillance mechanisms (Mentlak et al. 2012), and EPIC1 in *P. infestans*, which acts in the apoplast to inhibit cysteine proteases (Wang et al. 2017, 2018). In *M. oryzae*, cytoplasmic effectors are secreted into the specialized biotrophic interfacial complex (BIC), a focal plant lipid-rich structure that forms outside IH (but inside the EIHM) (Fig. 1). One BIC is formed in the first infected cell and then at the tips of IH that have spread to neighboring cells. Cytoplasmic effectors are packaged in the BIC into vesicle-like membranous effector compartments (MECs) that bud off from BIC outer layers and are translocated into the host cell via plant CME (Fig. 1); the accumulation of an effector in the BIC is thus the major predictor for its translocation into the rice cell (Oliveira-Garcia et al. 2023). Although BIC-less, cytoplasmic (but not apoplastic) effectors secreted from *P. infestans* haustoria are associated with host endosomes and are also translocated into host cells by plant CME (H. Wang et al. 2023), suggesting this is a conserved mechanism for the internalization of secreted cytoplasmic effectors.

The presence of N-terminal SP sequences historically suggested that most effectors were secreted via the conventional ER-Golgi secretion pathway, with the expectation that oomycete cytoplasmic effectors carrying RxLR motifs (and fungal cytoplasmic effectors carrying unspecified tags) would be further sorted for translocation into host cell cytoplasm. The SP is a leader sequence directing translating ribosomes to the ER, where the peptide is co-translationally folded before being transported to the Golgi and on to the plasma membrane. The prevalence of a SP and the assumption of conventional ER-Golgi trafficking has ensured that pathogen secretion routes remain poorly characterized in filamentous pathogens (Petre and Kamoun 2014). Nonetheless, BFA treatments that block conventional ER-Golgi secretion has verified the ER-Golgi route for effector secretion in *M. oryzae* (Giraldo et al. 2013), *Colletotrichum orbiculare* (Irieda et al. 2014), *Verticillium dahliae* (Zhou et al. 2017), and *P. infestans* (Wang et al. 2017, 2018). Mechanistically, gene knockouts show that in *C. orbiculare* (Irieda et al. 2014), the transport of effectors between ER and Golgi requires the v-SNARE protein SEC22, whereas in *M. oryzae*, a COPII cargo receptor, MoErv29, mediates efficient ER-to-Golgi trafficking of secreted proteins including at least the apoplastic effector Slp1 (Qian et al. 2022). However, BFA treatment also revealed BFA-insensitive trafficking of SP-carrying cytoplasmic (but not apoplastic) effectors in at least *M. oryzae* and *P. infestans*, indicating the use of UPS pathways. BFA-insensitive effectors are targeted to the ER but bypass the Golgi (Giraldo et al. 2013; Wang et al. 2017) and are secreted by what is known as the Type IV Golgi bypass UPS pathway (Li et al. 2023a; Rabouille 2017; Stuer et al. 2023). In at least *M. oryzae* and *P. infestans*, cytoplasmic effectors are translocated into host cells after first being secreted via the Type IV UPS pathway into the BIC or haustorium, respectively (Giraldo et al. 2013; Wang et al. 2017). In *C. orbiculare*, a BIC-like structure forming under appressoria and as a ringlike structure around primary hyphae has been described (Irieda et al. 2014), but effectors secreted into this structure traffic via the ER-Golgi network and, to our knowledge, unconventional secretion in *Colletotrichum* spp. has not yet been demonstrated.

A need for speed? Potential purposes and known determinants of the Type IV Golgi bypass UPS pathway

