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Tandem Protein Kinases Emerge as New Regulators of Plant Immunity

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Abstract

Plant–pathogen interactions result in disease development in a susceptible host. Plants actively resist pathogens via a complex immune system comprising both surface-localized receptors that sense the extracellular space as well as intracellular receptors recognizing pathogen effectors. To date, the majority of cloned resistance genes encode intracellular nucleotide-binding leucine-rich repeat receptor proteins. Recent discoveries have revealed tandem kinase proteins (TKPs) as another important family of intracellular proteins involved in plant immune responses. Five TKP genes—barley *Rpg1* and wheat *WTK1* (*Yr15*), *WTK2* (*Sr60*), *WTK3* (*Pm24*), and *WTK4*—protect against devastating fungal diseases. Moreover, a large diversity and numerous putative TKPs exist across the plant kingdom. This review explores our current knowledge of TKPs and serves as a basis for future studies that aim to develop and exploit a deeper understanding of innate plant immunity receptor proteins.

Keywords

fungus–plant interactions; intracellular perception proteins; plant defense response system; plant–pathogen interactions; plant responses to pathogens; *Pm24*; resistance gene; *Rpg1*; *Sr60*; wheat tandem kinase (WTK); *WTK4*; *Yr15*

Crop losses due to plant diseases significantly affect agricultural production. For example, the global cost of fungicide applications to control wheat stripe rust can exceed US$1

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AUTHOR-RECOMMENDED INTERNET RESOURCE
Disease Resistance Analysis and Gene Orthology: prgdb.org/prgdb/drago2

billion annually (Chen 2020). To resist pathogen infection, plants rely upon structural barriers, physiological differences, and preformed defense compounds as well as active defense mechanisms (Zhang et al. 2020a). The plant innate immune system includes receptors that actively recognize all pathogen classes and induce defense signaling responses that culminate in expression of host resistance. Surface-localized plant immune receptors encoding receptor-like kinases (RLKs) or receptor-like proteins (RLPs) are capable of recognizing diverse molecules and proteins of pathogen and plant origin (Boutrot and Zipfel 2017). Intracellular plant immune receptors frequently encode nucleotide-binding leucine-rich repeat (NLR) receptor proteins (Lolle et al. 2020). NLRs are able to recognize pathogen effector proteins that are delivered inside plant cells during infection. Recent discoveries highlighted overlap in immune signaling mediated by surface-localized and intracellular immune receptors as well as crosstalk between both receptor types (Ngou et al. 2021; Tian et al. 2020; Yuan et al. 2021). Although both receptor classes are structurally distinct and localize to different cellular compartments, they share significant overlap in immune signaling, including Ca^{2+} influx, activation of mitogen-activated protein kinases (MAPKs), production of extracellular reactive oxygen species, transcriptional reprogramming, and deposition of physical barriers for cellular fortification (Zhang et al. 2020a).

Posttranslational modification of proteins is an important component regulating all cellular signaling, including plant immune signaling. To rapidly regulate the induction, amplitude, and duration of defense, plant immune receptors and signaling proteins are frequently presynthesized and regulated posttranslationally (Liu et al. 2016). Surface-localized receptors such as the bacterial flagellin receptor FLS2 require auto- and transphosphorylation for activation (Chinchilla et al. 2007; Gómez-Gómez et al. 2001). Downstream of receptor activation, signaling networks controlled by calcium-activated kinases and MAPK phosphorylation act to regulate transcriptional reprogramming (Li et al. 2016). Receptor-like cytoplasmic kinases (RLCKs) are a subset of plant RLKs that lack both extracellular and transmembrane domains. Several members of the RLCK subfamily VII are required for robust immune responses, can interact with pattern recognition receptors (PRRs) and NLR immune receptors, act as an interface between surface-localized receptors and MAPK activation, and directly activate production of reactive oxygen species (Kadota et al. 2015; G. Wang et al. 2015; Wang et al. 2017; Yamada et al. 2016).

Plant cell walls not only act as a physical barrier for pathogens but also contain surface-localized receptors capable of recognizing pathogen effectors, conserved pathogen features, and damage induced during infection. An intriguing kinase family that is also involved in plant defense consists of wall-associated kinases (WAKs). WAKs are RLKs that encode a serine/threonine kinase domain and an epidermal growth factor-like domain (Shiu and Bleecker 2001). The WAK extracellular domains are capable of binding small pectic oligosaccharides that are released during pathogen-induced cell wall degradation with high affinity (Kohorn et al. 2014). Multiple research groups have posited that WAKs are involved in monitoring the integrity of the cell wall and activate intracellular signaling upon disruption by pathogen attack or abiotic stress (Kohorn 2015; Rui and Dinneny 2020). Tomato *WAK1* genome-edited lines were compromised in callose-induced cell wall fortification after perception of bacterial flagellin (Zhang et al. 2020c). Individual WAKs have also been demonstrated to be important regulators of disease resistance. *TaWAK6* is

effective in restricting pathogen growth, playing a role in adult plant resistance to leaf rust in wheat (Dmochowska-Boguta et al. 2020). *Stb6* encodes a WAK recognizing a small secreted protein from the wheat pathogen *Zymoseptoria tritici* (Brading et al. 2002; Kema et al. 2018; Saintenac et al. 2018; Zhong et al. 2017).

