Genome-Wide Analysis of the Cucumber Kinome: Uncovering and Analyzing the Expression of *Cucumis sativus* Protein Kinases in Response to Biotic Stress

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Abstract

Protein kinases (PKs) play a pivotal role in the regulation of diverse metabolic processes. However, a complete characterization of the PK family in cucumber remains lacking. This study aimed to characterize the cucumber PK family, shedding light on its genomic distribution, classification, and expression patterns in response to biotic stimuli triggered by pathogens like Powdery Mildew (PM), Alternaria Leaf Spot (ALS), and Root-Knot Nematode (RKN). The analysis of hidden Markov models (HMMs) uncovered 835 PKs in the cucumber kinome, distributed across its seven chromosomes, and categorized into 20 distinct groups and 123 families, with the RLK group being the most abundant. Evidence of tandem duplication of PK genes was also observed, enriching our understanding of cucumber PKs. The study delved into the functional characterization of cucumber PKs, scrutinizing their expression profiles in response to PM, ALS, and RKN. Notably, we observed a higher number of differentially expressed PK genes (PK DEGs) in susceptible genotypes when facing biotrophic pathogens (PM and RKN), while the greatest number of PK DEGs was found in the resistant genotype combating the necrotrophic pathogen ALS. We identified PK DEGs per genotype specific to each pathosystem. The number of DEGs ranged from 319 to 2,202, with PK DEGs numbering from 8 to 105. Our findings illuminated the role of cucumber PKs in regulating metabolic processes, particularly in the context of plant-pathogen interactions. This comprehensive characterization paves the way for further research into the intricate mechanisms underlying cucumber's responses to these major diseases.

Keywords: Gene expression, Kinase gene family, Metabolism regulation, Plant immunity, Protein evolution, Response to disease.

1. Introduction

Cucumber (*Cucumis sativus* L.) (Csa) is a species that belongs to the Cucurbitaceae family, holding significant importance for several industries worldwide (Pandey et al., 2022). This crop holds a prominent position in both large-scale and small-scale agricultural systems, making a substantial contribution to the economy. The demand for cucumbers, whether in their fresh or processed form, drives agricultural production and strengthens the development of international trade alliances, guaranteeing year-round availability. Cucumbers are essential to the food industry due to their nutritional potential and medicinal attributes, particularly their high potassium content, which is known to contribute to the alleviation of blood pressure (Jilani et al., 2009, Pandey et al., 2022). Given their applicability, the cultivation of cucumbers has emerged as an economically viable alternative for different market sectors, including those focused on the production of plant-derived pharmaceuticals (Khan et al., 2022). Furthermore, within the beauty and skincare industry, cucumbers are recognized for their moisturizing, refreshing, and soothing properties (Desam et al., 2021).

Despite its agronomic relevance, cucumber's susceptibility to diseases and pests results in reduced foliage, consequently leading to decreased productivity (Batta, 2003; Mukhtar et al., 2013; Sarhan et al., 2020). For instance, powdery mildew (PM), caused by the fungal pathogens such as *Podosphaera xanthii* (Castagne) U. Braun & Shishkoff (syn. *Sphaerotheca fuliginea* (Schlechtend.:Fr.) Polacci) and *Erysiphe cichoracearum* DC. (syn. *Golovinomyces cichoracearum*) (Abul-Hayja, 1982, Moustafa et al., 1990, Pérez-García et al., 2009), represents a significant threat. Alternaria leaf spot (ALS), caused by the fungus *Alternaria alternata* (Fr.) Keissl. (Vakalounakis, 1990; Vakalounakis et al., 1988) and *Alternaria cucumerina* (Ell. & Ev.) (Batta, 2003; Dey et al., 2022), manifests as dark circular lesions on the leaves, which can expand and result in premature defoliation. The Root-knot nematode (RKN) or gall nematode, specifically *Meloidogyne incognita* (Kofold & White) Chitwood, is a notorious pest that infests cucurbit roots, leading to the development of characteristic galls and impairing nutrient absorption (Cheng et al., 2022; Fassuliotis et al., 1963).

The use of resistant cultivars represents the most efficient approach to disease management, effectively reducing the symptom severity and avoiding the need for chemical control measures (Choudhary et al., 2002). Comprehending the defense mechanisms employed by Csa species is essential for the development of resistant cultivars, providing foundational knowledge to support the development of new plant biotechnology events. In this context, protein kinases (PKs) (Hanks et al., 1995) receive great attention due to their central role in regulating plant responses to disease defense processes, as well as in modulating plant immunity (Benschop et al., 2007; Peck, 2001; Tena et al., 2011). However, the genetic control of resistance mechanisms and the intricate molecular interactions between plants and pathogens remain critical research targets for elucidating the molecular mechanisms underlying plant immunity (Kamoun et al., 1999; Kliebenstein et al., 2008; Vidhyasekaran, 2014).

No comprehensive or comparative studies have been identified in the existing literature that encompass all families of PKs in Csa, particularly in the context of PM, ALS, and RKN interactions. Previous investigations have characterized the cucumber kinome as relatively small when compared to kinomes of other plant species, which is possibly attributed to the absence of recent whole-genome duplications in Csa (Huang et al., 2009; Lehti-Shiu et al., 2012). It is worth noting that several genomic analyses have already been conducted focusing on specific PK families within this species. To illustrate, the Casein Kinase (CK) family, which includes proteins responsible for phosphorylating casein (Meggio et al., 2003), plays roles related to the circadian cycle (Sugano et al., 1999), plant growth, and the regulation of light-modulated gene expression (Lee et al., 1999). On the other hand, the Mitogen-Activated Protein Kinase (MAPK) family constitutes a group of serine/threonine kinases that participate in diverse signal transduction pathways associated with hormonal responses and substantial developmental changes in organisms (Shoresh et al., 2006).

Conversely, the Lectin Receptor-Like Kinase (LecRLK) family emerges as notably crucial in the innate immunity of plants, especially in defense against biotrophic pathogens such as *P. xanthii*, the causal agent of PM (Haider et al., 2021). Furthermore, the Calcium-Dependent Protein Kinase (CDPK) and CDPK-Related Protein Kinase (CRK) families play roles in calcium-dependent signaling pathways in response to environmental stresses (Wei et al., 2019; Xu et al., 2017). Moreover, these kinase families are involved in pathways related to the inhibition of J2 (second-stage juvenile) invasion of *M. incognita* on cucumber roots, thereby enhancing cultivar resistance (Li et al., 2021). In general, although a comprehensive characterization of the Csa kinome remains limited, specific PK families have received attention in genomic analyses, contributing significantly to our understanding of their functions and potential involvement in various biological processes and stress responses.

PKs play a key role in modulating cellular responses to external stimuli (Peck, 2001; Tena et al., 2011), highlighting their importance in enhancing cucumber genetic improvement. Primarily, PKs function by facilitating phosphorylation, a critical biological process that enables signaling within gene expression networks in various cellular processes (Johnson et al., 1996). Thus, the comprehensive characterization of a kinome and its analysis holds substantial importance, particularly in the context of plant immunity. Moreover, the identification of PKs offers valuable insights into elucidating their specific functionalities and gives support to the elucidation of the evolutionary processes shaped by environmental pressures within different tissues of the organism.

The objective of this study was to define and evaluate the cucumber kinome, based on structural and evolutionary genomic assessments of the genes and their corresponding encoded proteins. We conducted a comprehensive analysis of cucumber PKs comprising the characterization, classification, and the gene expression evaluation in the context of stress caused by PM, ALS, and RKN. The inferences performed are intended to serve as foundational knowledge for future projects aimed at investigating the functional roles of specific PKs and developing strategies to enhance disease resistance in cucumber and related species of the Cucurbitaceae family.

2. Material and Methods

2.1. Genome-wide identification and classification of cucumber PKs

The identification of PKs was based on the alignment of the Pkinase (PF00069) and Pkinase_Tyr (PF07714) subfamilies against the cucumber Gy14 annotated proteins, available in the Cucurbit Genomics Database v2 (CuGenDbv2) (Yu et al., 2022). Such an alignment was performed with hidden Markov models (HMMs) obtained from the Pfam database (http://pfam.xfam.org/) (El-Gebali et al., 2019) together with the HMMER tool (Finn et al., 2011) considering an E-value cutoff of 0.01. The isoforms of the PKs were included for further analyses.

After identifying the cucumber PKs, we classified them into subfamilies using the HMMER tool together with the subfamily HMMs estimated from PKs of 25 plant species, as described and available as supplementary files by Lehti-Shiu et al. (2012): *Aquilegia coerulea, Arabidopsis lyrata, Arabidopsis thaliana, Brachypodium distachyon, Carica papaya, Citrus clementina, Citrus sinensis, Chlamydomonas reinhardtii, Cucumis sativus, Eucalyptus grandis, Glycine max, Manihot esculenta, Medicago truncatula, Mimulus guttatus, Oryza sativa, Populus trichocarpa, Prunus persica, Physcomitrella patens, Ricinus communis, Selaginella moellendorffii, Setaria italica, Sorghum bicolor, Vitis vinifera, Volvox carteri,* and *Zea mays*.

To confirm the subfamily classification obtained, we estimated a phylogenetic tree of PK sequences. PKs were aligned using the MAFFT v7.453 program (Katoh et al., 2013) and the tree was estimated with the FastTree 2.1.11 software (Price et al. 2010) considering 1,000 bootstrap replicates and an approximately-maximum-likelihood approach together with the Whelan Goldman (WAG) model for amino acid evolution (Whelan et al., 2001). Additionally, we included a PK outgroup sequence as a root in the phylogenetic tree. We selected a sequence from the *Chlamydomonas reinhardtii* v5.6 proteome (Cre07.g349540.t1.1) retrieved from Phytozome (https://phytozome-next.jgi.doe.gov) (Goodstein et al., 2012). We selected such a species because it does not have a multicellular ancestor (Ratcliff et al., 2013), which indicates that it must have evolved prior to Csa proteins.

2.2. Characterization of the cucumber PK sequences

The evaluation of cucumber PKs' gene structure was performed considering the number of exons and introns retrieved from the Gy14 GFF file, obtained from the CuGenDBv2. The chromosomal positions of PK genes were also obtained from the Gy14 GFF file, and a physical map of the chromosomes was constructed using the TBtools software (Chen et al., 2020). The domain composition analysis of PKs was performed using the Pfam database and the HMMER tool (Finn et al., 2011). For the physical and chemical characterization of PKs, we estimated using the ProtParam module from the SeqUtils subpackage (Cock et al. 2009): (i) the isoelectric point; (ii) the molecular weight; and (iii) the general average hydropathy (GRAVY). For the number of amino acids (aa), predictions of transmembrane helices and signaling peptides, the TOPCONS server (https://topcons.cbr.su.se/pred/) was employed (Tsirigos et al., 2015). We estimated the potential subcellular locations of PKs using the BUSCA web-server (http://busca.biocomp.unibo.it/) (Savojardo et al., 2018). Finally, for functional annotation, we used the Blast2GO (Conesa et al., 2008) tool and retrieved the associated Gene Ontology (GO) terms to create a treemap using the REViGO tool (http://revigo.irb.hr/) (Supek et al., 2011) together with the treemap R package (Tennekes, 2021).

2.3. Kinase gene expression patterns associated with biotic stress

Initially, we assessed the expression of PK genes using raw RNA sequencing (RNA-Seq) data derived from three independent experiments, all retrieved from the CuGenDBv2 database. Each dataset was chosen based on its response to specific biotic stresses: (i) PM (*P. xanthii*) (Xu et al., 2017); (ii) ALS (*A. cucumerina*) (Sa et al., 2020); and (iii) RKN (*M. incognita*) (Wang et al., 2018). A summary of the data used in this study, focusing on differentially expressed genes (DEGs), is presented in Table 1. Subsequently, after fitting the model with the complete gene set associated with each biotic stress, the dataset of PK-related DEGs underwent filtration for further in-depth analyses.

Table 1. Description of the data considered for the cucumber kinome construction.

