

Research

Diversity, classification and function of the plant protein kinase superfamily

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Eukaryotic protein kinases belong to a large superfamily with hundreds to thousands of copies and are components of essentially all cellular functions. The goals of this study are to classify protein kinases from 25 plant species and to assess their evolutionary history in conjunction with consideration of their molecular functions. The protein kinase superfamily has expanded in the flowering plant lineage, in part through recent duplications. As a result, the flowering plant protein kinase repertoire, or kinome, is in general significantly larger than other eukaryotes, ranging in size from 600 to 2500 members. This large variation in kinome size is mainly due to the expansion and contraction of a few families, particularly the receptor-like kinase/Pelle family. A number of protein kinases reside in highly conserved, low copy number families and often play broadly conserved regulatory roles in metabolism and cell division, although functions of plant homologues have often diverged from their metazoan counterparts. Members of expanded plant kinase families often have roles in plant-specific processes and some may have contributed to adaptive evolution. Nonetheless, non-adaptive explanations, such as kinase duplicate subfunctionalization and insufficient time for pseudogenization, may also contribute to the large number of seemingly functional protein kinases in plants.

Keywords: plant protein kinase; gene family evolution; lineage-specific expansion; comparative genomics

1. INTRODUCTION

The eukaryotic protein kinases are defined as enzymes that use the γ -phosphate of adenosine triphosphate (ATP) to phosphorylate serine, threonine or tyrosine residues in protein [1]. Protein kinases are highly similar in having a 250–300 amino acid protein domain that is responsible for the phospho-transfer reaction. Through alignments of protein kinase sequences available at the time, phylogenetic analysis revealed the sequence diversity in this superfamily and provided the first comprehensive classification scheme for protein kinases [2]. When the first plant genome, *Arabidopsis thaliana*, was sequenced, a surprising number protein kinases, over 1000, were identified [3]. Subsequently, an analysis of human genome sequences indicated the presence of 518 human protein kinases and 106 pseudogenes [4]. Thus, 1–2% of functional genes encode protein kinases, highlighting their importance in many aspects of cellular regulation in both plants and animals.

Plant protein phosphorylation was first detected in Chinese cabbage leaf discs after the application of a plant hormone, cytokinin [5]. Shortly thereafter, studies in common duckweed showed that plant ribosomes were phosphorylated on serine residues [6]. In

the same year, the first plant protein kinase was partially purified from pea [7]. However, it was not until the late 1980s and early 1990s that the first few plant protein kinase sequences became available. The first plant protein kinase sequences were identified in pea and in rice through the use of degenerate primers [8]. In 1990, the third plant protein kinase, ZmPK1, was cloned from maize, and was found to have a transmembrane region N-terminal to the kinase catalytic domain and a large putative extracellular domain [9]. This receptor-like kinase (RLK) resembles animal receptor kinases [10,11], but has a kinase domain belonging to a distinct family that is related to the fruitfly Pelle kinase and mammalian interleukin receptor-associated kinases (IRAK) [12], indicating that in plants a different class of kinases was co-opted for functions in transmembrane signal perception and transduction. Another major early finding was the sequencing through Edman degradation of a very abundant protein kinase with resemblance to both calmodulin- and calcium-dependent protein kinases [13], coupling calcium signalling and phosphorylation.

In the ensuing years, many more plant protein kinases homologous to multiple families of animal and fungal protein kinases were identified. However, the biological functions of plant protein kinases were first elucidated for protein kinases that play roles in plant-specific processes. The first published studies demonstrating plant protein kinase function genetically, to our knowledge, are on *Pto* [14], CTR1 [15] and Tousled [16]. *Pto*, a protein kinase with a catalytic

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One contribution of 13 to a Theme Issue ‘The evolution of protein phosphorylation’.

domain related to those of RLKs, was identified from a tomato cultivar resistant to a bacterial pathogen and confers resistance when introduced into an otherwise susceptible tomato cultivar [14]. CTR1, a kinase in the tyrosine kinase-like (TKL) group, is involved in the negative regulation of plant ethylene signalling [15]. Mutations in the Tousled protein kinase from *A. thaliana*, the founding member of the Tousled-like kinase group (TLK), were found to result in impaired floral organ development [16]. In the past 20 years, plant protein kinases have been found to be components of signalling networks such as the perception of biotic agents, light quality and quantity, plant hormones, and various adverse environmental conditions. They function in diurnal and circadian regulation, cell cycle regulation, developmental processes, modulating vesicle transport and channel activities, and in regulating cellular metabolism (for reviews, see earlier studies [17–32]).

Recently, a number of plant genome sequences have become available, allowing us to assess the evolutionary history of protein kinases from unicellular algae to land plants with significantly higher resolution than earlier studies that compared only two or four plant species [33,34]. Studies of protein kinases in other eukaryotes have led to detailed classification of this superfamily [35–39]. However, with the exception of studies of individual families, there is no comprehensive classification of plant kinomes using available plant genomic resources and knowledge of evolutionary relationships between kinases in other eukaryotes. Thus, we undertook a comparative study of protein kinases between plants and other eukaryote models. In addition to classifying plant protein kinases into families, we assess the diversity and evolutionary history of plant protein kinases in the context of their functions. The focus of this study is on the eukaryotic protein kinase superfamily. We have also identified other ‘atypical’ protein kinases not similar in sequence to eukaryotic protein kinases [40], but they are not discussed in detail.

2. DATA AND METHODS

(a) *Sequence data and identification of plant protein kinases*

To facilitate classification of plant sequences, protein kinase domain sequences and their classification schemes from nine eukaryotes were obtained from KinBase (<http://kinase.com/kinbase/FastaFiles/>). The nine species included are listed in figure 1a. To identify plant protein kinases, the annotated protein-coding sequences from 25 plant species (figure 1b) were obtained from PHYTOZOME (v. 7, <http://www.phytozome.net/>). For species with alternatively spliced form annotation, only the longest variant of each gene was analysed further. The putative protein kinases from plants were identified using HMMER v. 3.0 [41] with 16 PKinase clan hidden Markov models (HMM; <http://pfam.sanger.ac.uk/clan/pkinase>) from PFAM (v. 26; [42]). ‘Trusted cutoff’ values specified by PFAM were used as the thresholds to identify 30 431 putative protein kinase domain sequences. Among these sequences, 29 403 have significant matches to the

‘typical’ protein kinases (Pkinase and Pkinase_Tyr domains) and were analysed further. Both typical and atypical protein kinase information can be found in the electronic supplementary material, appendix S1.

Very few of the plant genomes analysed have been curated manually and there are probably annotation errors. In addition, the *A. thaliana* and *Oryza sativa* (rice) genomes have hundreds of pseudogenes that belong to the protein kinase superfamily [43]. Thus, the protein kinase domain sequences were not considered further if the domain alignments covered less than 50 per cent of the PFAM domain models. This resulted in the exclusion of 2841 sequences (see the electronic supplementary material, appendix S1). The same filter was applied to the protein kinase sequences from nine other eukaryotes because there are a few severely truncated or erroneous entries, particularly from *Tetrahymena thermophila*. The plant protein kinase domain sequences used in subsequent analysis can be found in the electronic supplementary material, appendix S2.

(b) *Classification of protein kinases from four model species*

To classify plant protein kinase sequences into families, a phylogenetic approach was used. First, the relationships between plant protein kinases and kinases from nine eukaryotes were established (figure 1a). The nine eukaryote protein KinBase dataset contains classification information, including group, family and, in some cases, subfamily levels. Plant kinases were classified based on this scheme. Because it is not feasible to build a phylogeny for all sequences from 25 species, four ‘plant model species’, *A. thaliana* (dicotyledon), *O. sativa* (monocotyledon), *Physcomitrella patens* (moss) and *Chlamydomonas reinhardtii* (a green alga), were chosen for the first round of classification. These four species were chosen because they are relatively well annotated and are representatives from major lineages in plant evolution. This first pass classification was then used to classify the other 21 plant species as outlined in §2c.