What is the purpose of the Golgi bypass UPS pathway, and what proteins and cellular processes are involved? The uptake of cytoplasmic effectors into the host cell via plant CME, followed by escape into the host cytoplasm, is the only known mechanism for fungi and oomycetes to deploy effectors against host cellular targets, suppress immunity, and overcome host resistance (Oliveira-Garcia et al. 2023; H. Wang et al. 2023). Therefore, it is imperative that the underlying mechanism(s) and rules governing the unconventional secretion of cytoplasmic effectors are established. However, while the Type IV Golgi bypass UPS pathway, like other UPS pathways, is prevalent across eukaryotic taxa, its purpose and machineries are largely unknown (Grieve and Rabouille 2011; Rabouille 2017). With regards to plant pathogens, it is also not clear why fungal and oomycete cytoplasmic effectors are secreted via the Golgi bypass route or how effectors are sorted into the UPS versus conventional secretion pathways. In other systems, the Golgi bypass UPS pathway is triggered by ER and mechanical stress, which may impair the functional integrity of the conventional ER-Golgi system, forcing some proteins into alternative secretory routes (Rabouille 2017). This trigger could be operational in *M. oryzae* because the unfolded protein response (UPR), an ER stress response, is activated during biotrophy (Qian et al. 2022) and because an ER-resident chaperone Lhs1, which functions as part of the UPR to facilitate protein folding in the ER lumen, is required for effector secretion (Yi et al. 2009). Furthermore, the Golgi bypass UPS may act faster than conventional ER-Golgi secretion (Grieve and Rabouille 2011), which would be important in deploying cytoplasmic effectors like Pwl2 that not only target host proteins in the cytoplasm of the first colonized host cell to modulate resistance but also migrate to neighboring cells ahead of IH (Khang et al. 2010; Oliveira-Garcia et al. 2023). In addition, trafficking through the Golgi bypass UPS pathway may modulate the activity of UPS cargo proteins by avoiding glycosylation in the Golgi stack (Grieve and Rabouille 2011), but this has not yet been demonstrated in plant pathogens. In *Aspergillus nidulans*, the Golgi bypass UPS pathway is a means to deploy transporters nonpolarly and homogenously, via the formation of distinct COPII subpopulations, along the hyphal membrane in contrast to polarly localized apical membrane cargoes secreted by the conventional ER-Golgi pathway (Dimou et al. 2020). This observation could be relevant to *M. oryzae* because although cytoplasmic effectors are secreted into a discrete BIC and are not localized homogenously along IH, the BIC itself is a subapical structure after the first few rounds of IH cell division, when it moves from contacting the primary hyphal tip to a location beside the first differentiated bulbous IH cells (Giraldo et al. 2013; Oliveira-Garcia et al. 2023). Thus, several, nonexclusive reasons for employing a UPS pathway are conceivable and testable, and future work should address which are the most likely drivers of cytoplasmic effector secretion in fungi and oomycetes.

The machinery of Golgi bypass UPS is almost entirely unknown in fungi and oomycete plant pathogens, although some molecular determinants have been characterized in *M. oryzae*. In *M. oryzae*, the ER-resident chaperone Lhs1 is required for the BIC accumulation of the cytoplasmic effector AVR-Pita (Yi et al. 2009) as well as for the secretion of other proteins, and this may be due to its role in translocating secreted proteins into the ER, suggesting Lhs1 is necessary for, but upstream of, the Golgi bypass UPS pathway. At the terminal end of the Golgi bypass UPS pathway, fusion to the plasma membrane of vesicles with Golgi bypass cargo has been functionally shown in *M. oryzae* to involve Exo70 and Sec5, which are two of eight exocyst components that tether vesicles to target membranes before fusion. The loss of Exo70 or Sec5 reduced the accumulation of the Bas1 and Pwl2 cytoplasmic effectors in the BIC and retained them in IH (Giraldo et al. 2013). Exocyst tethering occurs after vesicle delivery to the plasma membrane and proceeds soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-mediated vesicle docking and fusion (Heider and Munson 2012). SNAREs comprise vesicle (v-) SNAREs on the vesicle and target (t-) SNAREs on the membrane. Vesicles carrying Golgi-bypass cargoes may contain a different set of SNAREs than are needed for conventional secretion (Grieve and Rabouille 2011). Perhaps consistent with this notion, the t-SNARE complex component Sso1 is required for the accumulation of effectors at the BIC, and the loss of Sso1 results in a double BIC phenotype (Giraldo et al. 2013). In addition, the v-SNARE protein MoSnc1 is required for retromer-dependent vesicle docking at the plasma membrane, and its loss results in Pwl2 accumulating in IH in addition to impaired Bas4 ER-Golgi secretion, suggesting MoSnc1 is required for both secretion pathways (Chen et al. 2023b). The full complement of t- and v-SNAREs involved in the Golgi bypass UPS pathway and how they differ from those required for ER-Golgi secretion requires elucidation. Other global determinants of cytoplasmic effector secretion include ergosterol-enriched lipid rafts that foster the interaction of SNAREs, including MoSso1 and MoSnc1 (Guo et al. 2023), and fungal autophagy that maintains membrane homeostasis to ensure the integrity of BICs and the EIHM for effector deployment (Li et al. 2023b; Sun et al. 2018).