Disease	Pathogen lifestyle	Resistant	Susceptible	RNA extraction	Data origin
PM	Biotrophic	SSL508-28	D8	0 × 48 h	Xu et al. 2017
ALS	Necrotrophic	D1322	BJ204	2 × 6 dpi	Sa et al. 2020
RKN	Biotrophic	IL10–1	CC3	0 × 3 dpi	Wang et al. 2018

PM: powdery mildew (*P. xanthii*), ALS: alternaria leaf spot (*A. cucumerina*), RKN: root-knot nematode (*M. incognita*), BJ204: Beijing204, h: hours post-inoculation, dpi: days post-inoculation.

For the evaluation of resistance to PM, assessments were carried out for three consecutive years (spring 2013, spring 2014, and fall 2015) on the SSL508-28 (resistant) and D8 (susceptible) genotypes, conducted under greenhouse conditions (Yangzhou, China). Inoculations were performed by applying conidia collected from naturally infected D8 plants, as described by Xu et al. (2017). A total of 12 standard Illumina paired cDNA libraries were prepared, including three biological replicates for each genotype evaluated at 0- and 48-hours post-inoculation. These libraries were sequenced generating 125 base pairs paired-end reads through the Illumina Hiseq2500 platform. For mapping the reads to the cucumber reference genome, high-quality nucleotides were filtered according to their quality score (Q > 20) and aligned to the 9930 (Chinese Long) v2 reference genome (Li et al., 2019) using TopHat v2.0.9 (Trapnell et al., 2009). To count the number of reads mapped to reference genes, Cufflinks v2.1.1 (Mortazavi et al., 2008) was employed. The raw RNA-seq data was generated and available by Xu et al. (2017).

For ALS, the genotypes D1322 (resistant) and Beijing204 (BJ204) (susceptible) were evaluated. Spores of *A. cucumerina* were inoculated via spore suspension on the third true leaf of 3-week-old plants, as described by Sa et al. (2020). To obtain samples for RNA extraction and library preparation for sequencing, the first three true leaves were collected at 2- and 6-days post-inoculation (dpi) in three biological replicates, each consisting of one plant. Sequencing was performed using an Illumina HiseqTM-2000 system, which generated 125 bp/150 bp paired-end reads. Count data were obtained through the following steps: (i) removal of low-quality reads and reads containing polynucleotides using the FASTX-toolkit (Gordon et al., 2010); (ii) read mapping to the 9930 (Chinese Long) v2 reference genome (Li et al., 2019) using HISAT 2.0.4 (Kim et al., 2019); and (iii) counting the number of reads mapped to each gene using HTSeq v0.6.1 (Anders et al., 2015). The raw RNA-seq data was generated and available by Sa et al. (2020).

For RKN, 3-4 days old seedlings of two cucumber genotypes were employed, namely IL10-1 (resistant) and CC3 (susceptible). The raw RNA-seq data was generated and available by Wang et al. (2018). The inoculation involved the use of *M. incognita* race 1 eggs were extracted from infected 'Hezuo 903' tomato plants, as described by Wang et al. (2018). Root tips were collected at 0, 1, 2, and 3 dpi from 7 plants for each genotype and then pooled into a single biological sample, with 3 biological replicates for each sample. In total, 24 cDNA libraries were prepared and sequenced on an Illumina Hiseq 2500 platform, generating paired-end reads. To ensure the generation of high-quality clean data, a filtering step was performed with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), including the removal of reads containing adapters, reads with poly-N sequences, and reads of low quality from the raw data. The remaining reads were aligned to the 9930 (Chinese Long) v2 reference genome (Li et al., 2019) using TopHat v2.0.12 (Kim et al., 2013).

To identify the whole set of DEGs, we employed the raw count data as input for DESeq2 package (Love et al. 2014). We considered the following contrasts: PM between genotypes (time as a covariate), D8 between time points (0h vs 48h), SSL508-28 between time points (0h vs 48h); ALS between genotypes (time as a covariate), BJ204 between time points (2 dpi vs 6 dpi), D1322 between time points (2 dpi vs 6 dpi); RKN between genotypes (time as covariate), CC3 between time points (0 vs 3 dpi), and IL10-1 between time points (0 dpi vs 3 dpi). For each comparison, we conducted negative binomial tests to identify DEGs, with a significance threshold set at a maximum adjusted p-value (false discovery rate, FDR) of 0.05 and a minimum absolute log2 fold change (log2fc) of 1.5. After filtering the PK-related DEGs, they were visualized using Volcano Plots, generated using the tidyverse (Wickham et al., 2019), RColorBrewer (Neuwirth, 2014), and ggrepel (Slowikowski et al., 2018) packages in R.

For a better comprehension of the families that respond similarly to each disease, we employed Venn diagrams to illustrate the overlap between the total PK DEGs and those that were either up-regulated and down-regulated. Furthermore, we employed an UpSet plot to analyze PK DEGs that were shared or exclusive across all pathosystems, with a particular emphasis on distinguishing between down-regulated and up-regulated genes within the PK DEGs.

2.4. Kinase duplication and synteny analyses

To conduct the duplication analysis and identify duplication event categories, we employed the Multiple Collinearity Scan toolkit (MCScanX) (Wang et al., 2012). Calculations of synonymous (Ks) and non-synonymous (Ka) substitution rates were performed using the MCScanX module within the TBtools software. The elapsed time for each duplication event was determined using the formula $T = Ks/2\lambda$, where λ represents the mean synonymous substitution rate (6.5×10^{-9}) (Gaut et al., 1996).

A comprehensive synteny analysis was performed to explore the relationships among kinase genes in the Gy14 cucumber and DHL92 melon genomes, both sourced from the CuGenDBv2 database. The identification of syntenic blocks was accomplished through the application of the MCScanX toolkit, while the subsequent visualization of the obtained data was achieved using the Dual Synteny Plot package, seamlessly integrated into the TBtools software. This methodological approach allowed for a detailed examination of conserved genomic regions and facilitated a visually informative representation of the syntenic relationships between kinase genes in the cucumber and melon genomes.

3. Results

3.1. Genome-wide identification and Classification of cucumber PKs

To identify potential cucumber PKs, the alignment of the cucumber proteome (Gy14) against two HMM profiles, Pkinase (PF00069) and Pkinase_Tyr (PF07714) identified 837 putative PKs, and after a domain check two atypical PKs were removed, remaining 835 true PKs. The PK isoforms were kept for further analysis. Our analysis revealed that most kinases (828 PKs) possessed both domains simultaneously, while seven PKs had only one of these domain types (5 Pkinase and 2 Pkinase_Tyr). Specifically, we identified 828 PKs with varying combinations of both types (676 PKs had one of each domain, and 152 PKs had between three to six Pkinase and Pkinase_Tyr domains) (Supplementary Table S1).

The domain composition analysis using the Pfam database and the HMMER tool identified two sequences as atypical PKs due to their absence of canonical kinase domains: (i) CsGy6G033750.2, which belongs to the ABC1 family (PF03109.16); and (ii) CsGy1G024940.2, associated with the Haspin_kinase family (PF12330.8). It is worth noting that atypical kinases, despite lacking canonical kinase domains, may still retain kinase functions, as reported in prior studies (Deshmukh et al., 2010; Manning et al., 2002). However, as our study primarily focuses on typical kinases, we have removed these atypical kinases from further investigations.

We identified PKs that contained multiple kinase domains within their sequences, comprising a total of 558 different domain types, including Pkinase and Pkinase_tyr. The number of distinct domains within each protein ranged from 1 to 97. Among these domains, seven were the most frequently observed across all PKs: Pkinase (PF00069), Leucine Rich Repeat 1 (LRR_1, PF00560), Pkinase_tyr (PF07714), Leucine rich repeat N-terminal domain (LRRNT_2, PF08263), Leucine Rich Repeat 4 (LRR_4, PF12799), Leucine Rich Repeat 6 (LRR_6, PF13516), and Leucine Rich Repeat 8 (LRR_8, PF13855). Other domains were less common (Supplementary Table S2).

Furthermore, we noted the frequent occurrence of other domains, such as Aminoglycoside Phosphotransferase (APH, PF01636) appearing 348 PKs, Kinase-like (PF14531) 297 times, Pkinase_fungal (PF17667) 243 times, and, especially, Leucine-rich Repeat (LRR) domains. The domains LRR_1 (PF00560), LRR_4 (PF12799) and LRR_8 (PF13855) were found 192 times each. Moreover, we observed the Haspin_kinase domain (PF12330) occurring 188 times. A complete description of these kinase domains is available in Supplementary Table S2.

Based on the HMM approach proposed by Lehti-Shiu et al. (2012), we were able to classify the 835 genes encoding cucumber kinases into 20 distinct groups and 123 families (Figure 1 and Supplementary Figure S1, Tables S3 and S4), namely: AGC (cAMP-dependent protein kinases, cGMP-dependent protein kinases, various types of protein kinase C, protein kinase B, 3-phosphoinositide-dependent protein kinase-1, and the ribosomal protein S6 kinases), Aur (aurora kinase), BUB (budding uninhibited by benzimidazoles), CAMK (calcium/calmodulin-dependent protein kinase), CK1 (casein kinase 1), CMGC (cyclin-dependent kinase, mitogen-activated protein kinase, glycogen synthase kinase, and cyclin-dependent-like kinase families), Plant specific (Group-Pl), IRE1 (inositol-requiring enzyme 1), NAK (NF-κB-activating kinase), NEK (never in mitosis gene-A), PEK (pancreatic eukaryotic initiation factor 2 α-subunit kinase), RLK (receptor-like kinase), SCY (*Saccharomyces cerevisiae* [yeast] kinase), STE (serine/threonine kinase), TKL (tyrosine kinase-like kinase); TLK (tousled-like kinase), TTK (threonine/tyrosine kinase), ULK (unc-51-like kinase), WEE (wee1, wee2, and myt1 kinases), and WNK (with no lysine-K). To confirm the subfamily classification, we used a phylogenetic approach that effectively grouped the genes into eight distinct clusters with strong support, as indicated by the 70% bootstrap threshold. These clusters were related to the AGC, CAMK, CK1, CMGC, RLK, SCY, STE, and TKL groups (Figure 1, and Supplementary Figure 1). The remaining proteins exhibited substantial functional diversity and expansion, forming different groupings of families within the same group. Furthermore, 13 genes could not be classified within their respective group (in instances where there was only one gene per family) or within their respective families (in cases where multiple genes belonged to a family). These unclassified genes were considered as belonging to an "unknown" category.

Among the groups defined for the cucumber kinome, the RLK group was the most numerous, with 530 genes (63.5% of the total number of PKs). The genes of this group were subdivided into 57 families. Notably, 33 of these families contained only one gene, while the RLK-Pelle_DLSV family was the most abundant with 68 genes, followed by the RLK-Pelle_LRR-XI-1 (40 genes), RLK-Pelle_LRR-III (38 genes), RLK-Pelle_RLCK-VIIa-2 (32 genes), and RLK-Pelle_L-LEC (28 genes) (Supplementary Tables S3 and S4).

3.2. Characterization of the cucumber PK sequences

When evaluating the chromosomal distribution of the 835 PK genes along the seven chromosomes, we did not observe any discernible pattern across chromosomes. However, we did observe a noticeable accumulation of PKs in the telomeric regions, aligning with a tendency for resistance genes to be in the distal regions of telomeres (Choulet et al., 2014, The International Barley Genome Sequencing Consortium, 2012). Additionally, genes from the same family tended to cluster together, suggesting potential tandem duplications (Figures 2 and 3A). It is noteworthy that two genes were associated with cucumber scaffolds and were not allocated to any chromosome in the Gy14 genome.

To analyze the gene organization of the 835 cucumber PKs, we assessed the number of introns. This analysis of gene structure revealed a variable number of introns, ranging from 0 to 28, with a median of 5 introns per gene (standard deviation of 5.46). The highest number of introns was observed in the CsGy6G003600 gene, a member of the PEK_GCN2 family, which presented 28 introns. Among the total of 835 PK genes, 123 (14.7%) presented no introns, while 554 (66.3%) contained between 1 and 10 introns, 139 (16.7%) had between 11 and 20 introns, and 19 (2.3%) exhibited a substantial range of 21 to 28 introns (Figure 3B, Supplementary Table S5).