The analysis pipeline for classifying protein kinases in the four model species includes three rounds of phylogenetic analyses (figure 1a). In the first round, a phylogenetic tree was generated for 3656 protein kinases from the four plant models and 218 protein kinase representatives from nine species with kinase classifications. One representative kinase was chosen from each protein kinase family and each of the following five major taxonomic groups: Amoebozoa—*Dictyostelium discoideum*, Alveolata—*T. thermophila*, Choanoflagellida—*Monosiga brevicollis*, Fungi—*Saccharomyces cerevisiae*, Metazoa—five species. Owing to annotation quality considerations, for metazoan protein kinases, preference was given to human and mouse sequences. The protein kinase domain protein sequences were aligned using CLUSTAL OMEGA [44] for generating a maximum-likelihood (ML) tree with RAxML-LIGHT (v. 1.0.5, <http://sco.h-its.org/exelixis/software.html>) using the CAT model (category approximation of GAMMA model of rate heterogeneity) to account for rate heterogeneities [45] and the JTT (Jones, Taylor, Thornton) substitution

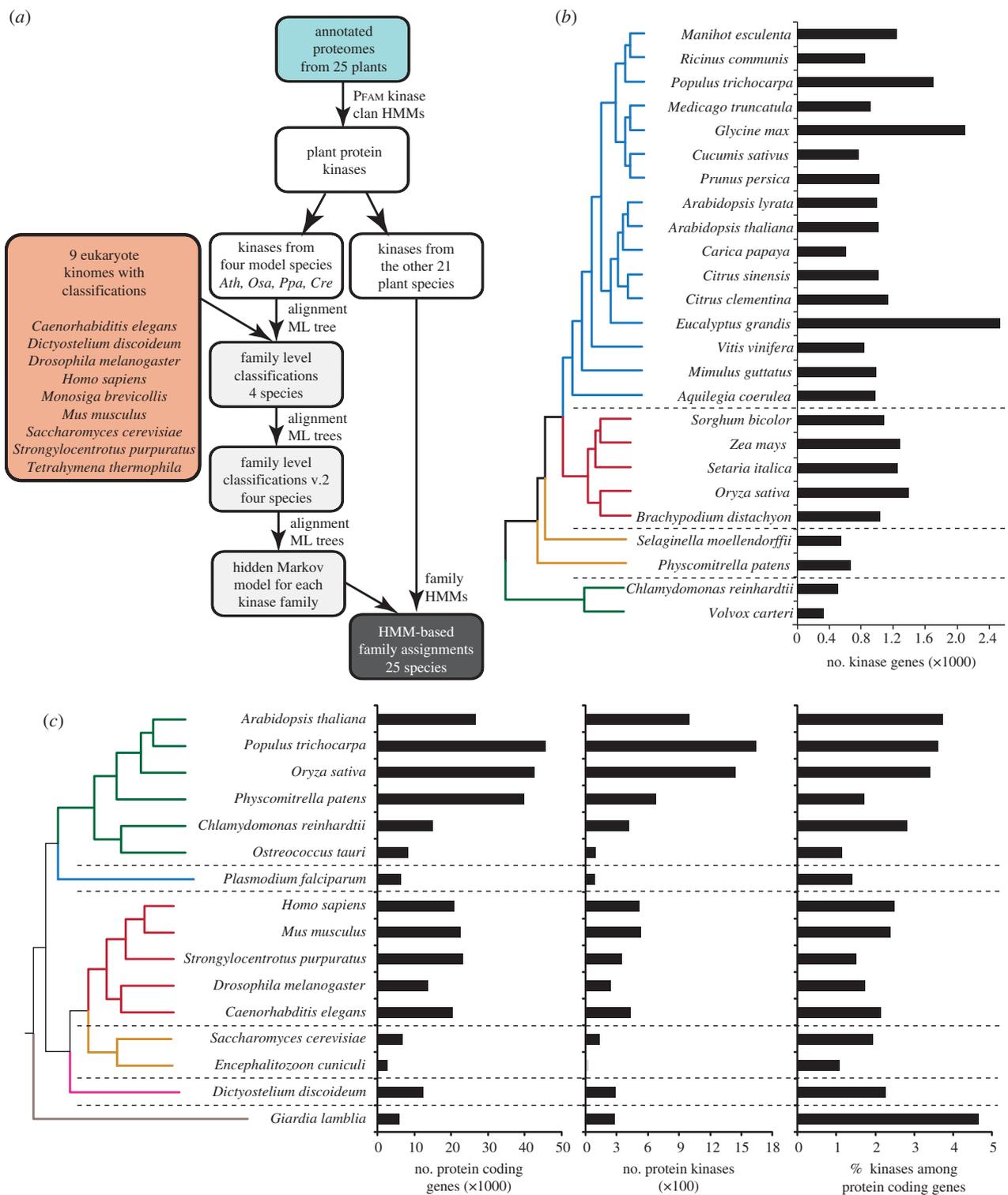


Figure 1. Protein kinase identification and number of protein kinases in eukaryotes. (a) Input data and analysis pipeline for identifying and classifying plant protein kinases. Model plant species: *Ath*, *Arabidopsis thaliana*; *Osa*, *Oryza sativa*; *Ppa*, *Physcomitrella patens*; *Cre*, *Chlamydomonas reinhardtii*. HMM, hidden Markov model; ML, maximum likelihood. (b) Phylogenetic relationships between and numbers of protein kinase genes in 25 plant species. Branch colour: blue, dicotyledon species; red, monocotyledon species; orange, bryophytes; green, green algae. (c) Phylogenetic relationships between selected eukaryotic species and sizes of protein kinase superfamily relative to gene numbers. Branch colour: green, Viridiplantae; blue, Apicomplexa; red, Metazoa; orange, Fungi and Microsporidia; magenta, Amoebozoa; grey, Excavata.

matrix [46]. On the basis of phylogeny, a plant protein kinase, K, from a model plant was assigned to a protein kinase family, F, if K is in a monophyletic group with all representative sequences from F. Plant sequences that could not be readily assigned were designated as plant-specific.

In the second round, the goal was to verify the family classification from round 1 by building an ML tree for each family. Alignment and tree building procedures were the same as round 1. The RLK/Pelle family has hundreds to more than 1000 members in plants [34]. Thus, instead of building a tree with all

Table 1. Accuracy of protein kinase family assignments.

subject ^a	classification ^a	total ^b	correct ^c	% correct
BLAST-based^d				
<i>Ath</i>	<i>Osa</i>	1015	983	96.85
<i>Ath</i>	<i>Ppa</i>	1015	904	89.06
<i>Ath</i>	<i>Cre</i>	1015	344	33.89
<i>Osa</i>	<i>Ath</i>	1422	1358	95.50
<i>Osa</i>	<i>Ppa</i>	1422	1053	74.05
<i>Osa</i>	<i>Cre</i>	1422	298	20.96
<i>Ppa</i>	<i>Ath</i>	689	574	83.31
<i>Ppa</i>	<i>Osa</i>	689	554	80.41
<i>Ppa</i>	<i>Cre</i>	689	240	34.83
<i>Cre</i>	<i>Ath</i>	530	113	21.32
<i>Cre</i>	<i>Osa</i>	530	115	21.70
<i>Cre</i>	<i>Ppa</i>	530	119	22.45
HMM-based^d				
<i>Ath</i>	HMM	1015	1014	99.90
<i>Osa</i>	HMM	1422	1420	99.86
<i>Ppa</i>	HMM	689	686	99.56
<i>Cre</i>	HMM	530	518	97.74

^aAbbreviations follow species names in figure 1b. Subject refers to the species where the protein kinases were to be classified. Classification refers to species where the family assignments were used to evaluate classification accuracy.

^bTotal number of kinase domain sequences examined.

^cNumber of cases where query kinase was in the same family as the subject kinase.

^dSee §2 for details.

putative RLK/Pelles, sequences in this family were first classified into subfamilies based on prior classification schemes [34] and a phylogenetic tree was built for each subfamily. During this step, some sequences were classified in a different family from that assigned in round 1. In the final round, alignments and phylogenetic trees were generated based on round 2 classifications to determine whether the classifications remained consistent. If not, the consistent family designation in at least two rounds of analysis was used. The final round alignments and phylogenetic trees and classification of protein kinases in the four model plants are compiled in the electronic supplementary material, appendix S3.

(c) Classification of protein kinases from 21 other plants

Two approaches were tested for classifying protein kinases from the other 21 plants into kinase groups, families and subfamilies. In the first approach, a protein kinase, K, from the 21 species dataset was first searched against the four model plant protein kinases with BLAST [47]. The classification of K was based on its top matching protein kinases from the model species (expect value < 1e - 5). To evaluate the classification accuracy, protein kinases from one model species were assigned into families based on classification of their top matches from one of the other three models (table 1). The classification accuracy differs widely depending on the evolutionary distances between species. Using rice protein kinases to classify *A. thaliana* ones, the accuracy is approximately 97 per cent. Using moss classification, more than 10 per cent of the *A. thaliana* protein kinases are mis-classified.

Using green algal classifications, 65–80% of protein kinases in *A. thaliana*, rice and moss are mis-classified.

In the second approach, an HMM was built for each family according to the family sequence alignment of model plants (see electronic supplementary material, appendix S4 for HMMs). The HMMs were then used to search against protein kinases from the other 21 plants. The family assignment of a protein kinase sequence was that of the top-scoring HMM (see electronic supplementary material, appendix S5 for classification results and numbers of members in each family). The accuracy of the HMM-based approach is very high, ranging from 98 per cent in classifying *C. reinhardtii* protein kinases to 99.9 per cent for *A. thaliana* kinases (table 1). This significant improvement is probably due to the fact that HMM better covers the family sequence space than a single top match sequence. Because of this significant improvement in classification accuracy over the similarity-based approach, in all subsequent analysis, the HMM-based assignments of plant protein kinase families were used.