Upstream of the exocyst, a molecular determinant of cytoplasmic (but not apoplastic) effector secretion, operating at the level of mRNA translation efficiency and protein folding and/or packaging into Golgi bypass vesicles, is the Uba4-Urm1 sulfur relay system (Li et al. 2023a). These two enzymes act together to add a thiol group to the 2-position (s^2) of wobble uridine (U_{34}) in tRNA anticodons that match cognate AA-ending codons specifying the positively charged (at physiological pH) amino acid lysine (AAA), the polar uncharged amino acid glutamine (CAA), and the negatively charged amino acid glutamic acid (GAA). The s^2 modification on U_{34} ensures stable base pairing between anticodon loops and their cognate codons, resulting in efficient decoding of AA-ending codons. The s^2U_{34} modification is almost universally conserved and is always found along with 5-methoxycarbonylmethyl (mcm^5) modifications attached to the same base (Pabis et al. 2020). The absence of the s^2 modification on tRNA anticodon wobble U_{34} impairs the decoding of AA-ending cognate codons, leading to ribosome pausing that affects the proteome (Nedialkova and Leidel 2015). The s^2 modification is not required for decoding synonymous AG-ending codons (i.e., AAG for Lys, CAG for Gln, and GAG for Glu). In *M. oryzae*, inefficient decoding of AA-ending codons in Δ*uba4* and Δ*urm1* mutant strains lacking the s^2U_{34} modification resulted in ribosome pausing and the loss of translation of mRNAs encoding cytoplasmic (Pwl2, AVR-Pita, and AVR-Pik) but not apoplastic (Bas4 and Slp1) effectors (Li et al. 2023a; Figs. 1 and 2), perhaps again pointing to speed (in terms of translational efficiency and protein folding accuracy) as an important factor in the deployment of cytoplasmic effectors via the Golgi bypass UPS pathway.

Cracking the Codon Code for Unconventional Effector Secretion by Filamentous Eukaryotic Plant Pathogens

A need for (translational) speed control?

How does *M. oryzae* use s^2U_{34} modifications to discriminate between apoplastic and cytoplasmic effector-encoding mRNAs at the translational level? Cytoplasmic effector mRNAs

in *M. oryzae* are enriched for AA-ending codons over synonymous AG-ending codons, while AG-ending codons predominate in apoplastic effector mRNAs and when summed across all *M. oryzae* protein-coding sequences (Li et al. 2023a; numbers shown for Pwl2 and Bas4 in Fig. 2). The loss of efficient AA-ending codon decoding in s^2U_{34}-deficient strains resulted in ribosome pausing on cytoplasmic (but not apoplastic) effector mRNAs. Pausing likely caused protein misfolding in the ER and triggered a ribosome-associated quality control pathway that eliminated the nascent peptides and associated mRNAs (Li et al. 2023a) before the proteins could be packaged into Golgi bypass secretory vesicles. However, the translation of a recoded version of the apoplastic effector-encoding *BAS4* mRNA, carrying all AA-ending codons, was not affected by the loss of s^2U_{34} (Li et al. 2023a), suggesting ribosomal pausing is tolerated for apoplastic effector translation during ER translocation and folding in the ER-Golgi pathway.

A recoded version of *PWL2* mRNA with all AA-ending codons changed to synonymous AG-ending codons, *PWL2* (*–AA → –AG*), restored Pwl2 production in Δ*uba4* (Li et al. 2023a). This raised the following question: why would *M. oryzae* evolve mRNAs of cytoplasmic effectors with predominantly AA-ending codons, requiring efficient decoding by a multi-gene tRNA modification system involving sulfur, when an all AG-ending codon transcript can be translated in the absence of this sulfur-relay system? To address this, *PWL2* (*–AA → –AG*) was expressed in the wild-type strain Guy11 (Li et al. 2023a). This resulted in huge, unstable BICs (Fig. 3)—due to the unregulated translation and super-secretion of recoded Pwl2—that destabilized the host-pathogen interface and abolished virulence (Li et al. 2023a). Without altering the protein sequence or transcript level, *M. oryzae* infection was thus abolished by expressing synonymously recoded *PWL2* (*–AA → –AG*), resulting in Pwl2 super-secretion and indicating that secretion information with biological relevance is encoded at the codon-usage level (Li et al. 2023a). Biased use of adapted, abundant codons in the genome (like AG-ending codons; 71% frequency in *M. oryzae*) facilitate the fast translation of highly expressed mRNAs, whereas the use of poorly adapted, rare codons in the genome (like AA-ending codons; 29% frequency in *M. oryzae*) result in slower but more accurate mRNA translation, which is especially needed for co-translational protein folding (Plotkin and Kudla 2011). So, the results from *M. oryzae* suggest that the precise control of translation speed, at the level of AA-ending (but not synonymous AG-ending) codon decoding, is critical for optimizing cytoplasmic effector protein folding and modulating secretion rates to maintain BIC integrity and facilitate pathogenicity. For apoplastic effectors, this translation speed requirement is less stringent, and pausing at AA-ending codons in apoplastic effector mRNAs in Δ*uba4* and Δ*urm1* strains is tolerated. Considering the Golgi bypass UPS pathway is the only known means to deploy effectors into host cytoplasm, the use of poorly adapted, rare AA-ending codons in cytoplasmic effector mRNAs (that are decoded by a rather elaborate system involving tRNA modification and the Uba4-Urm1 sulfur relay system) likely represents an evolutionary trade-off between a need to rapidly deploy effectors into host cells and a need to precisely control translation speeds to secure BIC integrity. These constraints are apparently absent for proteins secreted via the conventional ER-Golgi pathway.