The subcellular location prediction results revealed that approximately 44% of PKs are potentially located in the plasma membrane, 33% in the nucleus, 9% in the chloroplast, 7% in the endomembrane system, 3% in the cytoplasm, and 2% in the extracellular space (Figure 3C-D). Additionally, each of the following locations accounts for less than 1%: mitochondria, chloroplast outer membrane, organelle membrane, anchored component of plasma membrane, chloroplast thylakoid lumen, and mitochondrial membrane

(Figure 3C-D, and Supplementary Table S6). These results illustrate the importance of PKs in the plasma membrane and nucleus, since 77% of the proteins analyzed are in these locations.

The number of transmembrane (TM) domains per kinase exhibited significant variation (Figure 3E). Specifically, we observed 405 PKs lacking TM domains, 333 PKs with one TM domain, 68 PKs with two TM domains, 22 PKs with three TM domains, 6 PKs with four TM domains, and a single PK with 12 TM domains. We also were able to predict the presence of a signal peptide in 314 kinases, while it was absent in 521 kinases (Figure 3F Supplementary Table S6). The number of TM domains not only includes proteins located within the plasma membrane, but also encompasses those found in other internal membranes. Furthermore, the presence of a signal peptide suggests that the PK action is related to the secretory pathway, implying their likely extracellular site of action.

The physicochemical characteristics of the PKs were highly variable (Figure 3G-H). The number of amino acids ranged from 57 to 1694 (median: 645); molecular weight spanned from 6.42 to 191.01 kDa (median: 71.27 kDa); the isoelectric point ranged from 4.38 to 9.85 (median: 6.57); and the hydropathy (GRAVY) varied from -1.19 to 0.39 (median: -0.24). This diversity in physical and chemical properties highlights the wide range of sizes, charges, and hydrophobicity characteristics of the PKs, providing insights into the intricate mechanisms by which proteins harness a highly complex enzymatic system to modulate responses to environmental stress.

Functional annotation revealed a total of 386 different GO terms occurring 4,273 times. These GO categories encompass molecular function (F) (95 terms), biological processes (P) (227 terms), and cellular components (C) (64 terms). These annotations provide valuable insights into the functions and locations of PKs, highlighting their involvement in a wide range of metabolic processes and cellular components (Figure 4A-B; Supplementary Table S7). Notably, cucumber PKs play pivotal roles in processes such as protein phosphorylation, signal transduction, regulation of stress responses, and various biological processes, with molecular functions primarily associated with binding activities.

3.3. Kinase gene expression patterns associated with biotic stress

In the analysis of all 22,626 genes, the identification of DEGs involved the execution of three specific types of contrasts, as detailed in Table 2. These included: (i) a comparison between resistant and susceptible genotypes while incorporating time as a covariate; (ii) an assessment of gene expression changes in the resistant genotype at both initial and final time points; and (iii) an evaluation of gene expression alterations in susceptible genotypes at both initial and final time points.

For each comparison, we estimated a distinct model using the DESeq2 package and retained different numbers of genes for differential expression evaluation, ranging from 17,839 to 21,490 (Table 2). Following this, we identified DEGs specific to each pathosystem and subsequently narrowed our focus to the PK DEGs for further in-depth analyses. The number of DEGs varied from 319 to 2,202, while PK DEGs numbered from 8 to 105.

Among susceptible genotypes, we observed a higher number of DEGs in diseases caused by biotrophic pathogens (*P. xanthii* and *M. incognita*), whereas resistant genotypes exhibited more DEGs in response to the necrotrophic pathogen *A. cucumerina*. When considering time as a covariate, i.e., contrasting the gene expression between genotypes, we identified 319 DEGs (8 PKs) for the PM pathosystem, 929 DEGs (26 PKs) for ALS, and 498 DEGs (14 PKs) for RKN. These DEG numbers were lower in comparison to the other contrasts performed.

Table 2. DEG analysis of the compatible and incompatible interactions of cucumber genotypes against the pathogenic fungi *P. xanthii* (PM) and *A. cucumerina* (ALS), and the root-knot nematode *M. incognita* (RKN).

Condition	Contrast	Contrast time	Genes for modeling	DEGs	PK DEGs	Down	Up
PM	SSL508-28^R × D8^S	covariate	17,839	319	8	2	6
	SSL508-28 × SSL508-28	0h × 48h	17,522	1,428	87	50	37
	D8 × D8	0h × 48h	18,314	1,690	105	86	19
ALS	D1322^R × BJ204^S	covariate	19,317	929	26	15	11
	D1322 × D1322	2 dpi × 6 dpi	19,024	2,202	98	66	32
	BJ204 × BJ204	2 dpi × 6 dpi	19,001	1,847	64	40	24
RKN	IL10-1^R × CC3^S	covariate	21,490	498	14	9	5
	IL10-1 × IL10-1	0 dpi × 3 dpi	19,897	1,612	50	21	29
	CC3 × CC3	0 dpi × 3 dpi	21,188	1,863	63	41	22

DEG: differentially expressed genes, PK: protein kinase, PM: powdery mildew (*P. xanthii*), ALS: alternaria leaf spot (*A. cucumerina*), RKN: root-knot nematode (*M. incognita*), (R): resistant, (S): susceptible, dpi: days post-inoculation. Down: down-regulated, Up: up-regulated.

Of the general expression data, only values related to the expression of PK DEGs were considered among the total set of DEGs. To identify PK DEGs responsive to PM, three approaches were performed, in which the differential expression between resistant (SSL508-28) and susceptible (D8) genotypes at evaluation times (0 and 48 h, using the variable time as a covariate) were analyzed (Figure 5, Table 2). The second and third approaches were considered the expression of susceptible and resistant genotypes in relation to the initial and final expression times. Among genotypes, only 8 PK DEGs were significant, of which 2 were down-regulated, and 6 were up-regulated (Figure 5-A). In the resistant genotype, 87 significant PK DEGs were observed (50 down-regulated and 37 up-regulated). In the susceptible genotype, a total of 105 significant PK DEGs were identified (86 were down-regulated and 19 were up-regulated).

To identify the DEGs responsive to ALS infection, PM-like approaches were used. First, between the resistant (D1322) and susceptible (BJ204) genotypes with time as a covariate, 26 significant DEGs were identified (15 were down-regulated and 11 were up-regulated) (Figure 5-B). Subsequently, 98 significant DEGs were observed in the resistant genotype (66 DEGs down-regulated and 32 DEGs up-regulated). Finally, 64 DEGs were identified in the susceptible genotype at evaluation times 2 and 6 dpi (40 DEGs down-regulated and 24 DEGs up-regulated).

In case of RKN-responsive DEGs, 14 significant DEGs were identified between resistant (IL10–1) and susceptible (CC3) genotypes with the time of evaluation as a covariate (9 DEGs down-regulated and 5 DEGs up-regulated) (Figure 5-C). In the resistant genotype, 50 DEGs were observed in 0-3 dpi (21 down-regulated and 29 up-regulated). In the susceptible genotype, 63 significant DEGs were observed in 0-3 dpi (41 down-regulated DEGs and 22 up-regulated DEGs).

To study the DEGs in common, Venn diagrams were constructed with the PK genes common to the three pathosystems, with 312 DEGs in common among all of them, of which 225 down-regulated and 153 up-regulated (Figure 5D-F). To better understand the down- and up-regulated families common to each pathosystem, an UpSet plot (Figure 6-A) support the identification of 203 families (71 non-redundant PK families) distributed across all conditions analyzed. Members of the RLK families are most frequently observed, with the RLK-Pelle_DLSV family appearing 17 times (Figure 6-B-C, Supplementary Tables S8, and S9). The lists were categorized based on different combinations of proteins related to specific biological processes, containing proteins associated with responses to PM, RKN, and ALS. The number of families is varied, ranging from 1 to 41 members, and they contain proteins that belong to different RLK families, among others. These lists may represent significant groupings of proteins that play important roles in specific biological pathways or cellular regulatory processes. According to our results, 20 families were exclusive to PM, 6 were exclusive to ALS, and 4 were exclusive to RKN, 9 in common to PM and ALS, 4 common to PM and RKN, 8 in common to ALS and RKN, and 20 families in common to all (Figure 6, Supplementary Tables S10-S13).

3.4. Kinase duplication and synteny analyses

Through an in-depth examination of duplication events, our study yielded estimations for 833 protein kinases (PKs), considering that two kinases are still discernible at the scaffold level in the genome. The predominant origin of these PKs was attributed to whole genome duplication (WGD) events, encompassing approximately 69.4% (578 PKs) (Figure 7A). Tandem duplications contributed to ~28.5% (237 PKs), proximal duplications accounted for ~1.6% (13 PKs), singleton events represented ~0.36% (3 PKs), and dispersed duplications were observed at a minor frequency of 0.24% (2 PKs). For a subset of 40 genes, we calculated both the Ka and Ks substitution rates (Figure 7B). The Ka/Ks ratio, indicative of the interplay between purifying selection, neutral mutations, and advantageous mutations, ranged from 0.146 to 2.198, with a mean ratio of 0.146 (Supplementary Table S14). Ratios below 1 suggest selective pressure, values around 1 denote neutral effects, while ratios exceeding 1 signify advantageous mutations. Furthermore, the estimated ages of these duplication events were determined based on the Ks rate, spanning from ~ 50.42 to ~422.81 million years ago (MYA). This temporal insight provides a comprehensive perspective on the evolutionary dynamics of the PK landscape, shedding light on the selective forces and mutational processes that have shaped this vital component of the genome over extensive periods.

A total of 237 PKs exhibiting tandem duplications were identified across 54 families. Among these, one family belonged to the CAMK group, two to CMGC, 43 to RLK, 5 to TKL, and singular representatives from TLK, WEE, and WNK. Tandem duplications manifest when a chromosomal segment is replicated and integrates adjacent to the original segment, a result of the crossing-over process between chromatids. This mechanism plays a crucial role in amplifying the count of genes sharing identical or similar functions, contributing significantly to genomic diversity and functional specialization.

To investigate the evolutionary relationships between cucumber and melon species, we conducted a detailed synteny analysis, focused on cucumber kinase genes. Of the 835 kinase genes identified in cucumber, we identified remarkable syntenic relationships for 670 of these genes on all chromosomes of the two species (Figure 8, Supplementary Table S15). This finding encompasses 821 syntenic relationships with melon genes, of which 11 genes showed three relationships, 129 genes showed two relationships, while the remaining genes showed a single relationship. Analyzing the melon genome in relation to the cucumber PK array, we identified that 672 genes exhibited syntenic relationships, providing a comprehensive view of the genetic interconnections between these two species. These results provide insights into evolution and genetic relationships, highlighting complex patterns of conservation and divergence. The richness of these syntenic relationships reveals the intricate web of genetic connections between cucumber and melon, indicating not only shared ancestry but also specific duplication and rearrangement events throughout their evolutionary histories.

4. Discussion

4.1. Genome-wide identification and Classification of cucumber PKs

Cucumber was one of the first economically important crop to have its genome published. Over five years the genomes of the lineages ‘9930’ (Huang et al., 2009; Li et al., 2011), ‘PI 183967’ (Qi et al., 2013; Wóycicki et al., 2011; Yang et al., 2012), ‘Gy14’ and ‘B10’ were made available, which provided a significant scientific advance in the field of cucumber genetic research. In addition, the utilization of recently developed high-throughput long-read sequencing technology has improved the quality of genome assembly for the ‘Gy14’ v2.1, ‘9930’ v3.0, and ‘B10’ v3.0 (Weng, 2022). This improvement enabled us to conduct a genome-wide analysis, resulting in the construction of the cucumber ‘Gy14’ kinome.