Cysteine-rich receptor-like kinases (CRKs) possess extracellular cysteine-rich domains and have been implicated in plant immunity. In their extracellular region, most CRKs possess two DUF26 domains with a conserved C-X8-C-X2-C motif (Chen 2001).Quantitative trait loci corresponding to disease resistance map to CRK clusters, highlighting their importance in the plant kingdom (Chern et al. 2016; Larkan et al. 2016; Xu et al. 2016). High-level expression of some CRKs has been demonstrated to induce cell death, a hallmark of plant defense (Acharya et al. 2007; Yadeta et al. 2017). Although no CRKs have been identified as primary immune receptors, they are coordinately induced in response to activation of PRRs and NLRs, suggesting that they may act to amplify defense signaling (Elmore et al. 2012; Kadota et al. 2019; Yadeta et al. 2017).

Recently, a novel class of intracellular resistance (R) proteins with tandem kinase domain architecture has emerged. Tandem kinase proteins (TKPs) are a protein family that contains two kinase domains fused by a linker region. Diverse families of kinases can be present in different TKPs. In contrast to NLRs and surface-localized receptors, TKPs are less well known and less studied, and have not been included in previous models. This review provides an analysis of our current understanding of TKPs, justifies their important role in plant–microbe interactions, and advocates for their incorporation into current models of the plant immune system.

WIDE DISTRIBUTION OF TKPS IN THE PLANT KINGDOM

Because individual kinase domains found in TKPs vary, they cannot be identified in plant genomes based on homology alone and comprise a novel protein family. To date, approximately 100 putative TKPs have been predicted from the genome sequences of diverse plants, such as the Bryophyta *Physcomitrella patens*; monocots *Aegilops speltoides*, *A. tauschii*, *Triticum aestivum*, *T. turgidum* subsp. *durum*, *T. turgidum* subsp. *dicoccoides*, *T. urartu*, *Hordeum vulgare, Secale cereale*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor*, and *Brachypodium distachyon*; and dicots *Arabidopsis thaliana*, *Solanum tuberosum*, *Brassica napus*, and *Populus trichocarpa* (Klymiuk et al. 2018; Lu et al. 2020). Analyses of their structure revealed that most possess a putative tandem kinase-pseudokinase architecture (Klymiuk et al. 2018), determined by the presence or absence of the eight key conserved residues (Hanks et al. 1988) that are critical for phosphorylation activity. In addition, conserved residues in sequences neighboring the core motifs were described for putative kinase domains of TKP family members, suggesting that they share a common structure (Klymiuk et al. 2018).

Phylogenetic analyses of kinase and pseudokinase domains of the 92 predicted TKPs showed that they could be differentiated into 11 major clades (Klymiuk et al. 2018) that have clear relationships with specific, annotated *Arabidopsis* kinome families or subfamilies according to homology-based comparisons (Zulawski et al. 2014). These include

superfamilies of plant RLKs and soluble kinases (Klymiuk et al. 2018) that are distinguished based on their function, structure, and phylogenetic relationships (Zulawski et al. 2014). RLKs are represented by six families: concanavalin A-like lectin protein kinases (L-LPK), leucine-rich-repeat receptor-like kinases (LRR-RLKs), CRKs with extracellular DUF26 domains harboring a conserved cysteine-rich motif, RLCKs, WAKs, and other kinases with no published family (RK). WAK and LRR_8B (which represents the kinase domain in CRKs) form the majority of TKP domains (Klymiuk et al. 2018) and the protein structures of all functionally characterized TKP genes represent combinations of these kinases (Brueggeman et al. 2002; Chen et al. 2020; Klymiuk et al. 2018; Lu et al. 2020) (Fig. 1).

FUNCTIONALLY CHARACTERIZED TKPS

Currently, five resistance (*R*) genes with TKP structure have been functionally validated via mutagenesis, gene silencing, or transformation experiments: *Rpg1* (Brueggeman et al. 2002), *Wheat Tandem Kinase 1 (WTK1)* (Klymiuk et al. 2018), *WTK2* (Chen et al. 2020), *WTK3* (Lu et al. 2020), and *WTK4* (Gaurav et al. 2021) (Fig. 1). An additional TKP gene, MLOC_38442.1, has been proposed as a candidate gene (*Un8*) that confers barley true loose smut (*Ustilago nuda*) resistance (Zang et al. 2015) but its role in immunity has not yet been confirmed. All characterized TKPs confer resistance to biotrophic fungal pathogens in monocots. Key features of the validated TKP R proteins, including structure, expression, posttranslational regulation, and protein interactions mediating disease resistance, are described below.

Effectiveness of functional TKP genes.

All functionally validated TKPs provide resistance against biotrophic fungal pathogens, while effectiveness against necrotrophic and hemibiotrophic pathogens has not yet been demonstrated. The first cloned TKP gene, *Rpg1*, provides resistance to several races of *Puccinia graminis* f. sp. *tritici*, which causes stem rust disease of barley (*H. vulgare*) (Brueggeman et al. 2002; Horvath et al. 2003). The *Yr15* (*WTK1*) gene originates from wild emmer wheat, *T. turgidum* subsp. *dicoccoides* (Klymiuk et al. 2018), and is effective against more than 2,000 isolates of *P. striiformis* f. sp. *tritici*, the causal agent for yellow (stripe) rust disease of wheat (Ali et al. 2017; Liu et al. 2017; Sharma-Poudyal et al. 2013). It is effective in different genetic backgrounds, including many durum (*T. turgidum* subsp. *durum*) and bread (*T. aestivum*) wheats, as well as under numerous naturally occurring wild emmer wheat backgrounds (Klymiuk et al. 2018, 2019a and b, 2020; Yaniv et al. 2015). The *Sr60* (*WTK2*) stem rust *R* gene, originating from *T. monococcum*, is a race-specific gene conferring intermediate levels of resistance to some *P. graminis* f. sp. *tritici* races (Chen et al. 2018, 2020). *WTK3* (Lu et al. 2020) provides resistance to 93 genetically diverged Chinese isolates (McNally et al. 2018) of wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*) and was originally identified in three Chinese wheat landraces (Chiyacao, Baihulu, and Hulutou) as three different genes: *Pm24* (Huang et al. 1997), *Pm24b* (Xue et al. 2012), and *MlHLT* (Z. Wang et al. 2015). The recently cloned *WTK4* originating from *A. tauschii*, the diploid progenitor of bread wheat, provides resistance to *B. graminis* f. sp. *tritici* under genetic backgrounds of diverse *A. taushii* accessions and corresponding synthetic