3. PLANT PROTEIN KINASE SUPERFAMILY SIZE AND DIVERSITY

(a) Sizes of plant protein kinase superfamilies when compared with other eukaryotes

In the 10 years since the first plant protein kinase sequence was reported [8], the number of known plant protein kinases rose to over 500, and, because of the rapid progress in sequencing the *A. thaliana* genome, 175 of these plant kinases came from *A. thaliana* [18]. In the published annotation of the *A. thaliana* genome, there are approximately 1000 protein kinases [3], an number five times larger than that in budding yeast, and two to three times larger than various other eukaryotes, including mammals (figure 1c). Later analysis indicated that the protein kinase superfamily is even larger in other flowering plant species, with over 1500 members in rice and poplar [33,34,48]. Why are there more protein kinases in plants than in most other eukaryotes? The mechanisms underlying gene family expansion are similar between eukaryotes and involve tandem duplication in linked regions, retrogene formation, chromosomal duplications and whole genome duplication [49]. The differences in the size of the protein kinase repertoire between flowering plants and other eukaryotes suggest a relatively higher degree of lineage-specific expansion of this superfamily in plants. The main mechanisms contributing to expansion are elevated rates of tandem as well as whole genome duplication in plants relative to those in other eukaryotes [49]. Polyploidization is much more prominent in plants than in any other eukaryote; as many as 70 per cent of extant plant species are polyploids [50]. There have probably been at least three rounds of palaeo-polyploidization in the *A. thaliana* lineage after its split from monocotyledon species 150 Ma [51–53].

To determine whether there are more protein kinases derived from recent duplication events in plants compared with other eukaryotes, protein sequence identity between all paralogous protein kinases in a species was determined and used as a proxy

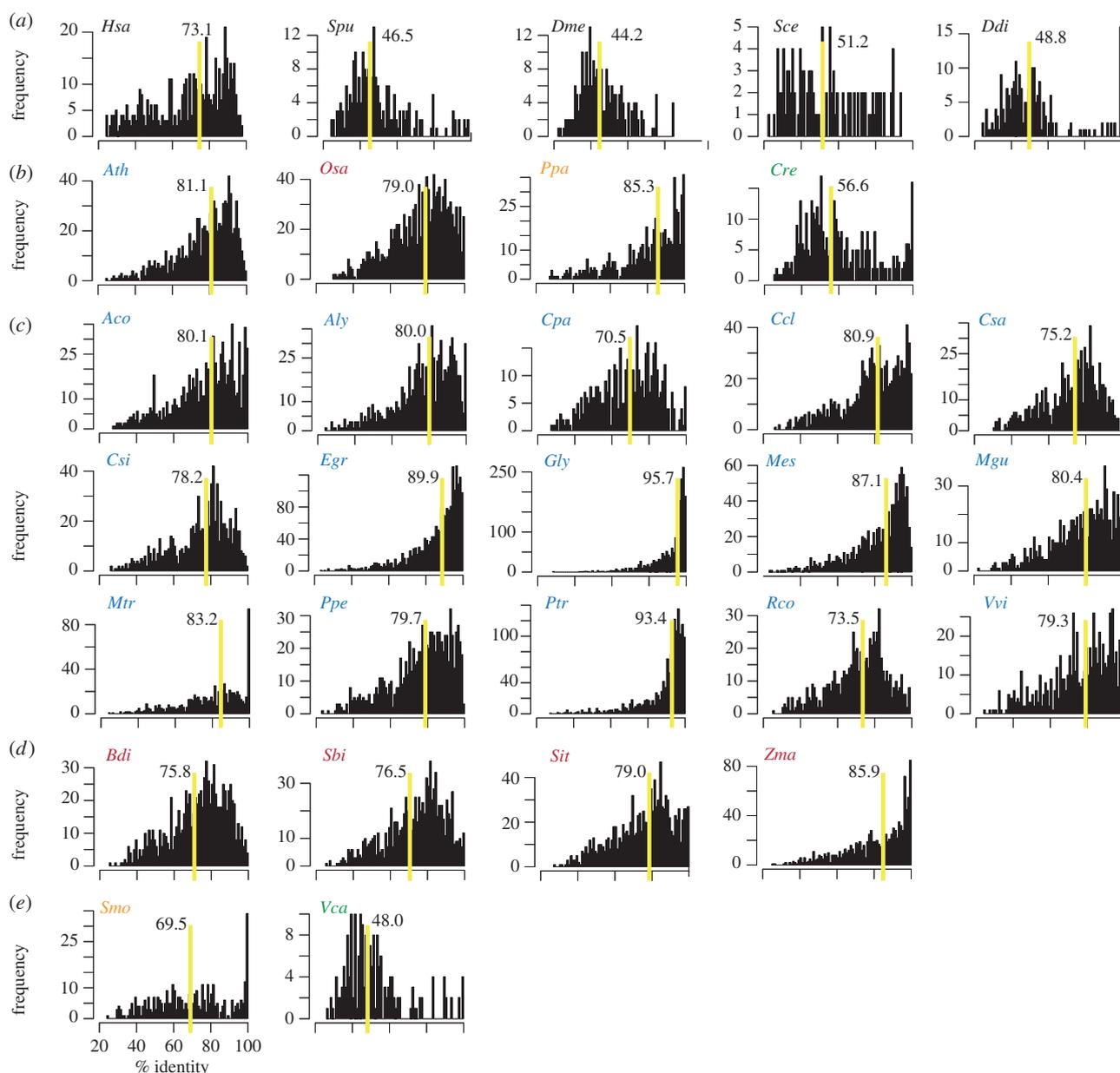


Figure 2. Distributions of pairwise identities between closest protein kinase paralogues. (a) Non-plant eukaryote representatives. (b) Four model plant species with species abbreviations coloured according to the convention in figure 1*b*. (c) Additional dicotyledon species. (d) Additional monocotyledon species. (e) Additional bryophyte and green alga. The x-axis indicates paralogue per cent identity, and the y-axis indicates the number of paralogous pairs in an identity bin. The species abbreviations follow the species names shown in figure 1. The yellow line indicates the median paralogue identity value (also shown) for each species.

for timing of duplication. Species that have more recently duplicated and retained protein kinases will have more paralogues with higher identities to each other. The paralogue identity distributions were compared between the four model plant species and five non-plant eukaryotes, including human (*Homo sapiens*), sea urchin (*Strongylocentrotus purpuratus*), fruitfly (*Drosophila melanogaster*), budding yeast (*S. cerevisiae*) and slime mould (*D. discoideum*). Among five non-plant eukaryotes, the median identity of protein kinase paralogues is highest in humans (73.1%), significantly higher than the other four species (Wilcoxon rank sum test, all $p < 1e - 11$; figure 2*a*). On the other hand, the three model land plants (*A. thaliana*, *Ath*; *O. sativa*, *Osa*; *P. patens*, *Ppa*; figure 2*b*) have significantly larger median identities compared with that of humans (all $p < 1e - 10$). In *C. reinhardtii* (*Cre*; figure 2*b*), which

does not have a history of whole genome duplication, the median identity of kinase paralogues is significantly lower than observed in the model land plants and in humans. This is consistent with the notion that there are more recent protein kinase duplicates in land plants than in other eukaryotes. In addition to elevated duplication rate, another explanation is that many plant protein kinase duplicates tend to be retained. This was shown in a comparative analysis of protein kinase families from *A. thaliana*, poplar, rice and moss where protein kinases were shown to have significantly higher expansion rates than other protein families [54].

(b) Expansion of the protein kinase superfamily in Viridiplantae

The expansion of protein kinases appears to be quite rapid, given that there are approximately 600 more

protein kinases in poplar than in *A. thaliana* (figure 1*b*) and that these two species diverged only approximately 70 Ma. On the other hand, the moss *P. patens* has 685 protein kinases, substantially less than those of flowering plants (figure 1*b*). In green algae, the protein kinase superfamily is even smaller with 426 and 93 members in *C. reinhardtii* and *Ostreococcus tauri*, respectively [34]. These findings indicate that within Viridiplantae (green plant species, including land plants and green algae), the protein kinase superfamily has steadily grown in size in the lineage leading to flowering plants. In addition, such expansion correlates with an increase in developmental complexity. Both *C. reinhardtii* and *O. tauri* are unicellular Chlorophyta green algae hypothesized to have diverged from the Charophyta green algae and land plant lineage approximately 1200 Ma [55]. The divergence time between *C. reinhardtii* and *O. tauri* is unclear. Although both of these green algae are free-living unicellular organisms, *C. reinhardtii* has 333 more protein kinases than *O. tauri*.