Some projects have already been carried out considering some families of PKs in Csa species (Lehti-Shiu et al., 2012; Xu et al., 2015). In this study, we performed analyses of the cucumber proteome to identify and characterize the PK family (Hanks et al., 1995). Using the profiles of Hidden Markov Models (HMM) PF00069 (Pkinase) and PF07714 (Pkinase_Tyr) to scan the cucumber annotated proteome, a total of 835 proteins containing kinase domains were identified, which provided the opportunity for a holistic view of the biological processes played by PKs. Among these, 517 sequences were classified as Serine/Threonine kinase (Pkinase) and 318 sequences as Tyrosine kinase (Pkinase_Tyr) This number represents 3.69% of the total proteins of the Csa species, slightly below the average (3.85%) of other species already studied, such as Arabidopsis, common beans, corn, cowpea, soybean and vine (Aono et al., 2023; Champion et al., 2004; Ferreira-Neto et al., 2021; Liu et al., 2015; Wei et al., 2014; Zhu et al., 2018; Zulawski et al., 2014).

For a better understanding of the evolutionary relationships between cucumber PKs, a phylogenetic analysis was obtained, which successfully grouped the members of eight well-established groups: AGC, CAMK, CK1, CMGC, RLK, SCY, STE and TKL (Lehti-Shiu et al., 2012). These groups represent distinct families of PKs with known functions in several biological processes. However, there was significant functional diversity and expansion within these groups, as evidenced by the presence of different families within the groups. The expansion of these families may be related to the evolutionary process, since PKs are central regulators of stress in plants (Benschop et al., 2007; Peck, 2001; Tena et al., 2011), acting like a “central processor unit” (CPU), processing external signals and converting them into specific response in the cell metabolism (Hardie, 1999) and, therefore, need to stand fine-tuned to recognize a plethora of pathogenic effectors that trigger the immune response processes. As observed by Lehti-Shiu et al., (2012), some PKs of the remaining groups (AUR, BUB, PLANT-SPECIFIC, IRE1, NAK, NEK, PEK, TLK, TTK, ULK, UNKNOWN, WEE, and WNK) observed in our analysis are composed of PKs that probably arose after events that led to the divergence of plants, fungi and animals, with these families having few members, as they are absent in the algae *Chlamydomonas reinhardtii*, such as BUB, IRE1, PEK. This is an aspect that needs to be elucidated, regarding which families arose before and after the divergence of eukaryotes into fungi, plants and animals. Some specific families still have not their functions clarified in some plant species, such as the PKs of the Group-Pl (Lehti-Shiu et al., 2012), NEK in both plants and animals (Baker et al., 2023, Dahan et al., 2009, Zhang et al., 2011).

According to the rules proposed by Lehti-Shiu et al. (2012), the 835 PKs of the cucumber were classified into 20 groups. Out of these, the RLK group was the most numerous, comprising 527 genes, representing approximately 63.5% of the total kinases identified. This result is consistent with previous studies highlighting the abundance of RLKs in common bean, corn, Arabidopsis, vine, cowpea, and soybean plants, averaging 66.9%, where RLKs play crucial roles in signal transduction and various developmental and stress response processes (Aono et al., 2023; Champion et al., 2004; Ferreira-Neto et al., 2021; Liu et al., 2015; Wei et al., 2014; Zhu et al., 2018; Zulawski et al., 2014). Notably, Lehti-Shiu et al. (2012) did not detect the AGC_PKA-PKG, BUB, PEK_PEK, and TKL-PL-8 families in the Csa species; however, our study identified their presence. Discrepancies in analytical methodologies and the absence of genome collinearity among the studied cucumber lineages may account for these differences.

According to Lehti-Shiu et al. (2012), the PK groups could be subdivided into 123 families, with 34 of them composed of only one PK gene (Supplementary table S6). Notably, in our studies, the RLK-Pelle_DLSV family was the most numerous, consisting of 68 proteins, a result compatible with that obtained for the Csa species by Lehti-Shiu et al. (2012), this being one of the most numerous families of the species reported. Similar results have also been found in other species, such as Gossypium (Yan et al., 2018), rice (Dardick et al., 2007), Arabidopsis (Champion et al., 2004; Zulawski et al., 2014) and corn (Wei et al., 2014). The expansion and establishment of this family probably occurred before the divergence of Viridiplantae species (Lehti-Shiu et al., 2012), as no evidence that the Csa species has undergone whole genome duplication (Huang et al., 2009). However, after a new assembly of the cucumber genome, Li et al. (2019) reported 239 tandemly duplicated genes, and Yu et al. (2022), reporting on 15 pairs of tandem duplication genes and 31 pairs of segmental duplication in the cucumber genome.

Not only does the RLK-Pelle_DLSV family stand out, but within the PKs of the RLK group, the RLK-LRR forms the most abundant subgroup, comprising 202 genes and further subdivided into 23 families (refer to Supplementary Table S4). These findings align with those presented by Yu et al. (2022), who identified 189 genes distributed across 22 families RLK-LRR in the Chinese Long cucumber genome v3. The substantial presence of genes within this family underscores their significance in stress response mechanisms, as highlighted in studies by Dambroz et al. (2023), Fischer et al. (2016), Magalhães et al. (2016), and Sakamoto et al. (2012).

4.2. Characterization of the cucumber PK sequences

This investigation delves into various facets of PK genes, providing insights into the diversity and functional characteristics of cucumber's PK gene repertoire. The analysis commenced by scrutinizing the structural attributes of the 835 PK genes, with a particular focus on the determination of intron numbers. The presence of introns holds significance as it may be intricately linked to the regulation of gene expression, facilitating processes such as alternative splicing. Notably, intronic genes, which encompass specific intron fragments undergoing expression, as well as factors such as intron length and intron phase, assume crucial roles in the regulation of gene expression (Baulin et al., 2020). These findings underscore the structural diversity inherent in cucumber's PK genes and shed light on the intricacies of their regulatory landscapes (Rose, 2008). Moreover, the presence of introns in a gene can exert an influence on its susceptibility to silencing through microRNAs (miRNAs). Furthermore, miRNAs have the ability to bind to mRNA sequences that include introns, and the quantity of introns can impact the efficiency of miRNA-mediated silencing (Huberdeau et al., 2018; Lin et al., 2006). Hence, the number of introns plays a role in the regulatory process. Then, the occurrence of introns in miRNA target genes can impact mRNA maturation and translation efficiency, thereby exerting a downstream effect on overall gene expression dynamics (Lin et al., 2006).

The uneven distribution of PK genes on the seven chromosomes of the cucumber genome represents a non-random pattern, which may be a result of the evolutionary process undergone by the species (Yang, et al., 2012). However, the distribution of RLK genes in clusters was observed, supporting the hypothesis that this family originated by tandem duplication and later subfunctionalization (Holub, 2001), although no duplication was evidenced in cucumber species (Huang et al., 2009). On the other hand, the results of Li et al. (2019), and Yu et al. (2022) contradict previous studies, giving evidence of the cucumber genome duplication.

Considerable variation was observed in all the characteristics examined, indicating specialized functions in their respective microenvironments, similar to patterns also observed by Dambroz et al. (2023). Most PK functions are associated with the plasma membrane and nucleus, comprising about 77% of the PK genes in cucumber. The kinase domains of nuclear and membrane proteins are intricately linked to pathogen identification and the plant defense response. The predominant functions of PKs are associated with the plasma membrane and the nucleus, playing a pivotal role in the regulation of gene expression and the response to environmental stimuli, such as stress and disease resistance in plants (Delormel et a., 2019; Luan et al., 2002). This dual functionality, spanning both nuclear and membrane realms, underscores the versatility of PKs in performing essential cellular processes (Romeis, 2001). At the plasma membrane, PKs are instrumental in perceiving external signals, transducing them into intracellular responses, and modulating various physiological pathways crucial for plant growth and adaptation (Ni et al., 2017). Simultaneously, within the nucleus, PKs contribute significantly to the intricate machinery of gene regulation, influencing transcriptional processes that ultimately shape the plant's responses to environmental challenges (Carbonnel et al., 2010). A pattern of specialized functions localized predominantly in the plasma membrane and nucleus emerges, aligning with observations in cucumber, common beans (Aono et al., 2023), *Hevea brasiliensis* and *Manihot esculenta* (Santos et al., 2023). This consistency across diverse plant species suggests a fundamental role for PKs in essential cellular processes and stress responses. However, the observed diversity in subcellular locations among PKs also hints at their involvement in specific signaling pathways, highlighting the versatile and intricate nature of their functions. Similar results were also observed in common beans (Aono et al., 2023), *Hevea brasiliensis*, and *Manihot esculenta* (Santos et al., 2023). However, other genes can be found in various subcellular locations, suggesting their involvement in specific cellular processes, and signaling pathways in various cellular biological processes together with the stress responses.

In principle, PKs are responsible for the processes of phosphorylation and binding to specific targets in the cell related to activation processes and can be listed as interesting targets for genetic breeding, in the hypothesis of improvement of central metabolic processes, such as increased efficiency of enzymatic conversion or modifications at the binding site that recognize pathogen effectors, preventing its infection. As an example, regulation of gene expression through transcription factors led to overexpression of mutant rice genotypes, increasing nitrogen use efficiency and productivity (Wei et al., 2022). However, studies detailing these processes involving phosphorylation remain scarce.

4.3. Kinase gene expression patterns

The study of differential gene expression is one of the main methodologies for understanding the nuances of pathogen-plant interaction (PPI). This interaction involves several processes that include the proteins phosphorylation and the expression of various defense-related genes, among others. In this study, we investigated the expression profiles of the 22,626 genes encoded in Gy14 genome in response to biotic stress conditions of PM, ALS, and RKN, using transcriptome data from different cucumber genotypes interacting with different pathogens. After modeling with DESeq2, some genes did not fit and were removed to further analyses. Then, PK DEGs were identified from the DESeq2 fitted pool. It is possible to observe that susceptible genotypes present a greater number of down-regulated DEGs when the pathogens have a biotrophic lifestyle (PM and RKN); on the other hand, in the necrotrophic pathogen (ALS), a greater number of down-regulated genes is observed in the resistant genotype (Table 2, and Figure 5).

Our analysis identified PK DEGs in response to *P. xanthii* infection (PM), showing similar expression patterns but at different response times. However, the process is still elusive and needs to be investigated, since some genes with differential expression are present in both resistant and susceptible genotypes. Some DEGs act at the cell nucleus level (~25% of DEGs) in processes related to protein phosphorylation and mitotic cell cycle regulation; while most of the DEGs (~55%) function in the nucleus, with general functions related to phosphorylation and participation in some more specific processes such as Peptidyl-Tyrosine Phosphorylation, MAPK Cascade, Hormone-Mediated Signaling Pathway, Phragmoplast Assembly

(Supplementary Table S10). Some proteins in smaller numbers were also located in chloroplasts, endomembrane system, extracellular space, mitochondrion, and mitochondrion membrane with functions related to Polysaccharide Binding, Nucleotide Binding, Transmembrane Receptor Protein Tyrosine Kinase Activity, Polysaccharide Binding; and metabolic processes, such as Defense Response to Bacterium, and Peptidyl-Tyrosine Phosphorylation (Figures 5 and 6 and Supplementary Table S6). These findings suggest that PKs play important roles in the defense response against PM, with similar expression patterns observed between resistant and susceptible genotypes and major expression differences related to response time to infection, because in general, susceptible genotypes show an immediate response, while resistant genotypes show a later response after infection. The shallow response time may be related to the time of cell maturation, time of development of infection-induced apoptosis, rates of infection spread, and recovery of infected cells. In addition, these factors may express themselves with different intensity among different genotypes, suggesting that uninfected cells do not have sufficient acquired immunity to recover the host from infection (Goyal et al., 2022). In this way, resistant genotypes have a more effective immune response responding more quickly against infection, due to several factors including localized immune efficacy (Escobar et al., 2003, Groenenboom et al., 2005). The difference in stress response time also involves the perception of changes in the environment by plant tissues and directly interferes with the cell cycle. This perception involves the activation of CDKs, which are the central regulators of this cycle. Cyclin-dependent kinases (CDKs) play important roles as it relates to cell division and response to many intracellular and extracellular signals (Malumbres, 2014). This process results in the activation of a signaling cascade that prolongs the S phase with delayed entry into mitosis (Kitsios et al., 2011). This reveals the importance of the PKs of the CMGC families to cucumber resistance to PM and RKN.