hexaploid lines developed by hybridization of diploid wild accession with tetraploid durum wheat (Gaurav et al. 2021). Hence, the five known TKP genes possess diverse levels of effectiveness against different pathogens; that is, minor, race-specific resistance (*WTK2*); resistance to a wide range of isolates, except the most virulent (*Rpg1*); and high resistance to a wide range of pathogen isolates (*WTK1* and *WTK3*).

TKP structure.

The protein architecture of RPG1, WTK1, and WTK3 includes two kinase-like domains, of which one was classified as a kinase based on the presence of eight key conserved residues known to be important for kinase activity (Hanks et al. 1988), whereas the other kinase-like domain was classified as a pseudokinase due to the lack of one or a few key residues (Klymiuk et al. 2018; Lu et al. 2020; Nirmala et al. 2006) (Fig. 1). The relative order of the domains is variable for these proteins, with RPG1 possessing a pseudokinase (KinI)–kinase (KinII) structure, whereas WTK1 and WTK3 have a kinase (KinI)–pseudokinase (KinII) architecture (Klymiuk et al. 2018; Lu et al. 2020; Nirmala et al. 2006). Among these three, thus far, only the RPG1 kinase domain has been shown, experimentally, to be kinetically active, whereas the pseudokinase domain showed no activity (Nirmala et al. 2006). In RPG1, WTK1, and WTK3, both domains are necessary to provide resistance because mutations in either the kinase or pseudokinase domain lead to disease susceptibility (Klymiuk et al. 2018, 2020; Lu et al. 2020; Nirmala et al. 2006). A kinase-kinase structure was proposed for WTK2 because both domains share the eight key conserved residues (Chen et al. 2020; Hanks et al. 1988). This suggests that both WTK2 domains may be functional kinases but there is no experimental evidence to date (Chen et al. 2020).

The kinase domains of RPG1 (KinII), WTK1 (KinI), WTK3 (KinI), and WTK2 KinI are serine/threonine (S/T) nonarginine-aspartate (non-RD) kinases (Chen et al. 2020; Klymiuk et al. 2018; Lu et al. 2020; Nirmala et al. 2006), whereas WTK2 KinII falls into the RD-kinase category (Chen et al. 2020). All four proteins lack known receptor sequences or strong membrane-targeting motifs in their coding sequences (Brueggeman et al. 2002; Chen et al. 2020; Klymiuk et al. 2018; Lu et al. 2020). RPG1 and WTK1 have been experimentally shown to localize mainly in the cytoplasm, with only a small RPG1 fraction in the plasma or intracellular membranes (Nirmala et al. 2006) and a minor WTK1 fraction in the nucleus (Klymiuk et al. 2018). In summary, four known functional TKPs likely exhibit cytoplasmic localization and both of their domains, one of which is an S/T non-RD kinase, are essential for a successful immune response.

Gene families.

Rpg1 was identified in the barley cultivar Morex, which has five additional genes with different levels of homology to *Rpg1*; three of these have TKP structure and one appears in tandem with *Rpg1* (Brueggeman et al. 2006). The gene families of the other functionally characterized genes are even more complex due to the polyploidy of wheat (i.e., tetraploid [AABB] durum wheat *T. durum* and hexaploid [AABBDD] bread wheat *T. aestivum*). A search for *WTK1* family members yielded more than 20 copies (Klymiuk et al. 2019b) scattered across the three wheat subgenomes in a whole-genome assembly of the hexaploid wheat landrace Chinese Spring (The International Wheat Genome Sequencing Consortium

[IWGSC] et al. 2018). These included *WTK1* orthologs on the group 1 chromosomes and a cluster of *WTK1* paralogs on the group 6 chromosomes, all with analogous exon-intron structure but variable in terms of total genomic full-length and coding sequence due to large insertions (Klymiuk et al. 2019b). *WTK1* orthologs, paralogs, and homologs were also identified in whole-genome searches of other cereal species (i.e., *T. urartu*, *A. speltoides*, *A. tauschii*, *Secale cereale*, *H. vulgare*, and *Brachypodium distachyon*) (Klymiuk et al. 2018). Orthologs of *WTK3* were found on chromosome 1B but not on 1A of Chinese Spring (The International Wheat Genome Sequencing Consortium [IWGSC] et al. 2018) and durum wheat cultivar Svevo (Maccaferri et al. 2019), whereas wild emmer wheat Zavitan (Avni et al. 2017) and *T. urartu* (Ling et al. 2018) assemblies lacked any *WTK3* orthologs, suggesting that they may have been lost during wheat evolution (Lu et al. 2020). Alternatively, the genome assemblies of these two accessions do not represent the diversity of wild emmer or *T. urartu*, and *Pm24* may represent a presence-absence polymorphism that was not captured in the annotation of either assembly. *WTK3* homologs were found in *H. vulgare*, *S. cereale,* and *B. distachyon* (Lu et al. 2020). Thus, the presence of multiple orthologs, paralogs, and homologs is a common feature of the known TKP genes.