The explanation for this large difference is most likely to be due to extensive reduction in genome size that occurred in *O. tauri* [56]. The sizes of *O. tauri* intergenic regions are significantly shorter than other eukaryotes with similar genome sizes. In addition, there is substantial reduction of gene family size in general. Compared with other eukaryotes, only 1.1 per cent of annotated protein-coding genes in *O. tauri* are protein kinases, similar to two obligate intracellular parasites *Encephalitozoon cuciculi* (1.1%; [39]) and *Plasmodium falciparum* (1.4%; [38]). Interestingly, the *E. cuciculi* genome has undergone a significant degree of genome compaction and gene loss [57]. A recent analysis in another intracellular parasite, *Giardia lamblia*, indicates the presence of 278 protein kinases [37]. However, 198 of these *G. lamblia* protein kinases belong to the never in mitosis/*Aspergillus*-related kinase (NEK) family. Thus, excluding the dramatic expansion in NEKs, there remains only 80 protein kinases (1.3% of protein-coding genes) in *G. lamblia*. Some flowering plants have also undergone gene loss. For example, *A. thaliana* has probably lost DNA through rearrangement events [58]. However, *A. thaliana* also has a similar percentage of genes that are protein kinases compared with poplar, rice and moss (figure 1*c*). Thus, it appears that just as many protein kinases were lost compared with genes in general in flowering plant species with genome reduction.

(c) Protein kinase superfamily size variation among plant species

Although earlier studies have provided important clues on the expansion of the plant protein kinase superfamily, the number of species examined was small. Thus, it was not clear whether the patterns observed would remain consistent if more species were analysed. In the past few years, over 25 Viridiplantae genomes (including 21 flowering plants, one bryophyte, one moss and two algae; figure 1*b*) have been sequenced with draft annotation available. To further examine how the sizes of plant protein kinase superfamilies differ between plant species, protein kinase sequences were identified from the annotated protein sequences. Among them,

approximately 1 per cent are classified as ‘atypical’ protein kinases. Excluding the atypical protein kinases, 26 966 protein kinase domain sequences from 26 775 annotated genes were identified.

There is substantial variation in the protein kinase superfamily size among plant species. In addition to the differences between land plants and algae noted in §3*b*, the numbers of protein kinases among flowering plants differ by more than fourfold (figure 1*b*). The analysed species with the largest protein kinase superfamily is rose gum eucalyptus, *Eucalyptus grandis*, with 2532 kinase genes. By contrast, papaya (*Carica papaya*) has only 600 kinase genes. It should be noted that the lower numbers of kinases in some plant species are due to incomplete genome sequencing coverage and should be regarded as lower-bound estimates. To circumvent the incomplete genome issue, a comparison of protein kinase superfamily sizes can be conducted in consideration of annotated gene numbers. Interestingly, there exists a significant correlation between the number of protein kinase genes and the total gene number in a genome (Pearson’s correlation, $r = 0.63$, $p < 1e - 3$). This finding indicates that, despite the large variation in the size of the protein kinase repertoire among flowering plants, a similar proportion of genes encode protein kinases. The implication is that the protein kinase superfamily as a whole grows and shrinks with the rest of the gene families within each genome. Alternatively, this correlation may suggest that the propensity for expanding the protein kinase superfamily is similar among different plant species. In the following sections, we examine the approximate timing of plant protein kinase duplications and assess the degree of differential expansion among plant protein kinase families.

(d) Approximate timing of plant protein kinase duplications

To assess why these species differ so greatly in the number of protein kinase paralogues, an all-against-all similarity search was conducted for protein kinases in each species. The distributions of identities between the most closely related paralogous pairs are shown in figure 2*b*. In eucalyptus, *Glycine max* (soybean), and *Populus trichocarpa* (cottonwood), which have more than 1600 protein kinases, the paralogue identity distributions tend to peak at approximately 95 per cent, significantly higher than other plants. For example, the eucalyptus protein kinase paralogue identities are significantly higher than those of *A. thaliana* (Wilcoxon rank sum test, $p < 1e - 16$). Thus, very recent gene duplications contribute significantly to the larger protein kinase repertoire in some plants. Through this analysis, it also becomes apparent that some species, such as *Medicago truncatula*, *Selaginella moellendorffii* and *C. reinhardtii*, have an excess of protein kinases with 100 per cent identity (figure 2*b*). Some of these identical kinases may be the result of mis-annotation. Thus, an analysis eliminating sequences with 100 per cent similarity was also conducted (see electronic supplementary material, appendix S6), and our conclusion remained unchanged. In addition to identical sequences, there

are also a substantial number of nearly identical sequences. In some cases, these may be allelic variants that are identified as distinct loci, as noted in the *Selaginella* genome study [59].

On the basis of this expanded analysis, it is in general true that plant protein kinase genes experienced much more recent duplication compared with the other five eukaryotic species examined. However, there are some notable exceptions. For example, the protein kinase paralogue identities from castor bean (*Ricinus communis*), cucumber (*Cucumis sativus*) and papaya were not significantly different from those of human protein kinases ($p > 0.1$ in all cases). Interestingly, unlike plant species such as soybean [60] and cottonwood [61] with significantly higher paralogue identities and a recent history of polyploidization, there is no apparent recent whole genome duplication event detected in the cucumber or papaya genomes [62,63]. In addition, castor bean, cucumber and papaya have some of the lowest numbers of protein kinase genes among the plant species analysed (figure 1*b*). These findings reinforce the idea that whole genome duplication contributes significantly to the expansion of the protein kinase superfamily in plants. We should emphasize that the ancestral lineage leading to castor bean, cucumber and papaya probably experienced at least one round of whole genome duplication 70–100 Ma [51–53]. However, the protein kinase superfamily in papaya, cucumber and castor bean is substantially smaller than other species, particularly those with recent whole genome duplications. Thus, it is likely that many of the protein kinase duplicates derived from whole genome duplication were lost in a timeframe of tens to hundreds of million years. This timeframe for gene loss is consistent with that found in a global study of gene family expansion in four land plant species [54].

(e) Plant protein kinase families and their differential expansion

To evaluate the evolution of plant protein kinases at the family level, plant protein kinases were first classified using an established scheme based on phylogenetic analyses of animal, fungal and protist protein kinases [1,2,35]. The number of kinase sequences is large (more than 26 000), creating computing issues for both the multiple sequence alignment and the phylogenetic analysis phases. Thus, protein kinases were first classified in four model plant species (*A. thaliana*, rice, moss and *C. reinhardtii*), based on location in the same monophyletic group as kinases with known, consistent family designations (see electronic supplementary material, appendix S3 for alignments, phylogenies and classification). The model plant classification was then used to classify protein kinases from the other 21 species (see §2). The numbers of plant protein kinases in different families are shown in figure 3*a*.

Through this analysis, the majority of model plant protein kinases can be classified into known groups. Nonetheless, there remains a small number of plant kinases that cannot be assigned at the group level (figure 3*a*, plant-specific). Some of the plant-specific kinases are found only in green algae (Group-Cr-2),

or only in green algae and bryophytes (Group-Pl-1; here 'Pl' indicates plant). On the other hand, Group-Pl-3 and -4 are highly conserved across plants and have relatively few members. Most plant kinases that can be classified at the group level can also be readily classified into known families, indicating that many of these families were established prior to the divergence of the plant, animal and fungal lineages or earlier. Nonetheless, there remain unclassified plant-specific families in all major protein kinase groups (figure 3*a*, Pl families in different groups). There are also families that are shared only between green algae and fungi (CAMK1-Scer), between green algae, bryophyte and *T. thermophila* (CAMK1-Tthe); between green algae and animals (TK and several TKL families); and between land plants and *T. thermophila* (CMGC-Pl-Tthe). These 'mosaic' patterns suggest potential gene losses in other major lineages or horizontal gene transfers that remain to be further investigated.

Among families that are present in green algae, bryophytes and flowering plants, three major patterns emerge when comparing the variation in numbers of protein kinases across families and species. The first is protein kinase families that consistently have relatively low (1–5) copy numbers (figure 3*a*, blue series). Given that many of these families have animal or fungal orthologues, low copy numbers suggest that there has been no or limited changes in these families since the divergence of the plant and animal/fungal lineages approximately 1 Ga. Considering that there were repeated whole genome duplications in plants, these low copy number families had the opportunity to expand via duplication, but apparently most duplicates were not retained. For example, the CDK-CCRK, CDK-CDK7, and CDK-CDK8 subfamilies in most species consist of only one gene, except in soybean, where a very recent whole genome duplication occurred (approx. 59 and 14 Ma) [60]. In fact, soybean protein kinase families in general are larger than other species. Thus, the significantly larger protein kinase repertoire in soybean can partly be attributed to the possibility that there has been insufficient time for pseudogenization of duplicates.