We then analyzed the expression profiles of the PK genes in response to ALS caused by *A. cucumerina*. Among our findings, different patterns of DEGs were observed in different families of PK. The resistant genotype showed a small number of both down- and up-regulated DEGs compared to the susceptible genotype (Figure 5C). This pathogen exhibits a necrotrophic lifestyle, alike from the others. This means that the plant's response pattern is mainly based on the synthesis of toxins, such as, phenolic compounds, phytoalexins, and hydrogen peroxide, whose purpose is to stop the pathogen's development more quickly (Akter et al., 2015, Debona et al., 2012, Kaur et a., 2022). In fact, the response of the resistant genotype is related to the genes of quantitative effect, which involves processes related to controlling physical traits and developmental processes, enhancing the plant's fundamental defense mechanisms, encoding enzymes for neutralizing phytotoxins produced by pathogens, aiding in the transmission of defense signals, including genes associated with the circadian clock, and incorporating weaker variants of R genes (Zhang et al., 2013). The predicted function of the genes is related to chloroplast, chloroplast thylakoid lumen, cytoplasm, endomembrane system, extracellular space, nucleus and plasma membrane in biological processes, such as, activation of protein kinase activity, anatomical structure morphogenesis, aromatic compound biosynthetic process, blue light signaling pathway, brassinosteroid mediated signaling pathway, defense response to bacterium/fungus, positive regulation of abscisic acid-activated signaling pathway, proteolysis, reactive oxygen species metabolic process, and MAPK cascade (Supplementary Table S6).

Finally, we also analyzed the expression of PK genes in response to RKN (*M. incognita*). The response to this disease showed a pattern like that caused by PM, in which the same families participated in the responses, with differences observed only concerning response time (Figures 5 and 6). Genes of RLK family are more important in the response to RKN infection, with similar response patterns between resistant and susceptible genotypes, but with greater differences related to response time. The kinases of the RLK-Pelle_DLSV family play an important role in the regulation of gene expression, as it presented a greater number of genes, facilitating signal transduction when pathogens threaten. However, RLK genes were the only ones in common between the two pathosystems whose pathogens are biotrophic, PM and RKN. In addition to the RLK family, other DEGs only observed in response to RKN belonged to the AGC_PDK1 and TKL-Pl-6 families (Supplementary Table S13). AGC_PDK1 acts positively in regulating basal disease resistance in rice upstream of the OsOxi1-OsPti1a phosphorylation cascade (Matsui et al., 2010). CMGC genes were predicted only in the nucleus, unlike PM-responsive CMGC genes, which were also observed in the cytoplasm. They activate immune responses such as hypersensitivity reactions and systemic acquired resistance, phosphorylating immunity-related proteins to bolster defenses. In addition, these kinases regulate gene expression, favoring the synthesis of defense proteins. By phosphorylating defense-related proteins, including pathogen receptors, CMGC kinases increase a plant's ability to recognize and neutralize pathogens (Benschop et al., 2007; Tena et al., 2011), but it is important to note that other components of the cell determine the response time to the disease triggered by PK signaling, which still needs to be investigated. Further studies in this area are needed to elucidate these processes involving the response to diseases.

4.4. Kinase duplication and synteny analyses

Our detailed analysis of the duplication events revealed significant insights into the cucumber kinome. The estimates performed for 833 kinases identified the predominance of doubling events, highlighting notable contributions of total genomic duplications (WGD) and tandem duplications. The predominance of WGD events, which account for approximately 69.4% of kinases in cucumber, suggests a key role of these duplications in the evolution of the kinome. These events can be associated with specific spikes in synonymous substitution rates (Ks), indicating significant duplications around 50.41 and 422.81 million years ago (MYA). Notably, this last interval coincides with polyploidization events in the eudicot lineage, pointing to a possible influence of these events on the diversification of these kinases.

Analysis of the non-synonymous substitution rate (Ka) revealed that most kinases in cucumber are under purifying selection, indicating an evolutionary pressure to preserve the structure and stabilize the function of these proteins. This pattern is consistent with other studies on different gene families in common bean (Aono et al., 2023), suggesting a general trend in functional conservation across the genome. The identification of 237 kinases with tandem duplications is especially interesting. Among these, notable families, such as RLK-Pelle_DLSV, exhibited a significant number of protein duplications. These tandem duplications are known to be associated with stress responses, indicating a possible adaptive expansion of these subfamilies to address environmental challenges (Freeling, 2009; Hu et al., 2022; Leister et al., 2004; Panchy et al., 2016).

When comparing our results with studies in other plant species, we observed similar patterns regarding the presence of duplications under purifying selection. However, we highlight the uniqueness of our findings, especially in terms of high rates of Ks and distant dates of doubling for the kinases. These traits may be specific to the evolution of kinases in this species and may influence functional diversity and long-term adaptation.

5. Conclusion

We underscore the pivotal role of Protein Kinases (PKs) in cucumber immunity and disease resistance. Our comprehensive analysis delineates the characteristics of cucumber's 835 PKs, categorized into 123 families and 20 groups. Notably, only 312 PKs demonstrated significant relevance in response to biotic stress, further classified into 10 groups: AGC, BUB, CAMK, CMGC, RLK, STE, TKL, ULK, WEE, and WNK. This investigation establishes a solid groundwork for future exploration into the specific functional roles of these PKs, paving the way for the development of targeted strategies to enhance disease resistance in cucumbers and other Cucurbitaceae species. The outcomes of this research contribute significantly to the progression of agricultural practices, offering valuable insights for bolstering crop resilience against diverse disease challenges.

Data availability

Data in pre-publication were analyzed in this study relative to the *Cucumis sativus* Gy14 genome with permission for publication from Dr. Yiqun Weng (USDA-ARS Vegetable Crops Research Unit). The data from the *Cucumis sativus* Gy14 genome, and public data of RNA-seq projects PRJNA321023 (PM), PRJNA563186 (ALS), and PRJNA419665 (RKN) are available in the Cucurbit Genomics Databsase v2 (CuGenDBv2) (http://cucurbitgenomics.org/v2/).

Conflict of Interest

The authors assert that they do not possess any identifiable financial conflicts of interest or personal associations that might have seemed to impact the findings presented in this manuscript.

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Figure 1. Phylogenetic tree of *Cucumis sativus* PKs with the approximately-maximum-likelihood Likelihood approach with bootstrap values (> 70%), using the Whelan Goldman (WAG) model with gamma parameter. The clades highlighted are referred to the groups RLK, SCY, TKL, STE, CMGC, AGC1, CK1, and CAMK (from up to down)**.** The members of each family are collapsed to a better visualization. The gene names displayed with square brackets represent branches with single genes. The tree is rooted with a kinase (Cre07.g349540.t1.1) from the *Chlamydomonas reinhardtii* v5.6 proteome. Some genes from expanding groups can be found within other consolidated groups.

Figure 2. Chromosome physical localization of kinase genes from *Cucumis sativus*. The kinase genes are presented by their names followed by the family name, and the colors represent different groups. Chromosome zero (0) contains genes that have not been assigned to specific chromosomes.

Figure 3. **(A)** Number of cucumber kinases per chromosome. **(B)** Number of introns per kinase per chromosome. **(C)** Number of kinases per subcellular location. **(D)** Distribution of kinase per subcellular location per kinase group. **(E)** Number of transmembrane domains and signal peptides. **(F)** molecular weight (MW), and isoelectric point (IP) in function of grand average of hydropathy (GRAVY).

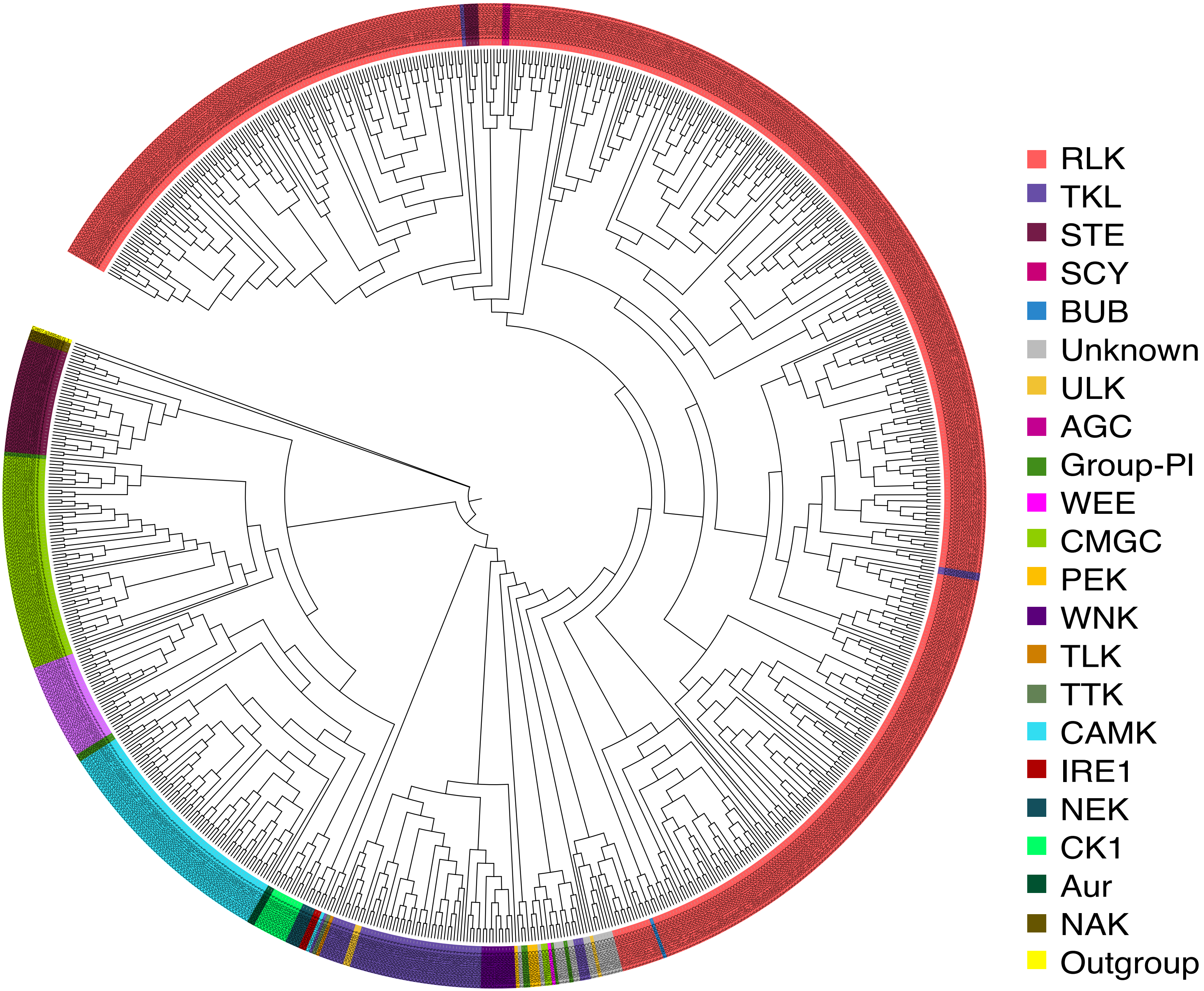
Figure 4. (A) Biological processes (227) predicted in the Gene Ontology analysis. **(B)** P: biological process, F: molecular function, C: cellular component. Figure shows the 40 top GO terms; a full list is available in Supplementary Table S7.

Figure 5. Differential expression of kinase genes (kinase DEGs) of the **(A)** the genotypes resistant (SSL508-28) and susceptible (D8) to powdery mildew (PM) (*Podosphaera xanthii*), **(B)** the genotypes resistant (D1322) and susceptible (BJ204) to alternaria leaf spot (ALS) (*Alternaria cucumerina*); and **(C)** the genotypes resistant (IL10-1) and susceptible (CC3) to root-knot nematode (RKN) (*Meloidogyne incognita*). **(D)** DEGs in common to all pathosystems; **(E)** DEGs down-regulated in common to all pathosystems; **(F)** DEGs up-regulated in common to all pathosystems; **(G)** Family DEGs in common to all pathosystems.