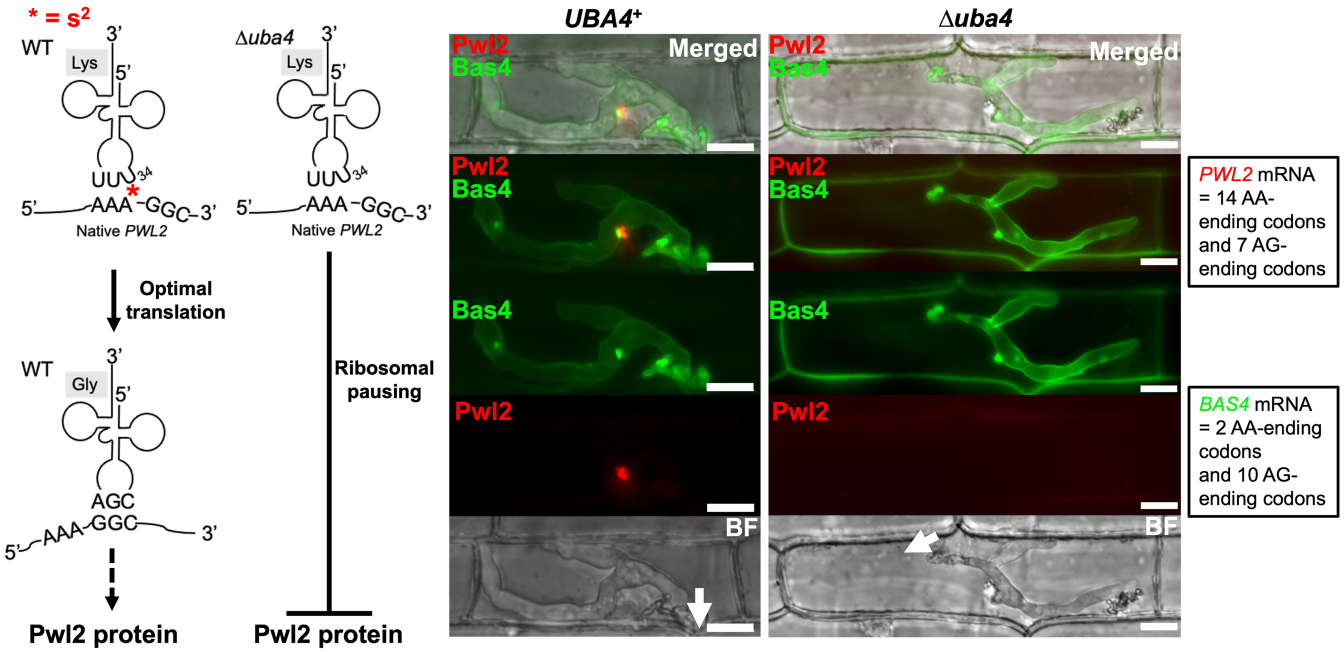

Fig. 2. The loss of wobble uridine (s^2U_{34}) tRNA modification abolishes cytoplasmic but not apoplastic effector production. For optimal mRNA translation, anticodons of tRNAs decoding cognate AA-ending codons (shown here for AAA specifying Lys) require a thiol modification on position 2 of their wobble uridines. Loss of the s^2U_{34} tRNA modification caused ribosome pausing at AA-ending codons, resulting in activation of a ribosome-associated quality control pathway that destroyed the nascent peptide and associated mRNA (Li et al. 2023a). In *Magnaporthe oryzae*, *PWL2* mRNA (like other cytoplasmic effector-encoding mRNAs) is enriched for AA-ending codons and depleted for AG-ending codons, whereas the reciprocal is true for *BAS4* mRNAs and other apoplastic effector-encoding mRNAs. Consequently, the loss of wobble uridine thiolation (s^2U_{34}) in, for example, Δ*uba4* strains abolished Pwl2 production and unconventional secretion into the biotrophic interfacial complex (BIC) but did not affect the production and conventional endoplasmic reticulum (ER)-Golgi secretion of apoplastic effectors like Bas4 into the space around invasive hyphae (IH). In the example shown here, *UBA4*$^+$ and Δ*uba4* are near-isogenic strains carrying a vector expressing *PWL2-mCherry:NLS* and *BAS4-GFP*. Live-cell images of detached rice leaf sheaths inoculated with the indicated strains were obtained at 36 h postinoculation using a Nikon Eclipse Ni-E upright epifluorescence microscope and NIS-Elements software (N. Dulal and R. A. Wilson, *unpublished*). The scale bar is 10 μm; BF = bright field; white arrows indicate penetration sites. Note that ribosome pausing on the codon shown on the left is for illustration purposes only, and it is not known on which AA-ending codon(s) the ribosome pauses in s^2U_{34}-deficient strains. WT, wild type.

Effector prediction

High AA-ending codon rates are selected in cytoplasmic effector mRNAs to tune translation, optimize secretion through the Golgi bypass UPS pathway, and safeguard the integrity of the BIC. Experimentally determined MAX effectors in *M. oryzae*, grouped together based on conserved but sequence-unrelated structural folds, have higher AA-ending codon rates in cytoplasmic MAX effector mRNAs than apoplastic MAX effector mRNAs, which are depleted for AA-ending codons in favor of synonymous AG-ending codons (Li et al. 2023a). Cytoplasmic MAX effectors