Expression patterns.

Although the four functional TKP genes are constitutively expressed at all tested stages of phenological development, differential expression when challenged by avirulent pathogens varies considerably between them (Chen et al. 2020; Klymiuk et al. 2018; Lu et al. 2020; Rostoks et al. 2004). After inoculation with an avirulent *P. graminis* f. sp. *tritici* race, the expression level of *Rpg1* did not change significantly and was comparable with non-inoculated and mock-inoculated control plants (Rostoks et al. 2004). *WTK1* expression is downregulated in plants infected with an avirulent *P. striiformis* f. sp. *tritici* race compared with mock-control plants (Klymiuk et al. 2018). In contrast, *WTK2* and *WTK3* are both transiently upregulated in the presence of avirulent pathogen races, responding with a peak of expression (2.5 to 3.7-fold for *WTK2* and 6-fold increase for *WTK3* compared with mock-inoculated plants) at 1 day postinoculation (dpi) (Chen et al. 2020; Lu et al. 2020). Expression of the nonfunctional *WTK3* allele was similarly upregulated and followed the same pattern as the functional allele, with only a slight reduction in expression compared with the functional allele at 1.5 dpi (Lu et al. 2020). Thus, it is difficult to draw solid conclusions regarding the importance of this expression peak for the resistance response. As with many NLRs, the WTK3 activity could be regulated by other regulatory mechanisms at the RNA or protein levels such as alternative splicing, nonsense-mediated RNA decay, small RNAs, protein folding, compartmentalization, nucleocytoplasmic trafficking, posttranslational modification, and so on rather than by transcriptional regulation (Borrelli et al. 2018). A feedback loop might actively downregulate *WTK2* transcription after its upregulation in response to the presence of the pathogen and transmission of the signal triggering the activation of the immune response (likely by phosphorylation of a downstream target) (Chen et al. 2020).

The expression of *Rpg1* was not correlated with variation in stem rust resistance in transgenic plants of barley cultivar Golden Promise (Horvath et al. 2003). Moreover, some transgenic plants exhibited greater resistance than native carriers of *Rpg1* such as Morex,

despite having nearly identical *Rpg1* copy number variation and expression levels (Horvath et al. 2003). At the same time, high-copy-number (five to six copies) *Rpg1* transgenic plants were susceptible to inoculation with an avirulent *P. graminis* f. sp. *tritici* isolate (Chai et al. 2012), suggesting that an appropriate level of RPG1 suitable for fast degradation (see the posttranslational modifications sections below) is required for conferring resistance.

In contrast, even though one *WTK2* copy in a transgenic line of bread wheat was sufficient to provide resistance, the transgenic line with four *WTK2* copies exhibited better resistance than the *T. monococcum* donor line (Chen et al. 2020). Similarly, WTK1 was shown to confer variable levels of resistance that are more dependent on the genetic background of the carrier than on the expression level (Klymiuk et al. 2020). Such different resistance phenotypes served as the basis for distinguishing between *Yr15*, *YrH52,* and *YrG303* genes, all originating from different wild emmer wheat accessions but shown to carry an identical *WTK1* allele (He et al. 2020; Klymiuk et al. 2020). Taken together, TKP expression levels and their patterns after inoculation with corresponding avirulent pathogen races are diverse and are genetic background dependent.

Posttranslational modifications.

A different kinetic activity of the two TKP domains has motivated research on posttranslational modification in response to pathogen attack. Rapid (within 5 to 15 min) phosphorylation of RPG1 was shown for all *Rpg1*-carrier lines after their exposure to spores of an avirulent *P. graminis* f. sp. *tritici* race (Chai et al. 2012; Gill et al. 2016; Nirmala et al. 2010), whereas in vitro inactive-kinase-domain transgenic mutants were susceptible to stem rust and failed to phosphorylate (Nirmala et al. 2010). This demonstrated that RPG1 phosphorylation was required for stem rust resistance and recognition of *P. graminis* f. sp. *tritici* spores is a rapid response.

Following phosphorylation, the RPG1 protein degrades via specific ubiquitination within the first 22 to 28 h after inoculation with avirulent *P. graminis* f. sp. *tritici* spores in all resistant *Rpg1* carriers (Chai et al. 2012; Nirmala et al. 2007). In contrast, RPG1 is not fully degraded in susceptible, high-copy-number *Rpg1* transgenic plants even though they express a higher level of *Rpg1* expression and rapid protein phosphorylation (Chai et al. 2012). Thus, RPG1 degradation has been identified as one of the key steps necessary to confer resistance. However, it should be noted that RPG1 degradation itself is not sufficient to provide resistance in those cases where downstream genes required for *Rpg1*-dependent disease resistance (e.g., *Rpr1*) are nonfunctional (Zhang et al. 2006). The *rpr1* mutant line shows degradation of RPG1 but is still susceptible to the *Rpg1*-avirulent *P. graminis* f. sp. *tritici* race (Nirmala et al. 2007). Nirmala et al. (2007) discussed possible roles for RPG1 degradation in stem rust resistance—such as regulation of the hypersensitive response (HR), initiation of the disease resistance-signaling pathway by removing a negative regulator from the R protein complex, or release of a retained peptide that de facto serves as the initiator of the disease resistance response—but empirical tests have not yet been conducted.

TKP-mediated resistance.