Figure 6. (A) UpSet plot illustrating the shared 312 Protein Kinases Differentially Expressed Genes (312 PK DEGs) across three pathosystems: RKN (Root-knot nematode, *M. incognita*), ALS (Alternaria leaf spot, *A. cucumerina*), and PM (powdery mildew, *P. xanthii*), for both down-regulated (RKN_down, ALS_down, and PM_down) and up-regulated (RKN_up, ALS_up, and PM_up) genes. In the plot, black dots connected by vertical black lines represent the count of PK DEGs shared among the respective conditions. Vertical bars indicate the number of DEGs in specific conditions, and horizontal bars show the sum of down- or up-regulated DEGs. **(B)** Word cloud represents the diversity and abundance of Protein Kinase families reported in these pathosystems. **(C)** Absolute frequency of Protein Kinase families reported in the three pathosystems studied.

Figure 7. **(A)** Classification of duplication types of 833 Protein Kinases of Cucumis sativus (Gy14). **(B)** Range of Ks values for the Protein Kinases of Cucumis sativus (Gy14).

Figure 8. Synteny analysis of Protein Kinases between Cucumis sativus (Gy14) and Cucumis melo (DHL92). The chromosomes of C. sativus are represented in orange color and the prefix Gy14, and the chromosomes of C. melo represented in green and the prefix chr.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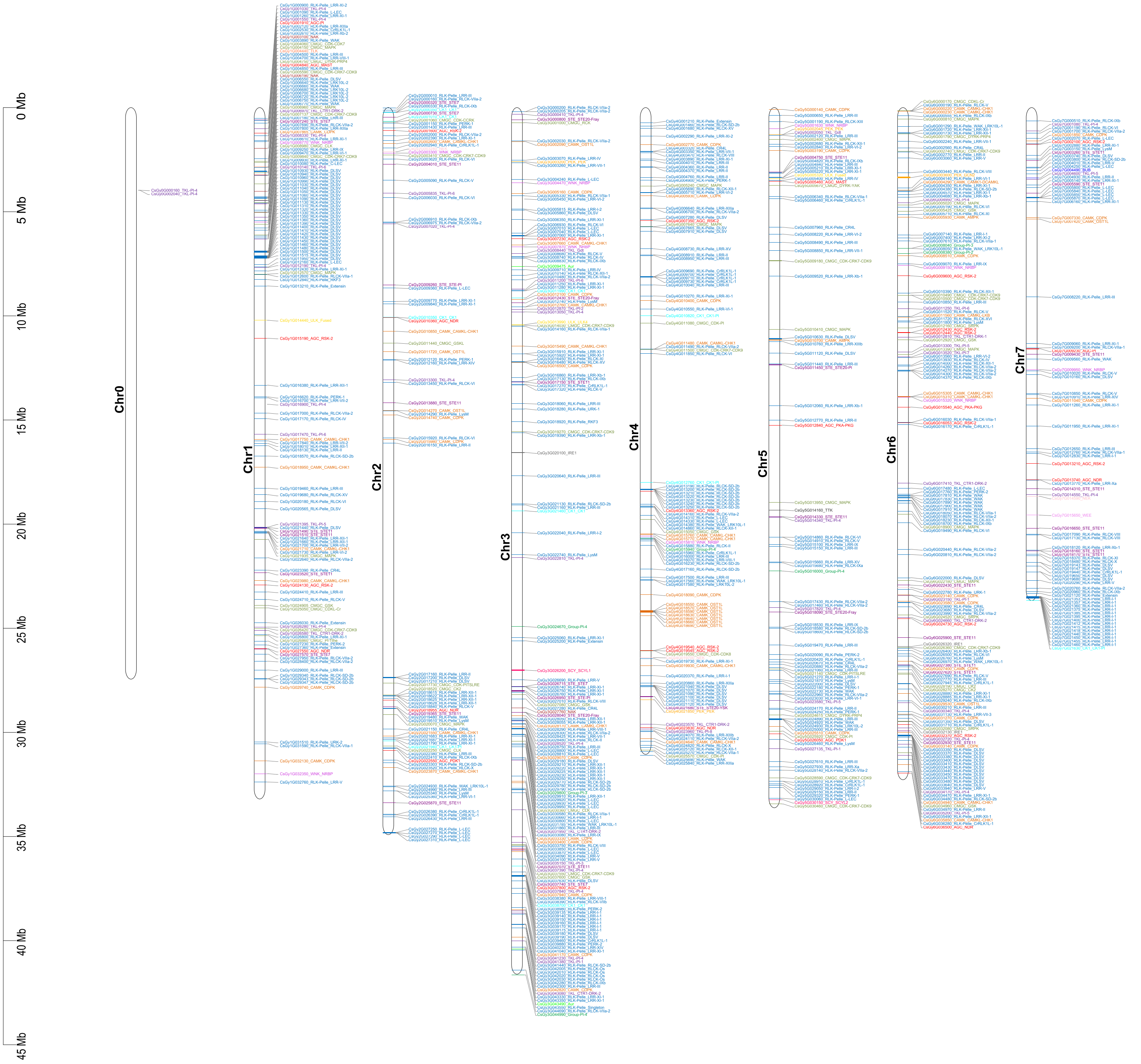
TKL
STE
SCY
BUB
Unknown
ULK
AGC
Group-PI
WEE
CMGC
PEK
WNK
TLK
TTK
CAMK
IRE1
NEK
CK1
Aur
NAK
Outgroup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25 Mb
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35 Mb
40 Mb
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Chr0
Chr1
Chr2
Chr3
Chr4
Chr5
Chr6
Chr7
CsGy0G000160_TKL-Pl-4
CsGy0G000340_TKL-Pl-4
CsGy1G014440_Ulk_Fused
CsGy1G015190_AGC_RSK-2
CsGy1G032130_CAMK_CDPK
CsGy1G032350_WNK_NRBP
CsGy1G032760_RLK-Pelle_LRR-V
CsGy3G020100_IRE1
CsGy3G024670_Group-Pl-4
CsGy3G026200_SCY_SCYL1
CsGy5G012840_AGC_PKA-PKG
CsGy6G009600_AGC_RSK-2
CsGy6G015540_AGC_PKA-PKG
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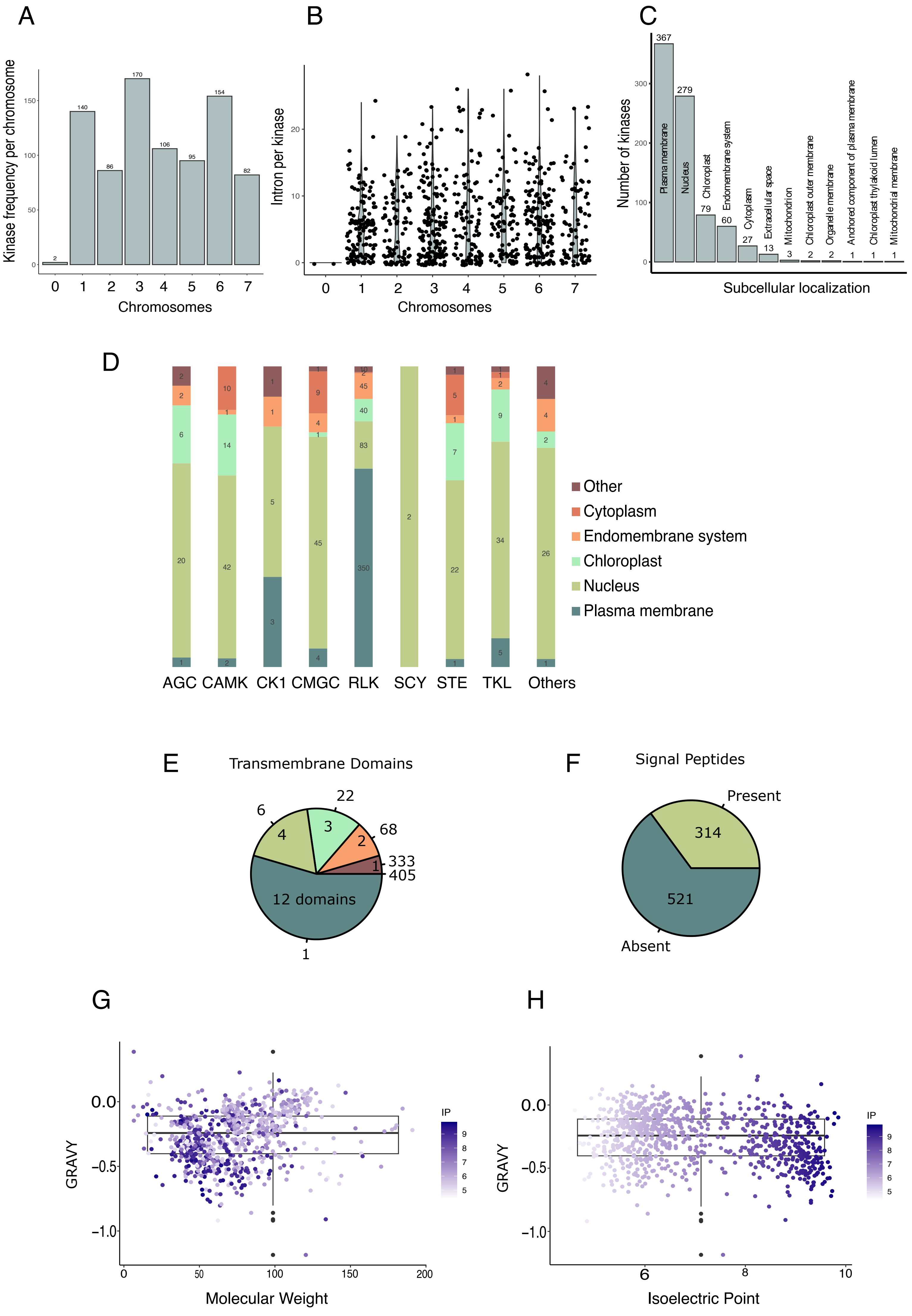
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Kinase frequency per chromosome
Chromosomes
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2
3
4
5
6
7
0
50
100
150
2
140
86
170
106
95
154
82
B
Intron per kinase
Chromosomes
0
10
20
C
Number of kinases
Subcellular localization
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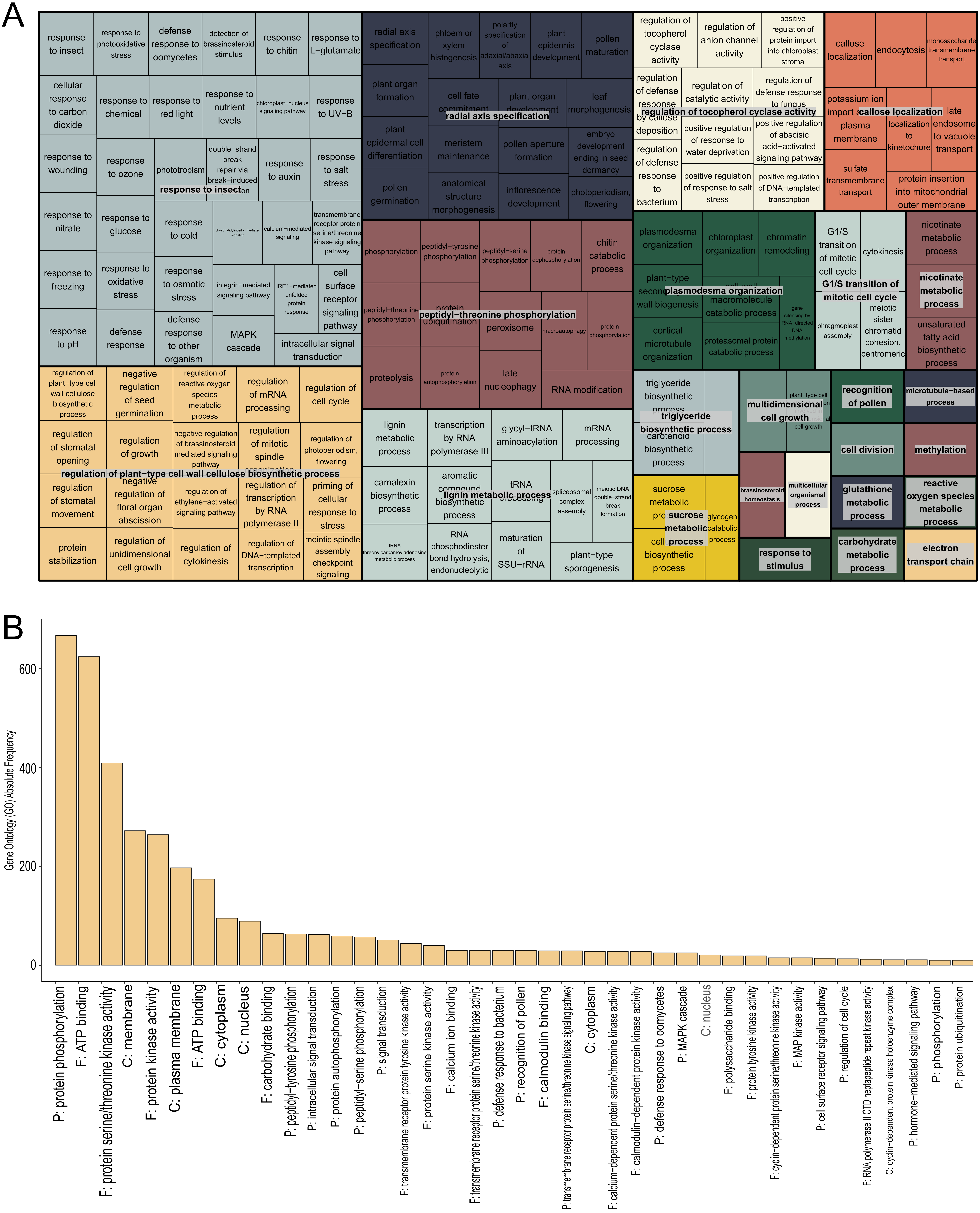
279
79
60
27
13
3
2
2
1
1
1
Plasma membrane
Nucleus
Chloroplast
Endomembrane system
Cytoplasm
Extracellular space
Mitochondrion
Chloroplast outer membrane
Organelle membrane
Anchored component of plasma membrane
Chloroplast thylakoid lumen
Mitochondrial membrane
D
AGC
CAMK
CK1
CMGC
RLK
SCY
STE
TKL
Others
Other
Cytoplasm
Endomembrane system
Chloroplast
Nucleus
Plasma membrane
E
Transmembrane Domains
12 domains
4
3
2
1
1
6
22
68
333
405
F
Signal Peptides
Present
314
521
Absent
G
GRAVY
Molecular Weight
0.0
−0.5
−1.0
0
50
100
150
200
IP
9
8
7
6
5
H
GRAVY
Isoelectric Point
6
8
10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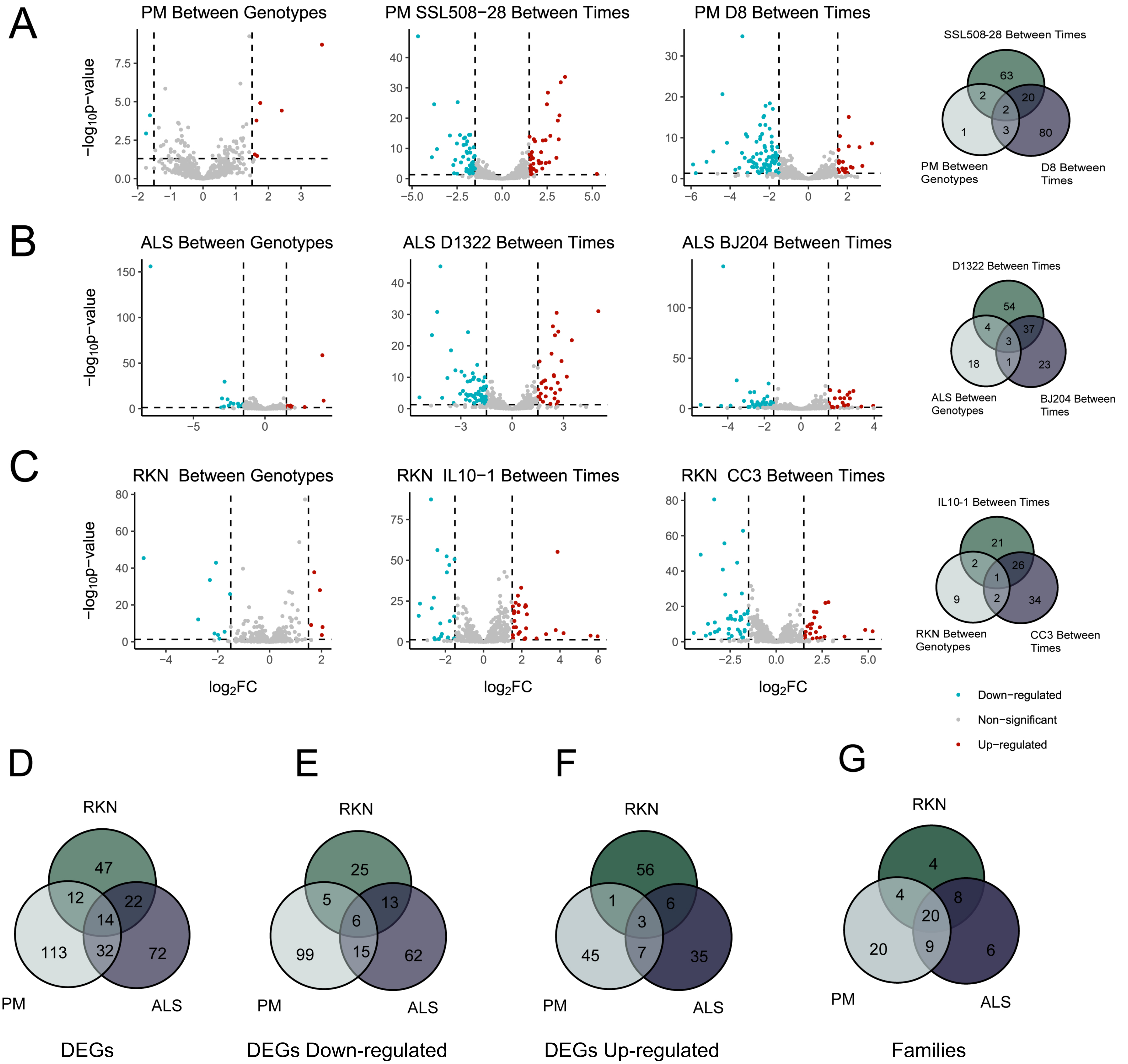
response to insect
response to photooxidative stress
defense response to oomycetes
detection of brassinosteroid stimulus
response to chitin
response to L−glutamate
cellular response to carbon dioxide
response to chemical
response to red light
response to nutrient levels
chloroplast−nucleus signaling pathway
response to UV−B
response to wounding
response to ozone
phototropism
double−strand break repair via break−induced replication
response to auxin
response to salt stress
response to insect
response to nitrate
response to glucose
response to cold
phosphatidylinositol−mediated signaling
calcium−mediated signaling
transmembrane receptor protein serine/threonine kinase signaling pathway
response to freezing
response to oxidative stress
response to osmotic stress
integrin−mediated signaling pathway
IRE1−mediated unfolded protein response
cell surface receptor signaling pathway
response to pH
defense response
defense response to other organism
MAPK cascade
intracellular signal transduction
radial axis specification
phloem or xylem histogenesis
polarity specification of adaxial/abaxial axis
plant epidermis development
pollen maturation