may therefore be under dual evolutionary pressure to both adopt similar sequence-unrelated protein folds that determine their activity in rice cells and to evolve high AA-ending codon content that determines their secretion into host cells. Conversely, also in line with this general rule, mRNA of the apoplastic effector ToxB from *Pyrenophora tritici-repentis* is depleted for AA-ending codons in favor of AG-ending codons (Li et al. 2023a). AA-ending codon usage rates (the number of AA-ending codons in an mRNA as a fraction of the total number of AA- and AG-ending codons in the same mRNA) may thus be used to improve the prediction of cytoplasmic effectors, and this was demonstrated using *AVR-Pik* (92% AA-ending codon usage rate; actual number = 12 AA-ending codons and 1 AG-ending codon), which was predicted and subsequently shown to encode a cytoplasmic effector secreted into the BIC via the Golgi bypass UPS pathway in a s^2U_{34}-dependent manner (Li et al. 2023a). Such ability to predict cytoplasmic effectors based on AA-ending codon rates may extend to oomycete effectors. Figure 4 shows AA- and synonymous AG-ending codons in the protein-coding regions of the cytoplasmic *Phytophthora infestans* effector Pi04314 and the apoplastic effector EPIC1 (Wang et al. 2017). Consistent with observations in *M. oryzae*, the AA-ending codon usage rate is much higher in *Pi04314* mRNA (44%) than in *EPIC1* mRNA (14%), demonstrating AA-ending codon enrichment in *Pi04314* relative to *EPIC* and the *P. infestans* transcriptome (the AA-ending codon usage rate across *P. infestans* protein-coding sequences is 21% based on 400 open reading frames [ORFs] in Randall et al. 2005). Furthermore, the coding sequence of the Pi04314 RxLR-dEER motif carries three clustered AA-ending codons flanked by two AG-ending codons (Fig. 4), suggesting that translational speed control across the RxLR-dEER motif-encoding section of *Pi04314* mRNA may be important for cytoplasmic effector secretion in oomycetes. While this role needs to be experimentally determined, these observations nevertheless suggest that AA-ending codon decoding underlies cytoplasmic effector secretion across kingdoms, which may enable the further dissection of the role of AA-ending codons in protein secretion. Furthermore, incorporating AA-ending codon usage rates may help improve machine learning-based effector prediction methods such as EffectorP 3.0 (Sperschneider and Dodds 2022).