The second pattern is families with moderate sizes (6–30 members; figure 3*a*, green series and yellow), and the third is large families (more than 30 members; figure 3*a*, red series, magenta series). In general, the sizes of these families are in the order: algae < bryophytes < flowering plants, consistent with the notion that the protein kinase superfamily has expanded over the course of plant evolution. Among plant species, the family with the largest size differences is the RLK/Pelle family with two to three genes in green algae, approximately 300 genes in bryophytes and 374–2205 members in flowering plants. The RLK/Pelle family was established because the kinase domains of various RLKs are more closely related to fly *Pelle* and human IRAKs than to protein kinases from any other families [12]. This family was later defined as the IRAK family. For historical reasons, we use the 'RLK/Pelle designation' throughout. Earlier studies have established that this family has experienced extensive expansion in the land plant lineage [33,34,64]. When the RLK/Pelle family is classified at the subfamily level (figure 3*b*), a

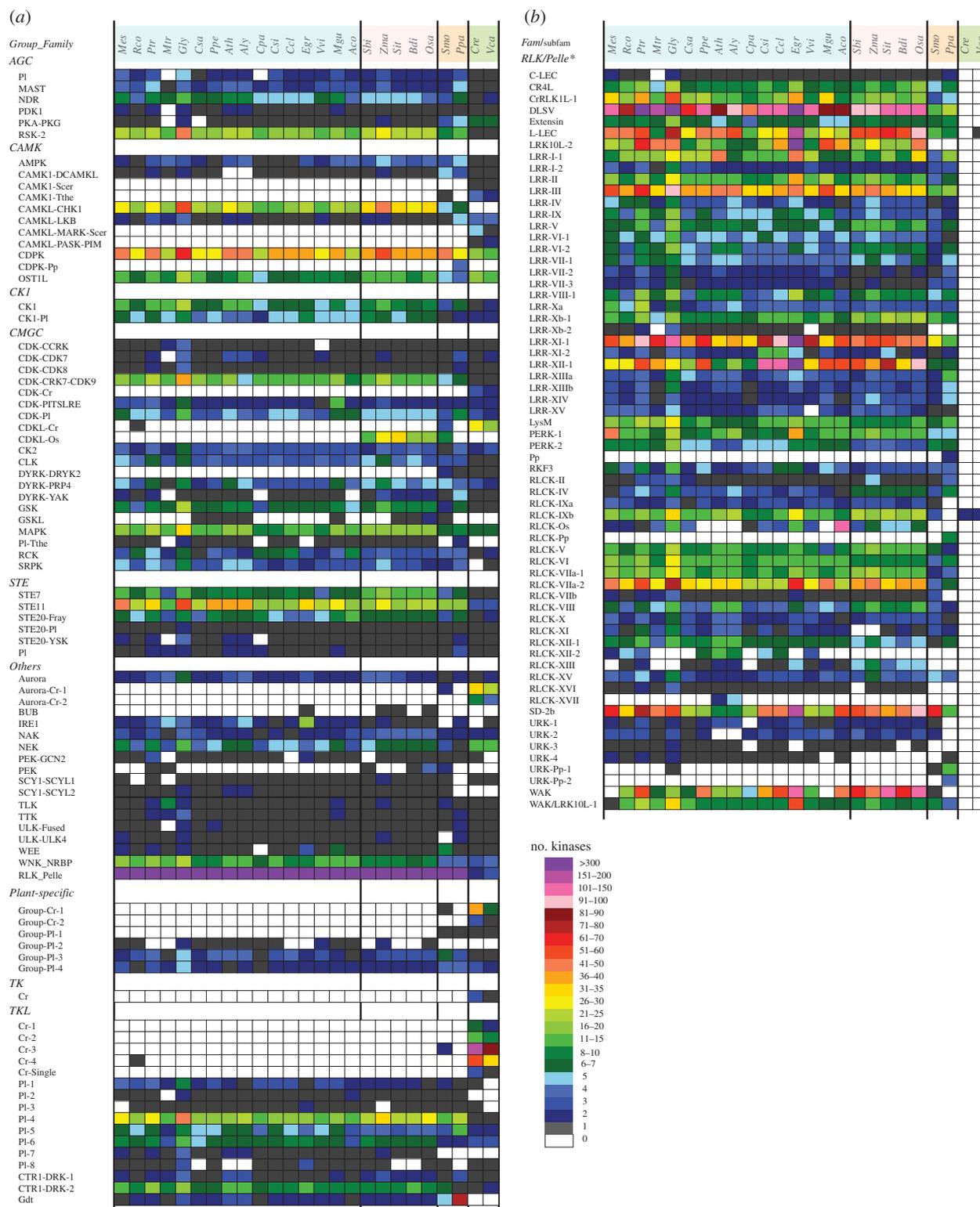


Figure 3. Sizes of protein kinase families in 25 plant species. *(a)* Major groups and families. *(b)* RLK/Pelle subfamilies. The species abbreviations follow the species names shown in figure 1*b*. The number of protein kinases in each family/subfamily is colour coded according to the colour key on the lower right. Kinases that cannot be readily assigned to a group are classified as ‘plant-specific’. Within each group, some plant or algal kinases cannot be assigned to an existing family. They are designated as ‘PI’ or ‘Cr’ for Plant and *C. reinhardtii*, respectively. Similarly, some kinases can be assigned to a family but not at the sub-family level. In addition to ‘PI’ and ‘Cr’ designations, they are designated as ‘Pp’ or ‘Os’ specifying *P. patens* and *O. sativa*-specific kinases, respectively.

few subfamilies have low copy number (e.g. RLCK-II) but most others have moderate to large sizes. RLK/Pelle subfamilies in soybean are in general larger than in other species, consistent with the patterns observed

for other protein kinase families. The most striking expansion of the RLK/Pelle family is seen in eucalyptus where multiple subfamilies have more than 300 members (figure 3*b*). However, other protein kinase

families in eucalyptus are of similar size as other plant species (figure 3a). Thus, the very large number of protein kinases in eucalyptus is mostly a consequence of expansion in the RLK/Pelle gene family.

4. FUNCTIONAL DIVERSITY OF PLANT PROTEIN KINASES

(a) Kinase families that have remained low in copy number in both plants and other eukaryotes

(i) Conserved central roles in metabolic signalling and stress response

As shown in figure 3a, many protein kinase families remain low in copy number, despite ample opportunity to duplicate. Considering that the flowering plant species studied are either palaeo- or recent polyploids, the implication is that many of these protein kinases were duplicated but the duplicates were subsequently lost. Most of these small families are conserved between plants and animals, suggesting that they play roles in conserved processes. Several subfamilies that have undergone limited expansion in most eukaryotic lineages have central roles in metabolic signalling and regulating metabolic changes in response to stress, and include AMPK/SNF1, PDK1, S6K, IRE1 and GCN2. Here, we discuss how the functions of these genes have remained conserved and/or diverged in the plant lineage.

AMPK/SNF1 and LKB1

The AMP-activated protein kinase (AMPK)/SNF1 (sucrose-non-fermenting 1) kinases are conserved across eukaryotes and play central roles in sensing energy status and maintaining energy homeostasis [65]. Like their yeast and animal counterparts, the *A. thaliana* AMPK homologues SnRK1.1/AtKIN10 and SnRK1.2/AtKIN11 also play roles as central regulators of energy metabolism and homeostasis under stress conditions [66,67]. The involvement of SNF1 kinases in response to starvation also appears to be evolutionarily conserved, and in moss, AMPK/SNF1 kinases enable plants to cope with periods of dark [65,68]. SNF1/AMPK functions and interaction partners are probably broadly conserved across kingdoms. AMPK/SNF1 is activated by LKB1, and PAK1, TOS3 and ELM1 in mammals and yeast, respectively [69,70]. The two *A. thaliana* LKB subfamily members, GRIK1 and 2, can complement a yeast *pak1/tos3/elm1* mutant and can phosphorylate AtSNF1 [71,72].

PDK1

In metazoans and yeast, phosphoinositide-dependent kinase-1 (PDK1) serves as a master regulator of AGC kinase activity, phosphorylating several AGC kinases through interaction with the PDK1 interacting fragment domain in response to 3-phosphoinositide generation [73,74]. *A. thaliana* PDK1 activates RSK-2 AGC kinases in response to a different lipid signal, phosphatidic acid [75–77]. *A. thaliana* plants lacking both PDK1-related genes are dwarf and defective in reactive oxygen species-mediated signalling, but still viable [78]. This is in contrast to mice where loss of PDK1 results in embryo lethality [79]. It appears that the activation of

AGC kinases by PDK1 is conserved in plants, animals and yeast, but that the details of that regulation as well as downstream effects have diverged.