plant organ formation
cell fate commitment
plant organ development
leaf morphogenesis
radial axis specification
plant epidermal cell differentiation
meristem maintenance
pollen aperture formation
embryo development ending in seed dormancy
pollen germination
anatomical structure morphogenesis
inflorescence development
photoperiodism, flowering
regulation of tocopherol cyclase activity
regulation of anion channel activity
positive regulation of protein import into chloroplast stroma
regulation of defense response by callose deposition
regulation of catalytic activity
regulation of defense response to fungus
regulation of tocopherol cyclase activity
positive regulation of response to water deprivation
positive regulation of abscisic acid−activated signaling pathway
regulation of defense response to bacterium
positive regulation of response to salt stress
positive regulation of DNA−templated transcription
callose localization
endocytosis
monosaccharide transmembrane transport
potassium ion import across plasma membrane
callose localization
protein localization to kinetochore
late endosome to vacuole transport
sulfate transmembrane transport
protein insertion into mitochondrial outer membrane
phosphorylation
peptidyl−tyrosine phosphorylation
peptidyl−serine phosphorylation
protein dephosphorylation
chitin catabolic process
peptidyl−threonine phosphorylation
protein ubiquitination
peptidyl−threonine phosphorylation
peroxisome
macroautophagy
protein phosphorylation
proteolysis
protein autophosphorylation
late nucleophagy
RNA modification
plasmodesma organization
chloroplast organization
chromatin remodeling
plant−type secondary cell wall biogenesis
plasmodesma organization
cell wall macromolecule catabolic process
gene silencing by RNA−directed DNA methylation
cortical microtubule organization
proteasomal protein catabolic process
G1/S transition of mitotic cell cycle
cytokinesis
G1/S transition of mitotic cell cycle
phragmoplast assembly
meiotic sister chromatid cohesion, centromeric
nicotinate metabolic process
nicotinate metabolic process
unsaturated fatty acid biosynthetic process
regulation of plant−type cell wall cellulose biosynthetic process
negative regulation of seed germination
regulation of reactive oxygen species metabolic process
regulation of mRNA processing
regulation of cell cycle
regulation of stomatal opening
regulation of growth
negative regulation of brassinosteroid mediated signaling pathway
regulation of mitotic spindle organization
regulation of photoperiodism, flowering
regulation of plant−type cell wall cellulose biosynthetic process
regulation of stomatal movement
negative regulation of floral organ abscission
regulation of ethylene−activated signaling pathway
regulation of transcription by RNA polymerase II
priming of cellular response to stress
protein stabilization
regulation of unidimensional cell growth
regulation of cytokinesis
regulation of DNA−templated transcription
meiotic spindle assembly checkpoint signaling
lignin metabolic process
transcription by RNA polymerase III
glycyl−tRNA aminoacylation
mRNA processing
camalexin biosynthetic process
aromatic compound biosynthetic process
lignin metabolic process
tRNA processing
spliceosomal complex assembly
meiotic DNA double−strand break formation
tRNA threonylcarbamoyladenosine metabolic process
RNA phosphodiester bond hydrolysis, endonucleolytic
maturation of SSU−rRNA
plant−type sporogenesis
triglyceride biosynthetic process
triglyceride biosynthetic process
carotenoid biosynthetic process
multidimensional cell growth
plant−type cell wall organization
unidimensional cell growth
recognition of pollen
microtubule−based process
cell division
methylation
sucrose metabolic process
sucrose metabolic process
glycogen catabolic process
cellulose biosynthetic process
brassinosteroid homeostasis
multicellular organismal process
glutathione metabolic process
reactive oxygen species metabolic process
response to stimulus
carbohydrate metabolic process
electron transport chain
B
Gene Ontology (GO) Absolute Frequency
0
200
400
600
P: protein phosphorylation
F: ATP binding
F: protein serine/threonine kinase activity
C: membrane
F: protein kinase activity
C: plasma membrane
F: ATP binding
C: cytoplasm
C: nucleus
F: carbohydrate binding
P: peptidyl−tyrosine phosphorylation