Conclusions and Open Questions

Knowledge of effector function and evolution, and effector prediction, may be enhanced by a better understanding of effector secretion. Cytoplasmic effectors destined for the host

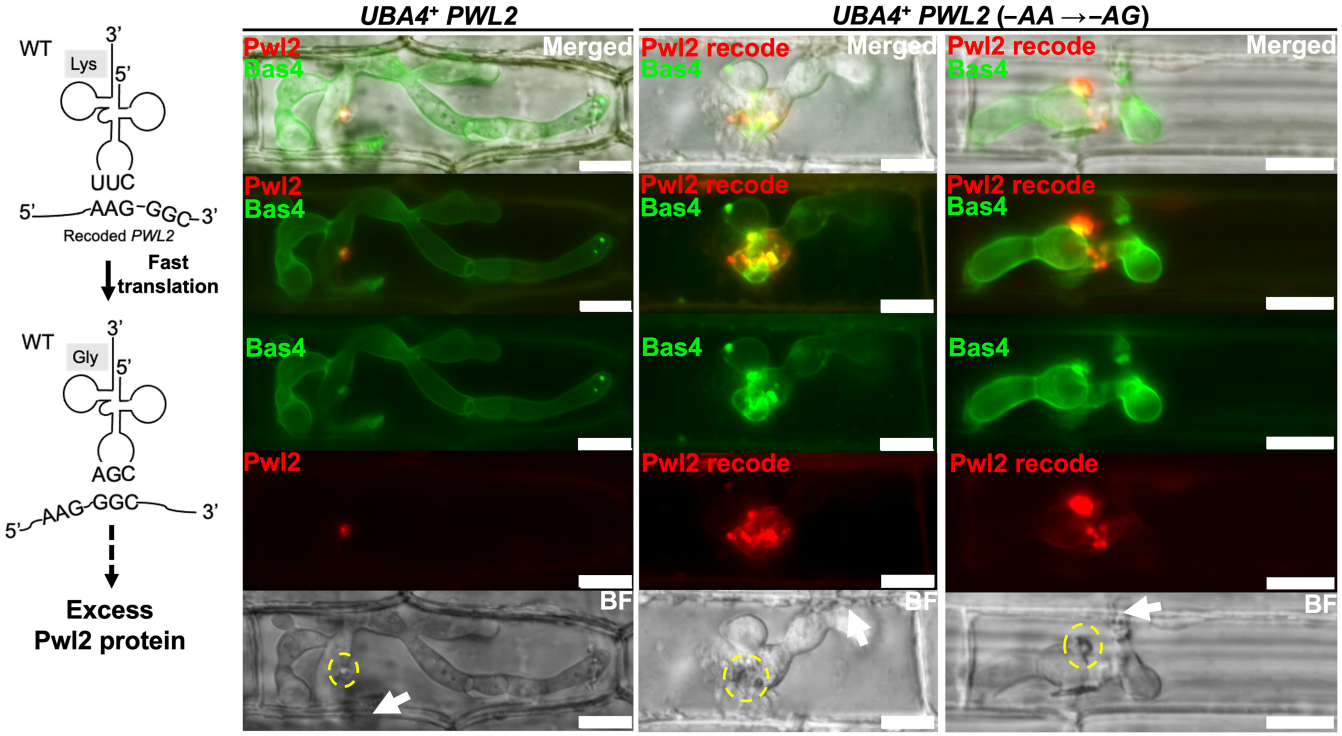

Fig. 3. Simply changing AA-ending codons to synonymous AG-ending codons in *PWL2* mRNA results in giant, unstable biotrophic interfacial complexes (BICs) that impair infection. Expressing a recoded version of *PWL2*, denoted as *PWL2* (*–AA → –AG*), where all AA-ending codons are exchanged for synonymous AG-ending codons, in wobble uridine thiolation (s^2U_{34})-sufficient strains resulted in unregulated translation leading to the overproduction and super-secretion of recoded Pwl2 into BICs, which became enlarged and fractured (two independent examples shown; N. Dulal and R. A. Wilson, *unpublished*). Such disruption of the host-pathogen biotrophic interface negatively affected virulence (Li et al. 2023a). Thus, pathogenicity is determined by the codon usage bias of cytoplasmic effector mRNAs and a hitherto unappreciated need for secretion fine-tuning encoded at the level of the individual codon. *UBA4*$^+$ *PWL2* is an s^2U_{34}-sufficient strain expressing native *PWL2-mCherry:NLS* and *BAS4-GFP*. *UBA4*$^+$ *PWL2* (*–AA → –AG*) is an s^2U_{34}-sufficient strain expressing recoded *PWL2-mCherry:NLS* and native *BAS4-GFP*. Live-cell images of detached rice leaf sheaths inoculated with the indicated strains were obtained at 36 h postinoculation using a Nikon Eclipse Ni-E upright epifluorescence microscope and NIS-Elements software. The scale bar is 10 μm; BF = bright field; white arrows indicate penetration sites; and yellow dotted circles indicate BICs in BF, which are enlarged in *UBA4*$^+$ *PWL2* (*–AA → –AG*) strains compared with the *UBA4*$^+$ *PWL2* strain. WT, wild type.