S6K/AGC-P1

Because we cannot clearly resolve the relationship between animal ribosomal S6 kinases (S6Ks) and their reported plant homologues, the plant S6K-like genes are designated AGC-P1 in this study. The p70 S6Ks are stimulated in response to nutrients and modulate protein translation by phosphorylating downstream targets such as ribosomal protein S6, EF2 kinase and eIF4B [77]. There are two p70S6K-related proteins in *A. thaliana*. The S6K2 gene, AT3G08720, can phosphorylate human and plant S6 ribosomal protein, suggesting that targets have been conserved [80]. AtS6K1 is located in the cytoplasm and nucleus, whereas S6K2 is only located in the nucleus and nucleolus; in animals, there are two isoforms of S6K with analogous distinct localization patterns [81]. S6Ks also are phosphorylated by PDK1 in both plants and animals [81]. As in animals, S6K1 is activated by an activation complex called TORC in response to stress, although plant S6Ks do not have the conserved TOR signalling motif found in animals [81]. *Arabidopsis thaliana* S6K1 interacts with the RBR1–E2F pathway to inhibit cell proliferation. Interestingly, the TSC/Rheb/Tor/S6k pathway in *Drosophila* was also shown to regulate E2F1 levels and to work with RB1 to regulate cell cycle progression and also cell survival [82]. Plants with reduced levels of AtS6K1 and 2 exhibit chromosome instability and a failure to repress cell proliferation under nutrient limiting conditions [83]. This contrasts with the absence of aneuploidy and effects on cell survival in fruitfly, and indicates that although there are similarities in regulation and targets, the functions of S6K have diverged in plants.

IRE1

One remarkable example of conservation is the mechanism by which IRE1 (inositol-requiring 1) senses endoplasmic reticulum (ER) stress and activates the unfolded protein response. Upon sensing unfolded proteins, IRE1 in yeast and its orthologues in metazoans and (presumably) in plants is activated by autophosphorylation [84,85]. Activated IRE1 then splices the mRNA of a transcription factor, Hac1 in yeast, XBP1 in metazoans [85] and bZIP60 in *A. thaliana* and rice [86,87]. In all cases, the spliced isoform of the transcription factor regulates the expression of stress response genes, but the mechanism by which splicing activates the transcription factor differs in yeast, animals and plants [85,87].

GCN2

GCN2 (general control nonrepressed 2) regulates translation in response to nutrient stress through its phosphorylation of eIF2 α [88]. Yeast GCN2 is also activated by ultraviolet radiation and is required for a checkpoint delaying progression from G1 to S phase [89]. In mammals and *Schizosaccharomyces pombe*, there are GCN2 orthologues as well as three and two additional eIF2 kinases, respectively, that each respond to different stresses [88,90]. *Arabidopsis*

thaliana contains a single GCN2 gene that can complement amino acid starvation response in *gcn2* mutant yeast cells [91] and also EF2 α phosphorylation in response to multiple stresses, but not osmotic stress in contrast to yeast GCN2 [92,93].

(ii) Regulation of mitosis and cytokinesis

Another group of protein kinase families conserved between plants and animals that are low copy number includes Aurora and some cyclin-dependent kinase (CDK) subfamilies. Protein kinases in these two families play central roles in the regulation of mitosis, cytokinesis and cell cycle control.

Aurora

In yeast and metazoans, Aurora kinases play important roles in mitosis and cytokinesis (reviewed in Carmena *et al.* [94]). Plants have α - and β -type Aurora kinases, which are distinct from the A, B and C types in animals and B type found in yeast [95]. *Arabidopsis thaliana* Aurora kinases co-localize with mitotic structures, centromeres and/or the cell plate and also phosphorylate histone 3, a substrate of animal Aurora kinases, suggesting conservation of mitotic functions [96]. However, as suggested by sequence divergence, plant Aurora kinases have evolved plant-specific functions. A recent study showed that a double knockout of the α Aurora kinases *AtAUR1* and *AtAUR2* is gametophyte lethal and that the α kinases function in division plane orientation [97]. The β -type Aurora kinase could not complement the mutant, indicating that it has a different function. Interestingly, a distinct group of Aurora-related kinases has dramatically expanded in green algal species (figure 3a). A *C. reinhardtii* Aurora-like kinase, CALK, is required for flagellar disassembly and its phosphorylation state is used to measure flagellar length [98,99]. In mammals, Aurora kinase A, which regulates entry into mitosis, also has a non-mitotic role promoting disassembly of the primary cilium, a structure evolutionarily related to the motile flagella of *Chlamydomonas* [100]. In both cases, flagellar/ciliary disassembly requires microtubule destabilization, suggesting that signalling pathways leading to disassembly are conserved in *Chlamydomonas* and mammals; however, CALK is quite divergent from Aurora kinase A [98,100].

CDKA and CDKB

In plants, some CDK subfamilies are highly conserved with low copy number, such as CDKA, CDKB, while the others have expanded fairly significantly. In some cases, the functions of plant orthologues of small CDK gene families have remained highly conserved. For example, yeast requires one gene, *Cdc2/Cdc28*, to drive cell cycle progression, and in *A. thaliana*, this function is supplied by CDKA [101]. In our analysis, CDKA does not form a monophyletic group with *Cdc2/Cdc28* and is classified as a plant-specific CDK family (CDK-P1). In *A. thaliana*, there are three plant-specific CDKB genes that work with CDKA to regulate the G2/M transition [102].

CDK7

Similar to CDKA, the plant CDK7 subfamily members also seem to have conserved functions with their yeast and animal counterparts, the cyclin-activating kinases (CAKs). Animal CDK7, the major CAK, regulates transcription by phosphorylating the C-terminal domain (CTD) of RNA Pol II [103,104]. The CAK and transcriptional regulation functions are carried out by two separate CDK7 genes in yeast. The sole rice CDK7 orthologue appears to have both CAK and transcriptional regulation functions [105,106]. *Arabidopsis thaliana* has three CDK7-related genes, of which two (CDKD2 and 3) phosphorylate both the RNA Pol II CTD and human CDK2 (reviewed in Inagaki & Umeda [107]).

CDK20

In contrast to CDKA and CDK7, the functions of the small CDK subfamily CDK20 (originally named CCRK) have diverged in plants. In *A. thaliana*, a cell-cycle-related kinase (CRRK) subfamily member, CDKF1, functions as a CAK [108]. The mammalian CRRK, while required for cell proliferation, does not have CAK activity [109]. Interestingly, CCRK-related genes are involved in the regulation of cilia assembly in vertebrates [110] and flagellar length in green algae [111]. These examples indicate that even though CCRK remains largely a single copy gene in all lineages examined (figure 3a), it has been recruited for different functions in different organisms.

Other cyclin-dependent kinases

Other CDK members with non-mitotic roles have also remained low in copy number in the plant lineage. CDK8 subfamily members regulate transcription in metazoans and in yeast, functioning as part of a complex called Mediator, to modulate the activity of RNA Pol II [112,113]. The *A. thaliana* CDK8 orthologue, HEN3, is required for floral cell differentiation [114] and also functions as part of Mediator complex to regulate transcription in *A. thaliana* [115], indicating that this function is also conserved in plants.

(iii) Low copy number families with divergent or unknown functions

Similar to CDK20 discussed earlier, some subfamilies with low copy numbers in plants and animals can also diverge significantly in their functions. WEE1 and ULK are clear examples as they regulate plant-specific processes. On the other hand, functional similarities of RCK and TLK homologues between eukaryotes are not entirely clear.

WEE1

In fission yeast and metazoans, WEE1 plays a central role in the cell cycle, regulating cell cycle progression by phosphorylating and inactivating CDK1, which is in turn activated by the phosphatase Cdc25 (reviewed in Doonan & Kitsios [104]). In budding yeast, where there is no gap between S-phase and mitosis, the WEE1 homologue, Swel1p, functions in a morphogenesis checkpoint instead, monitoring the actin cytoskeleton [116,117]. On the basis of presence of homologous

genes, the same CDK1–WEE1–CDC25 regulatory loop was thought to operate in plants. However, recent evidence suggests that entry into mitosis is regulated differently in plants than in fission yeast and metazoans [118]. Supporting this, plant WEE1 functions in endoreplication and as a regulator of a DNA integrity checkpoint during the S phase rather than as a central regulator of cell cycle progression [119–121].

ULK

ULK (uncoordinated 51-like kinase) subfamily members regulate autophagy in metazoans and yeast, and have also been shown to regulate vesicle transport in neurons (reviewed in Chan [122]). The only ULK–ULK4 kinase in *A. thaliana*, RUNKEL (RUK), is an essential gene, but is involved in cell division rather than in autophagy [123], even though autophagy pathways appear to be conserved in plants and metazoans [124]. The ULK-fused subfamily members are components of the sonic hedgehog signalling pathway and regulate Gli transcription factors involved in cell proliferation and cell fate [125]. In *A. thaliana*, which lacks sonic hedgehog signalling components, the only ULK-fused homologue is located at the phragmoplast and regulates cytokinesis [126].