P: intracellular signal transduction
P: protein autophosphorylation
P: peptidyl−serine phosphorylation
P: signal transduction
F: transmembrane receptor protein tyrosine kinase activity
F: protein serine kinase activity
F: calcium ion binding
F: transmembrane receptor protein serine/threonine kinase activity
P: defense response to bacterium
P: recognition of pollen
F: calmodulin binding
P: transmembrane receptor protein serine/threonine kinase signaling pathway
C: cytoplasm
F: calcium−dependent protein serine/threonine kinase activity
F: calmodulin−dependent protein kinase activity
P: defense response to oomycetes
P: MAPK cascade
C: nucleus
F: polysaccharide binding
F: protein tyrosine kinase activity
F: cyclin−dependent protein serine/threonine kinase activity
F: MAP kinase activity
P: cell surface receptor signaling pathway
P: regulation of cell cycle
F: RNA polymerase II CTD heptapeptide repeat kinase activity
C: cyclin−dependent protein kinase holoenzyme complex
P: hormone−mediated signaling pathway
P: phosphorylation
P: protein ubiquitination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PM Between Genotypes
PM SSL508−28 Between Times
PM D8 Between Times
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SSL508-28 Between Times
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2
20
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80
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D8 Between Times
B
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D1322 Between Times
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4
37
3
18
1
23
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C
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RKN IL10−1 Between Times
RKN CC3 Between Times
IL10-1 Between Times
21
2
26
1
9
2
34
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CC3 Between Times
log2FC
Down−regulated
Non−significant
Up−regulated
D
RKN
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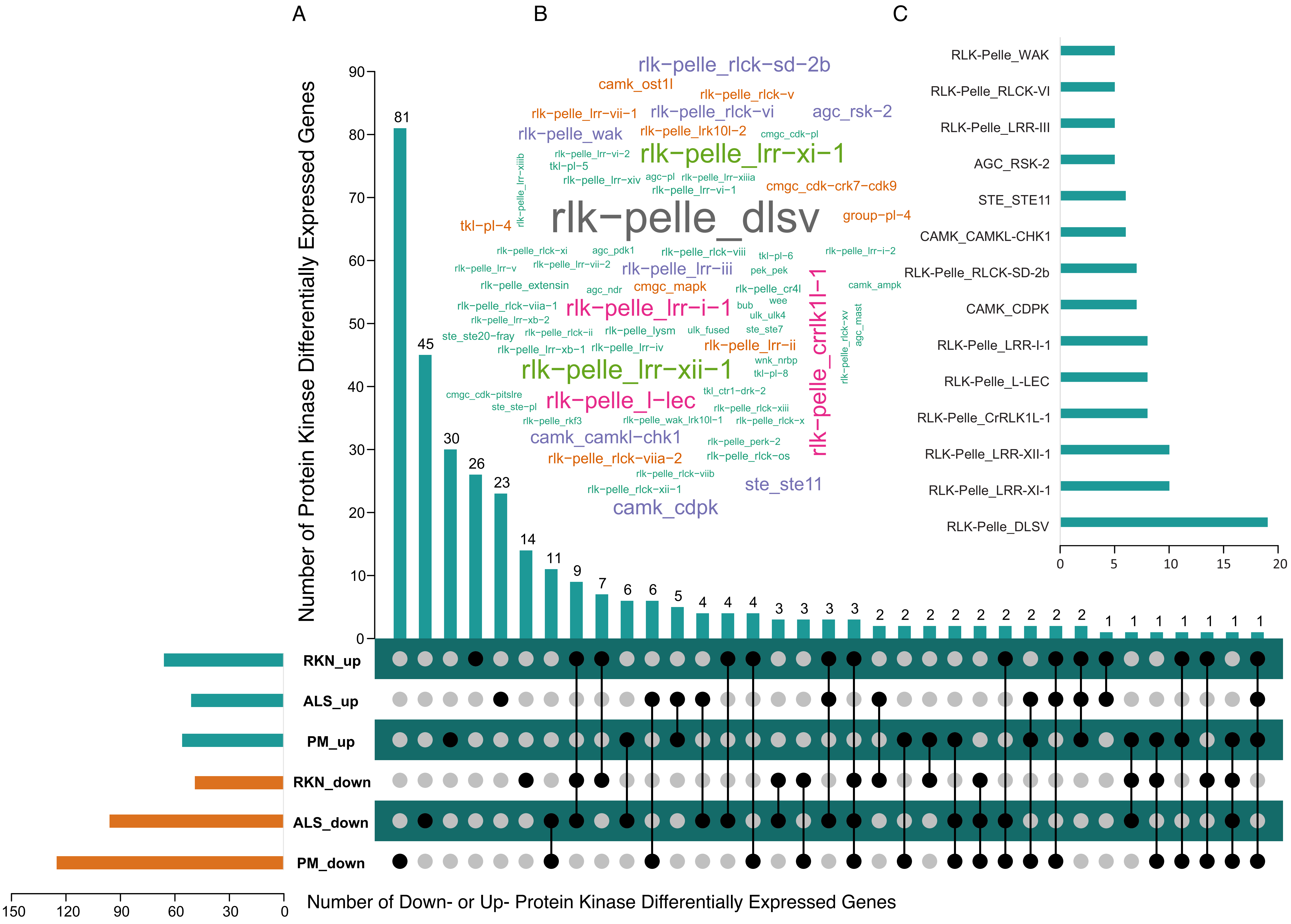
ALS
Families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B
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Number of Protein Kinase Differentially Expressed Genes
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45
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26
23
14
11
9
7
6
6
5
4
4
4
3
3
3
3
2
2
2
2
2
2
2
2
2
1
1
1
1
1
1
1
RKN_up
ALS_up
PM_up
RKN_down
ALS_down
PM_down
150
120
90
60
30
0
Number of Down- or Up- Protein Kinase Differentially Expressed Genes
rlk−pelle_rlck−sd−2b
camk_ost1l
rlk−pelle_rlck−v
rlk−pelle_lrr−vii−1
rlk−pelle_rlck−vi
agc_rsk−2
rlk−pelle_wak
rlk−pelle_lrk10l−2
cmgc_cdk−pl
rlk−pelle_lrr−vi−2
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tkl−pl−5
rlk−pelle_lrr−xiiib
rlk−pelle_lrr−xiv
agc−pl
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tkl−pl−4
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group−pl−4
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rlk−pelle_extensin
agc_ndr
cmgc_mapk
rlk−pelle_cr4l
camk_ampk
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rlk−pelle_lrr−i−1
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wee
ulk_ulk4
rlk−pelle_crrlk1l−1
rlk−pelle_rlck−xv
agc_mast
rlk−pelle_lrr−xb−2
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rlk−pelle_rlck−ii
rlk−pelle_lysm
ulk_fused
ste_ste7
rlk−pelle_lrr−xb−1
rlk−pelle_lrr−iv
rlk−pelle_lrr−ii
rlk−pelle_lrr−xii−1
wnk_nrbp
tkl−pl−8
cmgc_cdk−pitslre
rlk−pelle_l−lec
tkl_ctr1−drk−2
ste_ste−pl
rlk−pelle_rlck−xiii
rlk−pelle_rkf3
rlk−pelle_wak_lrk10l−1
rlk−pelle_rlck−x
camk_camkl−chk1
rlk−pelle_perk−2
rlk−pelle_rlck−viia−2
rlk−pelle_rlck−os
rlk−pelle_rlck−viib
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ste_ste11
camk_cdpk
RLK-Pelle_WAK
RLK-Pelle_RLCK-VI
RLK-Pelle_LRR-III
AGC_RSK-2
STE_STE11
CAMK_CAMKL-CHK1
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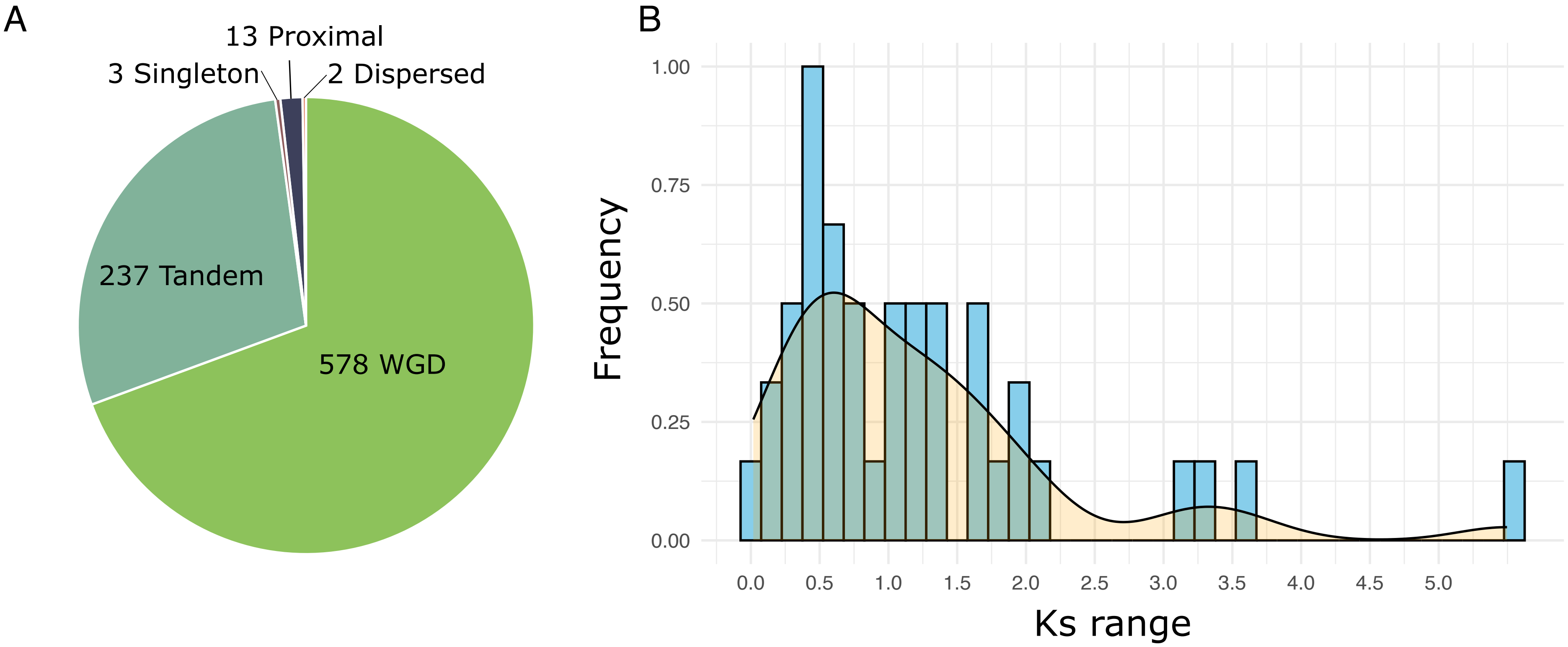
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3 Singleton
2 Dispersed
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5.0
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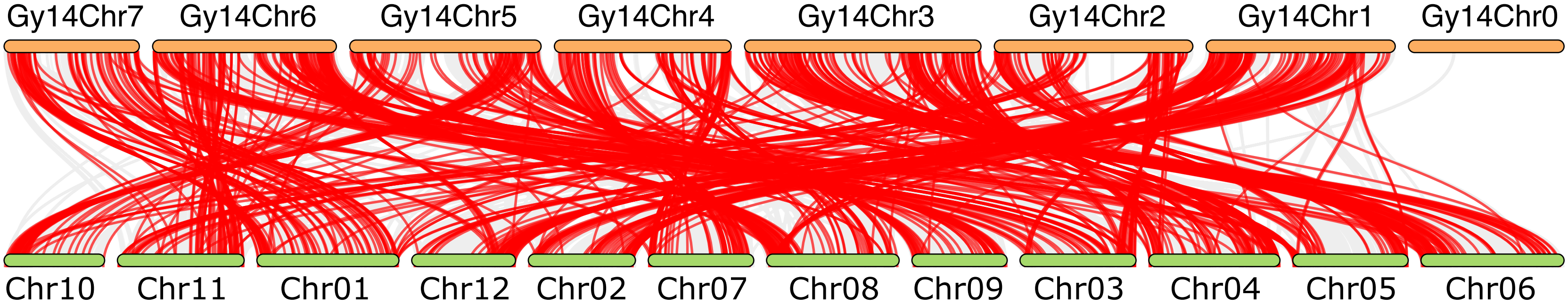
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ULK
AGC
Group-PI
WEE
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PEK
WNK
TLK
TTK
CAMK
IRE1
NEK
CK1
Aur
NAK
Outgroup