cytoplasm via plant CME are secreted in an unconventional manner via the Golgi bypass UPS pathway, which requires the exocyst complex, a t-SNARE, and mRNAs enriched for AA-ending codons that allows the precise tuning of mRNA translation rates. Efficient AA-ending codon decoding facilitates the co-translational folding of effector proteins after translocation through the ER and before or during packaging into Golgi bypass vesicles for transport to the plasma membrane. Without altering the protein sequence, *M. oryzae* infection was abolished by changing rare AA-ending codons in *PWL2* to synonymous, abundant AG-ending codons. These synonymous changes deregulated mRNA translation by accelerating codon decoding, leading to protein overproduction and super-secretion into BICs, which became inflated and unstable. Conversely, ribosome pausing at AA-ending codons in s^2U_{34}-deficient strains initiated a ribosome-associated quality control pathway that eliminated the nascent peptide and caused cytoplasmic effector mRNA instability (Li et al. 2023a). Ribosome pausing is tolerated for apoplastic effectors including recoded *BAS4* (Li et al. 2023a), suggesting that apoplastic and cytoplasmic effector proteins are folded and packaged in distinct compartments or interact with distinct co-translational machinery. The Golgi bypass UPS pathway is likely to be highly conserved and could be targeted to inhibit host infection as well as studied in a comparative manner to learn more about the rules governing cytoplasmic effector secretion into host cells. Outstanding questions regarding cytoplasmic effector secretion by the Golgi bypass UPS pathway include the following:

- How are Golgi bypass UPS cargoes recognized? Does recognition involve mRNA translational speed control, dictated by rare AA-ending codons, rather than a common motif? Does recognition occur before or after the regulation of translation speed by the first AA-ending codon? Does recognition involve co-translational proteins, vesicle coat proteins, or both?
- How is the ribosome-associated quality control pathway triggered in response to ribosome pausing? What proteins are involved? Which AA-ending codon(s) are involved?
- Ribosome pausing leads to nascent peptide destruction and cytoplasmic effector mRNA instability, with the latter likely via the No-Go Decay mRNA surveillance pathway (Li et al. 2023a). Does No-Go Decay play a role in controlling effector gene transcript levels under normal infection conditions or only in s^2U_{34}-deficient mutant strains?
- What machinery lies between protein folding in the ER and exocyst and SNARE-dependent docking and fusion of Golgi bypass vesicles to the plasma membrane? What other SNAREs or COPII subunits are involved? Are different COPII subpopulations involved compared with ER-Golgi secretion?
- Precisely why is ribosome pausing at AA-ending codons in apoplastic effector mRNAs tolerated?
- Why are there two or more pathways for effector secretion? Is the need for speed alone sufficient to explain the delivery of cytoplasmic effectors by UPS pathways when the ER-Golgi pathway is stressed?