RPG1 and *WTK1* resistance mechanisms are associated with HR development in host cells that are targeted by haustoria of biotrophic fungal pathogens (Klymiuk et al. 2018; Nirmala et al. 2011; Saleem et al. 2019). HR is observed in *WTK1*-carrier lines as early as 4 dpi (Klymiuk et al. 2018; Saleem et al. 2019). Avirulent *P. striiformis* f. sp. *tritici* races typically form colonies with substomatal vesicles, primary and secondary infection hyphae, haustoria mother cells, and haustoria within leaf tissues of *WTK1* carriers (Klymiuk et al. 2018). The size of colonies depends on the resistance level of the plant genetic background or the virulence of the *P. striiformis* f. sp. *tritici* race, and corresponds to the size of the HR area (Klymiuk et al. 2018, 2020; Saleem et al. 2019). *WTK3* provides high resistance to *Blumeria graminis* f. sp. *tritici* isolate E09 with no visible conidia produced, very mild cell death, and a robust H_2O_2 accumulation in the Chinese wheat landrace Hulutou background (Lu et al. 2020). In contrast, the resistance conferred by *WTK2* is partial and only slows but does not stop *P. graminis* f. sp. *tritici* infection (Chen et al. 2020).

RPG1 was shown to interact with two protein effectors of an avirulent *P. graminis* f. sp. *tritici* race, arginine-glycine-aspartic acid (RGD)-binding and vacuolar protein sorting (VPS)9. Infiltration of purified RGD and VPS9 effectors simultaneously on *Rpg1*-carrier leaves leads to rapid phosphorylation and degradation of RPG1, as well as visible HR (Nirmala et al. 2011). Yeast two-hybrid assays revealed an interaction of these two effectors both with RPG1 and with each other (Nirmala et al. 2011). However, experiments of silencing these effectors demonstrating loss of resistance have not been performed. Both *Rgd* and *Vps9* effectors are predicted to be secreted and expressed in spores, sporelings, and haustoria (Nirmala et al. 2011). Given the rapid phosphorylation of RPG1 upon treatment with avirulent *P. graminis* f. sp. *tritici* races (Nirmala et al. 2011), it is likely that these effectors can be recognized prior to haustorial development, or even prepenetration effector delivery may take place (Sánchez-Martín et al. 2021). It is possible that, when introduced into the apoplast, both effectors are internalized, where they can then associate with RPG1.

An analysis of sequence variation in *WTK3* haplotypes showed that the functional haplotype differs from all nonfunctional haplotypes by a 6-bp deletion in the fifth exon (kinase domain), leading to a two-amino-acid (lysine-glycine) deletion (Lu et al. 2020). The specific 6-bp deletion was proven necessary for *WTK3* functionality, representing a rare gain-of-function mutation. Separate 3-bp and 12-bp (6 target base pairs plus 3 bp from each side) deletions did not yield a gain of function (Lu et al. 2020). The 6-bp deletion does not involve key conserved residues of the active kinase domain and is located outside of the catalytic/activation loops, so that it should not affect the potential kinase activity of this WTK3 domain (Lu et al. 2020). Instead, this deletion was predicted to result in a more compact loop, shorter than for other homologous kinases, and is proposed to be important for a precise protein–protein interaction necessary for WTK3's downstream signaling (Lu et al. 2020). Taking into account that functionally validated TKPs confer resistance at diverse levels and have different kinase domains, they could potentially initiate diverse signaling pathways based on kinase type. Furthermore, these kinase subfamilies are enriched in immune signaling proteins; thus, TKPs could "short circuit" other surface- or NLR-immune signaling pathways by activation of downstream responses.

A MODEL FOR THE TKP FAMILY EVOLUTION

Two independent mechanisms have been suggested for the origin of TKPs (Klymiuk et al. 2018): (i) duplication, where the two kinase domains of the same gene show high sequence similarity, and (ii) fusion of two kinase domains homologous to two different kinase families or subfamilies, or sharing relatively low similarity. Over half of the putative TKPs across the plant kingdom resulted from gene duplications (Klymiuk et al. 2018). These findings are consistent with other studies showing that, during the evolution of multidomain proteins, duplications are more frequent than fusions (Forslund and Sonnhammer 2012). The known, functional TKPs are found in both groups: with fusion origin for WTK1 (WAK/RLCK_8) (Klymiuk et al. 2018) and WTK2 (LRR_8B/WAK) (Chen et al. 2020) and with duplication origin for RPG1 (LRR_8B/LRR_8B) (Brueggeman et al. 2006) (Fig. 1). The origin of WTK3 is not yet categorized as a duplication or fusion event because it is a combination of two kinases from the same family (LRR_8B/LRR_8B) which, however, share relatively low similarity at the protein level (29.3%). Although it is possible that WTK3 is derived from a fusion event, it is unlikely given its structure, and it may represent an ancient duplication event with substantial subsequent changes in one domain (Lu et al. 2020).

Neofunctionalization (Freeling 2009) has been suggested as the mechanism by which TKP members gain functionality (Klymiuk et al. 2018). Many TKPs that originated by duplication possess kinase-pseudokinase structure, suggesting that one member of the identical kinase domains deteriorates over evolutionary time to a pseudokinase and, as a result, loses its original phosphorylation-related function (Klymiuk et al. 2018). Probably this is the case of the ongoing process for WTK3, for which one of the hypothetically identical kinase domains already deteriorated to putative pseudokinase after duplication; however, it has not happened yet in WTK2, which still encodes two kinase domains with potential for activity after fusion of two kinases. This deterioration of one of the kinase domains to pseudokinase may be important for the molecular function of TKPs (Klymiuk et al. 2018) as pseudokinase gains a new key decoy function (see below, “A model for the molecular function of the TKPs”).