RCK

One interesting example of conservation of function is the role of the *ros* cross-hybridizing kinase (RCK) male germ-cell-associated kinase (MAK) homologues in flagella/cilia morphology [30]. Note that the plant MAK homologues were classified as mitogen-activated protein kinase (MAPK) in Rodriguez *et al.* [30], which is inconsistent with our analysis results. The *C. reinhardtii* MAK homologue [127], CrLF4, regulates flagellar length [128]. CrLF4 homologues in *C. elegans* and mouse regulate the morphology and length of cilia [129,130]. The three *A. thaliana* MAK-related genes are expressed in the male gametophyte and pollen tube [131], but their functions are not known.

TLK

The TLK was first identified in *A. thaliana* as a regulator of flower initiation and development [16] and transcriptional silencing [132]. TLKs are conserved throughout eukaryotes, but are absent from yeast. Metazoan and plant TLK orthologues appear to share functions in chromatin dynamics, indicated by their ability to phosphorylate the same substrates: Histone 3B and ASF1, a histone chaperone protein that functions in chromatin assembly [133–137].

CMGC_DYRK

Dual specificity yak-related kinases comprise three subfamilies: (i) DYRK, (ii) homeodomain interacting kinases (HIPKs), and (iii) pre-mRNA processing protein 4 kinases (PRP4). The first member of the DYRK family, YAK1, was identified in yeast, and since then members have been found in all eukaryotes (reviewed in Aranda *et al.* [138]). In yeast, Yak1 regulates stress and nutrient response transcription factors [139,140]. Interestingly, YAK1 genes are not found in animals, which have the related DYRK1 and DYRK2

genes [138]. Nearly all plants have one to three YAK1-related genes, but none of the DYRK genes in plants have been characterized. Plants do not have apparent HIPKs, which in animals regulate transcription by interacting with homeodomain proteins [141], but do have expanded PRP4 kinase subfamilies. The functions of these genes also remain uncharacterized. Given the importance of PRP4 kinases in splicing in yeast [142] and mammals [143], it would be interesting to investigate their function in splicing in plants.

(b) Protein kinase families with moderate degrees of expansion

(i) Families with divergent functions but conserved signalling network components

A number of families are found in most eukaryotes but have experienced moderate degrees of expansion in plants. Members in several of these families, including MAPK, MAPK kinase (MAP2K), MAPK kinase kinase (MAP3K) and RSK-2 have been shown to interact with homologous signalling network partners in plants, fungi and animals. However, they play apparently different roles in plants, presumably because of the morphological, developmental and physiological divergence and differences in life histories between plants and other eukaryotes.

MAPK

MAPKs in plants, metazoans and yeast link extracellular and intracellular signals to downstream responses. In plants, MAPKs play diverse roles in development, and response to abiotic and biotic stresses (reviewed in Rodriguez *et al.* [30]). Several signalling modules involving MAPKs in both plants and animals have been defined. Although there is a high degree of conservation of structure of these modules, MAPK signalling pathways in plants are different from those in other eukaryotes [30]. Furthermore, the MAPK family has expanded more in land plants (7–31 members) relative to yeast (6) and animals (6–14), suggesting more opportunities for diversification of functions in the plant lineage. In rice, poplar and *A. thaliana*, there is a high degree of conservation of MAPKs, and most orthologous relationships are clear [30].

STE7 and STE11

These two related families of protein kinases, STE7 (Sterility 7) and STE11, function as MAP2K and MAP3K, respectively. Note that Raf kinases also function as MAP3K but belong to the TKL group. Like MAPKs, the STE7 and STE11 subfamilies have expanded in the land plant lineage (3–19 and 14–55 members, respectively) relative to yeast (four) and metazoans analysed (4–10). The expansion of the STE11 lineage is particularly pronounced: there are three times as many STE11 kinases in *A. thaliana* than STE7. These two subfamilies have been reviewed extensively elsewhere [30]. The first complete MAPK signalling pathway in plants was determined for the innate immunity pathway activated by the flagellin peptide, flg22. Flg22 binds to the FLS2 receptor, an RLK, activating the MAP3K, MEKK, which in turn activates the MAP2Ks MKK4 and MKK5, which

activate MPK3 and MPK6 leading to downstream transcription of defence response genes [144]. Strikingly, this signalling framework is the same used in animals to signal innate immunity with the exception that the pathogen-associated molecular pattern (PAMP) is perceived by a Toll-like receptor that then acts through a cytosolic IRAK kinase [144]. Note that IRAK belongs to the RLK/Pelle family, which has undergone dramatic expansion in plants [34] but remains a small family in metazoans (1–4 members). Since this first example was published, MAPK pathways regulating cell division [145,146], stomata development (reviewed in Liu *et al.* [147]) and abiotic stress have been elucidated (reviewed in Sinha *et al.* [148]).

RSK-2

The most extensively studied AGC kinases in plants belong to the RSK-2 subfamily that is related to but evolutionarily distinct from RSK (p90 ribosomal S6 kinase). Members are involved in many aspects of cell growth and proliferation, and are activated by MAPK signalling cascades as well as by PDK1 (reviewed in Anjum & Blenis [149]). In land plants, the RSK kinases have undergone moderate expansion and are involved mainly in plant-specific roles, including blue light perception [150], polar auxin transport [151] and stress response [152,153]. Despite this divergence in function between plants and other eukaryotes, the regulation of RSK and RSK-2 activity by PDK1 is conserved (see §4a(i)).

(ii) Divergent functions but conserved regulatory mechanisms

The regulation of kinase activity of some moderately expanded families, OSTL1, CHK1 and CK1, is achieved by conserved mechanisms, even though the functions of and/or the signalling pathways regulating these kinases have diverged.

OSTL

The AMPK/SNF-related kinase family, OSTL (open stomata-like; SnRK2), which is related to but more divergent from the AMPK/SNF1 family discussed in §4a(i), has undergone pronounced expansion and divergence in plants [28]. Studies in rice, wheat and *A. thaliana* have revealed roles for SnRK2s in osmotic stress, the plant hormone abscisic acid (ABA) and/or sugar metabolism signalling (reviewed in Coello *et al.* [154]). There is evidence that SnRK2s are regulated by protein phosphatase 2C (PP2C), a mechanism used to regulate AMPK/SNF1 in animals and yeast. For example, the *A. thaliana* ABA-activated OST1/SnRK2.6 kinase is deactivated by the PP2C, HAB1, upon ABA binding to an ABA receptor [155]. The crystal structure of the SnRK2.6–HAB1 complex revealed that PP2C binds to both SnRK2s and ABA receptors through a common domain. Algae SnRKs lack this domain, indicating that SnRK2 ABA signalling is a land-plant-specific adaptation [156].

CHK1

In animals, CHK1 and the closely related CHK2 play roles in DNA damage response. Earlier studies indicated that there are no obvious plant CHK1

orthologues [157], but in our analysis, some plant kinases appear to be more closely related to CHK1 than to other CAMK families. CHK1, or the SNF1-related SnRK3, subfamily members are calcineurin B-like protein (CBL) interacting protein kinases (CIPKs). Plant CBLs are related to calcineurin B proteins in animals and yeast. However, CBL–CIPK signalling pathways are specific to plants; in yeast and animals calcineurin B binds to phosphatases, not kinases [158]. The plant CAMKL–CHK1 subfamily includes salt overly sensitive2 (SOS2), which interacts with the calcium-binding protein, SOS3, to regulate ion homeostasis and confer salt tolerance [159,160]. CIPKs have also been implicated in osmotic stress responses, ABA signalling, nitrate sensing and K⁺ transport, with the CBL-binding partner determining pathway specificity [161,162]. Like SnRK2s, CIPKs interact with PP2Cs, although whether CIPKs are dephosphorylated by PP2Cs has not yet been determined [156].

CK1

Casein kinase 1 (CK1) is evolutionarily conserved across eukaryotes and regulates a wide variety of cellular processes [163]. Many plant lineages have more than twice the number of CK1 paralogues compared with humans. Most plant CK1s have not been functionally characterized; however, there is evidence that *A. thaliana* CK1 proteins are involved in microtubule organization [164]. Not much is known about the regulation of plant CK1 activity, but achieving target specificity through the differential subcellular targeting of different isoforms appears to be a mechanism for controlling CK1 activity across eukaryotes [165,166]. Another group of CK1-related genes (CK1-CK1-PI) is found only in the plant lineage. Rice *EARLY FLOWERING1* encodes a plant-specific CK1 that negatively regulates gibberellic acid (GA) signalling by phosphorylating a DELLA domain protein, also a member of a plant-specific family of GA regulators [167], indicating that these family members participate in plant-specific processes.

(iii) Expansion and involvement in plant-specific processes

The plant-specific expansion of kinase families such as GSK3, NEK, CTR1, plant-specific TKLs and WNK/NRBP may take on functions in plant-specific processes that are probably adaptive. The plant-specific TKLs have clearly evolved to regulate processes that are specific to plants.