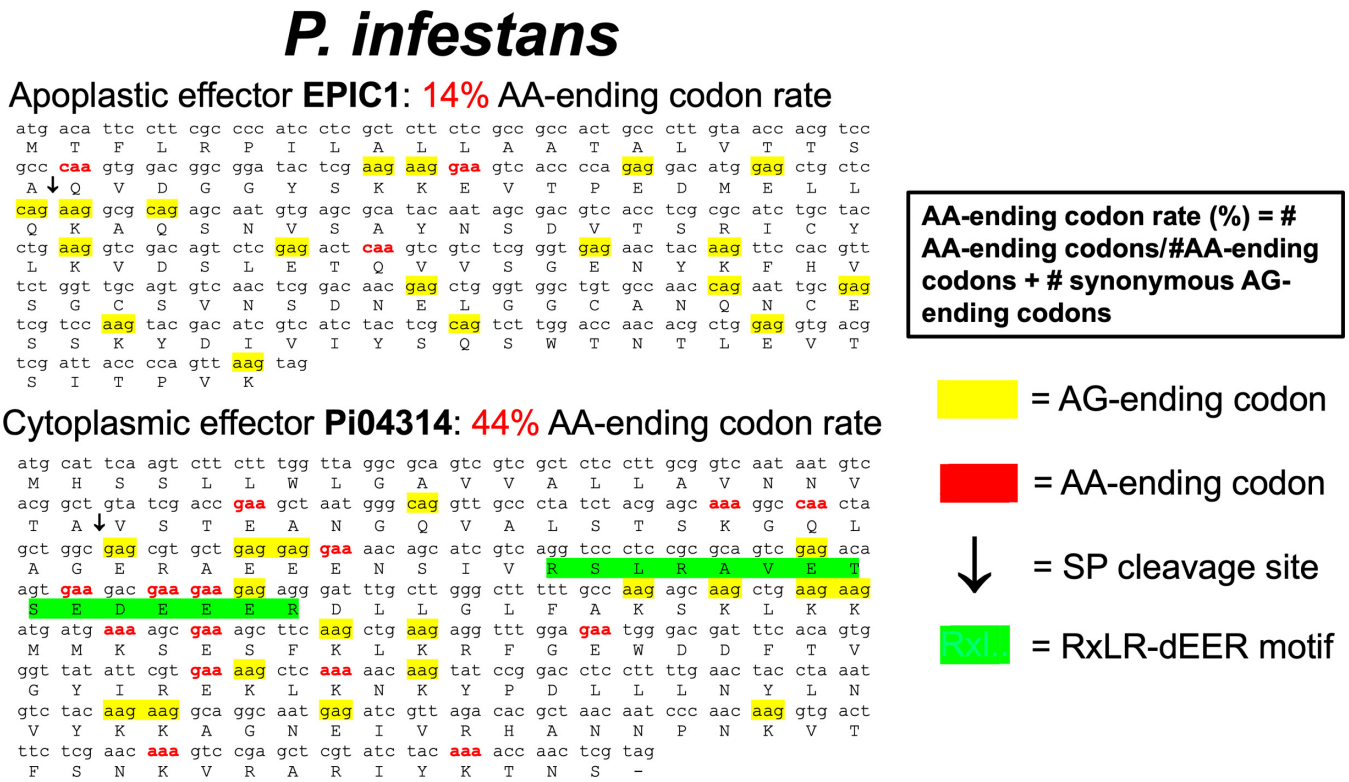

Fig. 4. Conservation across kingdoms of high AA-ending codon usage rates in cytoplasmic effector mRNA. In *Phytophthora infestans*, the AA-ending codon usage rate (the fraction of AA-ending codons as a percentage of the total number of AA- and synonymous AG-ending codons in a transcript) is low (14%) in the protein-coding sequence of the apoplastic effector EPIC1 (and 21% when averaged across 400 open reading frames [ORFs]; Randall et al. 2005), but it is considerably higher (44%) in the protein-coding sequence of the cytoplasmic effector Pi04314. *Pi04314* mRNAs carry 14 AA-ending codons compared with three AA-ending codons in *EPIC1*, and a cluster of AA-ending codons are prevalent across the Pi04314 RxLR-dEER motif-encoding sequence. These observations suggest that AA-ending codon usage rates may predict effector secretion routes across kingdoms, that AA-ending codon-dependent translational speed control may be important for fine-tuning unconventionally secreted effectors in oomycetes as well as fungi, and that, in oomycetes, translation of the RxLR-dEER motif-encoding codons may be particularly sensitive to AA-ending codon-dependent speed modulation. Demarcation of the Pi04314 RxLR-dEER motif (in green; amino acids 53 to 67) was obtained from Uniprot (D0N0Z8). Signal peptide (SP) cleavage sites were obtained from Uniprot for Pi04314 (D0N0Z8) and EPIC1 (A1L015). Coding sequences were obtained from NCBI for *Pi04314* (locus: NW_003303754) and *EPIC1* (locus: AY935250).

- Is efficient AA-ending codon decoding a requirement for secretion through other UPS pathways such as leaderless peptide secretion or for proteins packaged in exosomes? Relatedly, why is the Golgi bypass UPS pathway apparently favored for effector delivery over other UPS routes such as leaderless secretion or secretion via exosomes?
- Can AA-ending codon rates predict unconventionally secreted cytoplasmic effectors across all fungi and oomycetes?
- In *P. infestans*, what, if any, is the functional role of the AA-ending codons within the RxLR-dEER motif-encoding sequence?
- How is the Golgi bypass UPS mechanistically connected to BICs? Why does increased secretion of recoded Pwl2 into the BIC lead to BIC membrane expansion?
- Do the giant, unstable BICs resulting from recoded Pwl2 super-secretion imply impaired or overloaded packaging into MECs? Are MECs limiting when it comes to uptake into the host cell?
- Do all AA-ending codons contribute to fine tuning of the Golgi bypass UPS or just a subset? Are the decoding rates of AA-ending codons context dependent?

Answering these and other related questions—likely by employing molecular genetics, chemical inhibition, and super-resolution and cryo-EM approaches—may lead to a comprehensive understanding of effector deployment by the Golgi bypass UPS pathway that fosters effector and host target protein discovery and thus accelerates the development of innovative approaches to pathogen control.

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