An analysis of functional TKPs suggests different evolutionary histories. RPG1, WTK1, WTK2, and WTK3 are not homologs and do not share close phylogeny (Lu et al. 2020). The fusion event by which WTK1 was originated is considered to be ancient because its orthologous copies are present on group 1 chromosomes of all diploid (AA, BB, and DD genomes), tetraploid (AABB genomes), and hexaploid wheat and wheat-related species. Moreover, an old duplication of the whole tandem-kinase structure resulted in the formation of paralogs on group 6 chromosomes of all wheat and wheat-related species as well (Klymiuk et al. 2018). In contrast, a recent origin was suggested for RPG1 (Brueggeman et al. 2002) and WTK2 (Chen et al. 2020), because their homologs have been found only in the Triticeae tribe. WTK3 evolved before the Poaceae family diverged but became functional only recently (following the 6-bp deletion), after bread wheat was introduced into central China (Lu et al. 2020). These evolutionary histories of functional TKPs support a polyphyletic origin of the TKP family, with structural convergence of different family members evolved at various time points.

A MODEL FOR THE MOLECULAR FUNCTION OF THE TKPS

Plant–pathogen interactions have resulted in the coevolution of defense and counter-defense mechanisms, which involve specific R proteins, effectors, and cofactors in the host plants and their pathogens. Plants use PRR-associated kinases to trigger immune responses (Couto and Zipfel 2016). However, these defense kinases can be targeted by effectors and utilized by the pathogen to promote disease development (Irieda et al. 2019; Lei et al. 2020) (Fig. 2). Plants can also employ other kinases, structurally similar to kinase domains within PRRs, to serve as "decoys" for pathogen effectors and enable intracellular pathogen detection as part of the resistance response (Kroj et al. 2016; G. Wang et al. 2015) (Fig. 2). Successful intracellular detection of the pathogen by R proteins and decoys could be overcome via suppression of recognition by additional layers of pathogen effectors, which could explain the evolutionary need for a large repertoire of pathogen effectors and plant *R* genes (Thordal-Christensen 2020).

Kinases function in protein complexes that recognize the presence of the pathogen and trigger downstream signals to initiate defense responses. In some cases, these receptor complexes include two independent kinases or pseudokinases that work together; for example, the PBL2 (kinase)–RKS1 (pseudokinase)–ZAR1 (NLR) complex triggering immunity by detection of *Xanthomonas campestris* effector AvrAC in *Arabidopsis* (G. Wang et al. 2015). TKPs may represent another layer in R protein evolution when two independent genes acting together (one of which is structurally similar to the PRR defense kinase targeted by the effector) merge (fusion origin), or a structural homolog of a defense kinase duplicates and, following neofunctionalization, forms a single functional gene composed of two near-identical domains (duplication origin). A similar model of fusion of two proteins that are involved in the same receptor complex was suggested for NLR genes and their integrated domains, which can be found as separate genes in one species and as a single gene (NLR-ID) in other species (Sarris et al. 2016).

The shared kinase-pseudokinase domain architecture characteristic of most functional and putative TKPs may then be related to their mode of function. It has been suggested that the TKP pseudokinase domain is responsible for recognition of the pathogen, while the kinase domain is essential for downstream signaling (Kleinhofs et al. 2009; Klymiuk et al. 2018; Nirmala et al. 2007). In our current model of TKP molecular function, the pseudokinase interacts with the pathogen effector and sends a signal to the kinase domain, which activates downstream resistance cascades. The pseudokinase acts both as a decoy for the pathogen effectors and as a regulator of the kinase domain (Klymiuk et al. 2018; Nirmala et al. 2007) (Fig. 2). An analogous model has also been proposed for NLR pairs that consist of independent sensing (with an integrated domain that interacts with an effector) and a signaling partner working together to confer resistance (Feehan et al. 2020; Le Roux et al. 2015; Lolle et al. 2020; Sarris et al. 2015).

Similar to NLR proteins (Wang et al. 2019a), TKPs may also switch to an "active state" after conformational changes to reveal ATP-binding or functionally relevant TKP kinase domain regions only after the TKP pseudokinase domain has bound to the pathogen effector. The active state allows the kinase to form a "resistance complex" to serve as a signaling

platform (Fig. 2). Perturbations of a kinase-pseudokinase module have been suggested to activate plant immunity where modification of ZED1/ZRK pseudokinase may recruit PBL kinase for activation of ZAR1-mediated immunity (Bastedo et al. 2019). The regulation of the "nonactive state" of the kinase domain of TKPs has an important function, and an adaptive role, because the kinase activates a cascade of reactions leading to programmed cell death. Uncontrolled activation of this mechanism in the absence of the pathogen may result in an autoimmune response and even the death of the whole plant. Similar mechanisms of pseudokinase-mediated autoinhibition of the kinase domain were shown for some animal Janus kinases, which possess a multidomain structure with pseudokinase, kinase, and additional domains (Lupardus et al. 2014).

Although RPG1, WTK1, and WTK3 are combinations of kinase and pseudokinase in tandem, the WTK2 structure includes two kinases. Accordingly, here, we have used the more widely defined TKP terminology for this protein family instead of the previously proposed "tandem kinase-pseudokinase" (Klymiuk et al. 2018), with the same "TKP" abbreviation. Nevertheless, RPG1, with a kinase-pseudokinase architecture that was experimentally confirmed (Nirmala et al. 2006), is currently the most closely examined TKP and serves as a basis for our proposed model for the molecular function of TKPs (Klymiuk et al. 2018). RPG1 associates with two *P. graminis* f. sp. *tritici* effectors (Nirmala et al. 2011), advocating for a direct recognition mode of function (Dangl and McDowell 2006); however, both pseudokinase and kinase domains interact with these effectors and are required for resistance.

The described model is based on our current knowledge of TKPs, and it does not contradict other possible hypotheses; for example, possible formation of multilevel complexes of TKPs with other proteins, extracellular perception followed by TKP activation, or similarity with the recently discovered oligomerized ZAR (Shi et al. 2020; Wang et al. 2019a and b), ROQ1 (Martin et al. 2020), or RPP1 (Ma et al. 2020) resistomes. Further studies are required in phylogenetically representative plant species to fully elucidate the mechanisms of resistance conferred by this protein family.

IMPORTANCE OF TKP STUDIES

In the modern genomics era, the genomes of numerous plant species and accessions are routinely sequenced, generating large datasets. Many studies focus on available information to predict parameters for the most productive genome queries. In this case, uncommon, rare, and exceptional examples may be overlooked and only widespread models used. For example, genome-wide analyses of numerous plant species have focused on annotating NLR genes (Bettaieb and Bouktila 2020; Gu et al. 2015; Kim et al. 2017; Lozano et al. 2015; Meziadi et al. 2016; Seo et al. 2016; Shi et al. 2018; Walkowiak et al. 2020; Zheng et al. 2016). NLR-annotator (Steuernagel et al. 2020) prediction software and rapid cloning strategies for disease *R* genes such as RenSeq (Jupe et al. 2013), MutRenSeq (Steuernagel et al. 2016), and AgRenSeq (Arora et al. 2019) are built specifically to detect NLR *R* genes (Cesari 2018) and have already catalyzed NLR gene function characterizations (Zhang et al. 2020b) that have been collected in a reference dataset (Kourelis and Kamoun 2020). PRRs (RLKs and RLPs) have been investigated for some species (Fritz-Laylin et al. 2005; Kang

and Yeom 2018; Wang et al. 2008). Some computational tools account for kinase structures for R proteins (e.g., the Disease Resistance Analysis and Gene Orthology pipeline), and a method for RLP/RLK enrichment sequencing was also developed (Lin et al. 2020). Although these tools target the most abundant R protein types, this review highlights the need for a more widespread analysis of R proteins, not restricted to NLRs and PRRs (Zhang et al. 2020b).

Although, thus far, TKPs have been functionally validated only in barley and wheat species, they are abundant in numerous plant genomes. Taking into account rapid technical development and the urgent need for more sources of resistance, many more functional TKPs are likely to be discovered and validated soon, and there is a need to give more attention to TKP research. Currently, TKP searches in sequenced genomes are limited to customized scripts and no software exists for the rapid cloning of TKPs. The development of new pipelines for TKP discovery and cloning will facilitate their functional characterization and help to shed light on their evolution and molecular function.

CONCLUSIONS AND FUTURE PERSPECTIVES

Recent advances in functional characterization have brought more information to light regarding TKPs. Thus far, five barley and wheat TKP genes (*Rpg1*, *WTK1*, *WTK2, WTK3*, and *WTK4*) have been functionally validated to provide resistance to several fungal diseases. TKPs have been found across the plant kingdom, suggesting that many more functional TKPs remain to be discovered in different species. Here, the most recent information regarding TKPs' structures, possible origins, and modes of function is presented. TKPs represent an example of structural convergent evolution and are of polyphyletic origin through duplication or gene fusion. A decoy and regulator role is proposed for the pseudokinase domain; however, TKP molecular function is likely not limited to one protein alone and may involve formation of a complex with other proteins, or even formation of oligomers of such complexes. A better understanding of the evolution of TKPs and their molecular function is important to support future exploitation, including gene manipulation (e.g., via gene-editing approaches) and synthetic engineering of novel *R* genes because these approaches have now been approved or are under discussion (Wulff and Jones 2020) for many crops.

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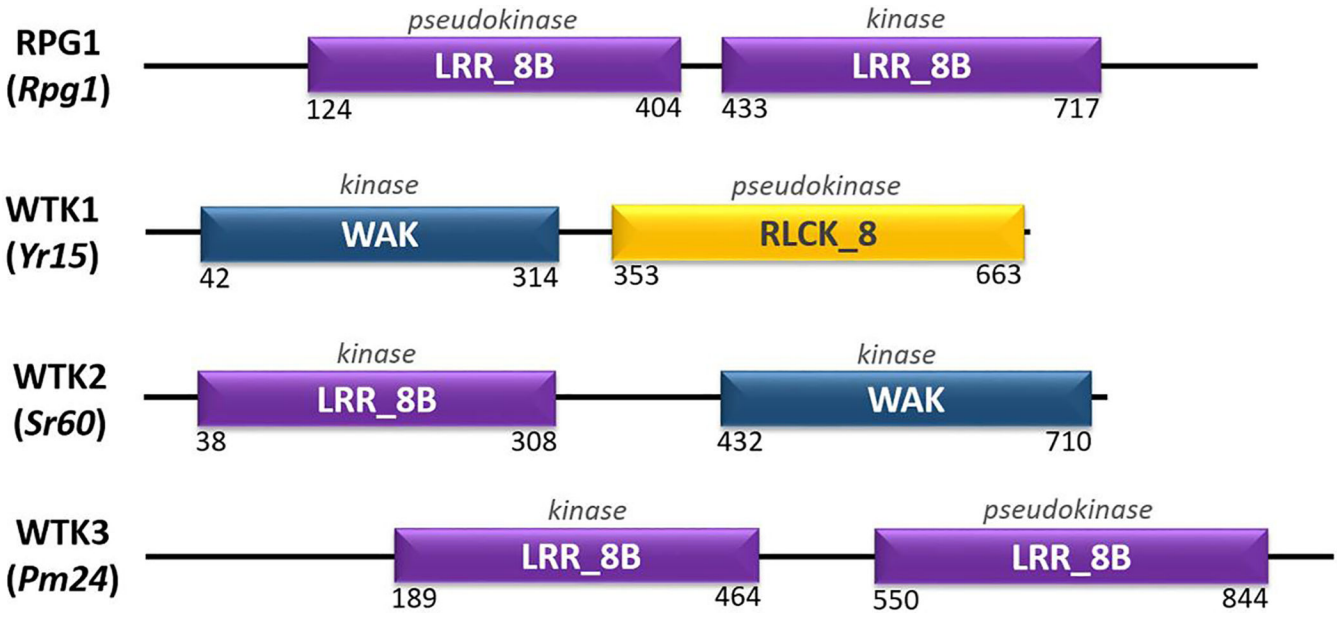

Fig. 1.

Protein architecture of functionally characterized tandem kinase proteins (TKPs). Kinase families and subfamilies are defined based on the relationships with specific, annotated *Arabidopsis* kinome families or subfamilies according to homology-based comparisons (Zulawski et al. 2014). LRR_8B = the kinase domain in cysteine-rich receptor-like kinases, WAK = wall-associated kinase, and RLCK_8 = receptor-like cytoplasmic kinase. Numbers correspond to start and end amino acid positions for each kinase-like domain.

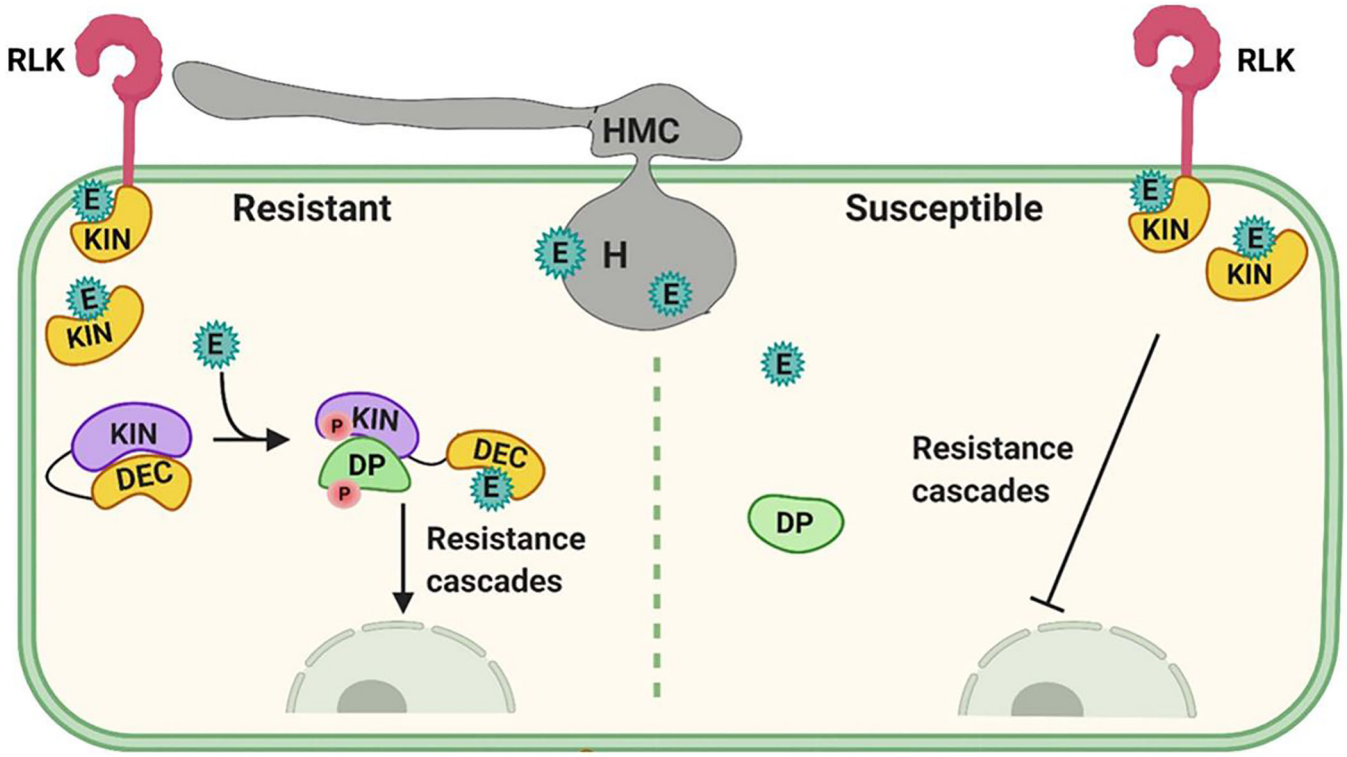

Fig. 2.

Model of the molecular function of tandem kinase proteins (TKPs). Pathogen effectors target receptor-like kinases (RLKs) and receptor-like cytoplasmic kinases to advance disease development. The plant utilizes TPKs with domains exhibiting similarity to effector defense kinase targets as decoys for pathogen effector recognition, which triggers a defense response. HMC = haustorial mother cell, H = haustorium, KIN = kinase domain, DEC = decoy domain (pseudokinase), E = effector, DP = defense protein, and P = an ATP-binding site.