GSK3

Glycogen synthase kinase 3 (GSK3) is a key regulator of several developmental processes, including cell migration [168], metabolism [169] and cell proliferation. CMGC_GSK3 kinases are conserved in animals, fungi and plants, but while mammals have two copies of GSK3, this subfamily has expanded (up to more than 20 members) in land plants. In *C. reinhardtii*, the only GSK3 homologue is required for flagellar assembly [170]. In plants, GSK3 kinases function in brassinosteroid hormone signalling, abiotic and biotic stress pathways, and flower development (reviewed in Saidi *et al.* [32]). The diversification of GSK3 genes several times during land plant evolution suggests that they have been important for adaptation [32].

NEK

NimA was first isolated from *Aspergillus niger*, and NimA-related kinases (NEK) are found in all eukaryotes, ranging from one family member in yeast to 14 in mammals. Although NEKs have divergent functions, a common feature is their regulation of microtubules (reviewed in Moniz *et al.* [171]). NEK genes have roles in cell cycle regulation as well as ciliogenesis, and it is thought that expansion in organisms with cilia/flagella might be due to the need to coordinate the cell cycle with cilia development [171,172]. In *G. lamblia*, the NEK family has expanded to an astounding 198 of the 278 protein kinases, 70 per cent of which are likely to be catalytically inactive [37]. The dramatic expansion of the NEK family may be due to the fact that *G. lamblia* has eight flagella and two nuclei, requiring more regulatory kinases; however, the expanded NEK genes are not orthologous to NEKs in expanded subfamilies in ciliates, which are also binucleate [37]. Notably, green algae *C. reinhardtii* and *Volvox carteri* have twice as many NEKs as land plants, suggesting that expansion may also be related to the presence of flagella in these algae. In plants, *A. thaliana* NEK6 has been the most extensively characterized. It interacts with microtubules to regulate epidermal cell morphogenesis [173,174] and is also involved in stress responses [175,176].

DRK-1 and DRK-2

In *A. thaliana*, expansion of TKLs has occurred in families whose members are involved in plant-specific processes. Because of high sequence divergence, the classification of TKLs is challenging. Particularly, in our analysis, many plant kinases that are regarded as Raf homologues (or referred to as MAP3Ks on some occasions) belong to the TKL group but do not appear to be in a monophyletic group with metazoan Raf kinases. One such example are the DRKs (downstream of receptor kinases) originally found in the slime mould *D. discoideum* [177] that resolved into two clades: DRK-1 and DRK-2. The DRK-2 family includes EDR1, which regulates SA-inducible defence signalling [178] and CTR1, which regulates ethylene responses [15]. Originally, on the basis of sequence similarity with Raf kinase, which has MAP3K activity, CTR1 was proposed to activate MAPK signalling cascades in response to ethylene (reviewed in Colcombet & Hirt [25]). However, a biochemical function as a MAP3K has not been demonstrated. EDR1 is recruited to the ER via its association with the plant-specific E3 ligase/kinase KEEP ON GOING, a member of the small TKL_PI-1 subfamily; this complex may regulate signalling complexes located at the ER [179]. Rice DSM1, another DRK-2 member, mediates drought stress response [180]. The function of other DRK-2 and all DRK-1 members remains unclear.

TKL: plant-specific subfamilies

The TKL_PI-4 kinases belong to multiple expanded, little studied subfamilies. HIGH TEMPERATURE1 is expressed in guard cells and regulates stomata in response to CO₂ [181]. Three additional members (STY 8, STY17 and STY46) phosphorylate the transit

peptides on chloroplast-targeted preproteins and are required for chloroplast differentiation [182]. The TKL_PI-5 subfamily member, VH1-interacting kinase (VIK), interacts with VH1/BRL2, and mutations lead to defects in leaf venation and auxin and BR response [183]. VIK also phosphorylates the *A. thaliana* tonoplast monosaccharide transporter to regulate vacuolar sugar accumulation [184].

WNK/NRBP

WNK (with no lysine) and nuclear receptor binding protein (NRBP) families are considered together because they consistently form a monophyletic group. In addition, the duplication event that led to these two families appears to have taken place in the animal/fungal lineage after its divergence from plants (see electronic supplementary material, appendix S3). WNK kinases are distinct from other kinases because a conserved lysine in the catalytic cleft is found in subdomain I instead of subdomain II. They are found in most eukaryotes, but were lost in yeast [185]. In mammals, WNKs have been extensively studied owing to their effects on renal ion transport (reviewed in Huang *et al.* [186]). The *C. elegans* WNK1 gene functions in cell volume recovery after hypertonic stress [187]. Not much is known about the functions of WNKs in plants. Plant WNK genes in plants are transcriptionally regulated by the circadian clock and abiotic stress [188,189]. Soybean GmWNK1 regulates root architecture in response to ABA and osmotic signals [190]. On the basis of common functions of WNKs in osmotic stress responses [190], members in this family may be retained owing to their adaptive roles in protecting cells against water loss.

(c) Families with significant degree of expansion

(i) Significantly expanded families tend to play roles in stress response

The CDPKs and RLK/Pelle have undergone the most significant degree of expansion among protein kinase families in all land plant lineages. On the basis of our knowledge of their functions, their expansion and subsequent functional divergence may have allowed plants to perceive and/or respond to various environmental signals.

CDPK

The calcium-dependent protein kinases (CDPKs) comprise a large family of Ca²⁺ regulated kinases found in plants [13] and alveolates [38] (see Talevich *et al.* [191], in this issue) that have a domain similar to calmodulin and bind Ca²⁺ directly. Different CDPKs bind to Ca²⁺ with differing affinities, potentially allowing different CDPKs to respond to different Ca²⁺ signals [20]. CDPKs play roles in development, for example regulating transcription in response to hormone levels [192]. Several CDPKs are known to function in abiotic stress response and ABA signalling pathways through phosphorylation of targets, including ion channels and transporters (reviewed in Das & Pandey [193]). CDPK activity in guard cells regulates the opening and closing of stomata in response to ABA [194,195] and methyl

jasmonate [196]. Recently, CDPKs have also been found to be key components of innate immunity signalling pathways [197,198].

RLK/Pelle

Plant RLK/Pelle family members play diverse roles in development, regulating cell-type specificity and organ identity (reviewed in De Smet *et al.* [199], as well as in defence response [200] and, to a lesser extent, in abiotic stress response [201–203]. It has been shown that RLK/Pelle subfamilies with developmental regulatory functions tend to have lower degrees of expansion than those involved in defence response [33]. However, subsequent study has shown that this distinction is not as clear-cut as was once believed [34]. Involvement in basal immunity is a function shared by both animal and plant RLK/Pelle genes, and the functions of RLK/Pelles in innate immunity pathways as pattern recognition receptors have been particularly well-studied [204]. Several lines of evidence suggest that RLK/Pelle expansion is tied to the need for plants to adapt to changing biotic conditions [34]. Although little domain gain within RLK extracellular domains has occurred since the divergence of the vascular plant lineage from moss, the domains that have been acquired have been implicated in sensing biotic signals. In addition, there is evidence that RLK/Pelle genes have been co-opted for roles in sensing biotic signals. For example, LRR-I subfamily members have evolved legume-specific roles in symbiosis. Finally, RLK/Pelle subfamilies show substantial differential expansion in different plant lineages, and subfamilies showing differential expansion, such as DLSV (DUF26, SD-1, LRR-VIII and VWA, a moss-specific new RLK subfamily), L-LEC, LRR-XII, SD2b, WAK and WAK/LRK10L-1 also tend to be enriched in genes that have been implicated in biotic stress response based on either function or expression evidence [34].

5. WHY THERE ARE SO MANY PLANT PROTEIN KINASES?

In this study, plant protein kinases were identified and classified based on assignments from other eukaryotes. Many of these families and even subfamilies have clear plant homologues, suggesting their early establishment during the course of eukaryote evolution. The plant protein kinase superfamily is in general larger than that in other eukaryotes. Our findings indicate that the larger protein kinase repertoire is the consequence of recent duplications. Species such as soybean and cottonwood, where very recent genome duplications have taken place, have more kinases than most other plant species. Together with frequent tandem duplication found in this gene family [12,33,34], these duplication mechanisms are the main proximal cause for the higher degree of expansion of the protein kinase superfamily in plants.

The propensity to expand, however, differs greatly among protein kinase families and subfamilies because clearly not all duplicates were retained. Despite frequent duplication, many plant protein kinases remain low in copy number. In some cases, such as WEE1

and TLK, there are only single members within the families established prior to the divergence of eukaryotic species. Considering their functions, many are involved in ‘house-keeping’ functions such as regulation of metabolism, cell cycle and mitosis. Their low copy number and house-keeping role is consistent with the finding that duplicability of house-keeping genes tends to be low [205]. However, the plant lineage diverged from the animal and fungi lineages over a billion years ago. Extensive developmental and physiological adaptation and life-history differences have led to significant differences in how these highly conserved kinases function. As a result, many of them now play roles in plant-specific processes.

Aside from families with low copy number, some families have been moderately or highly expanded. What was the evolutionary force(s) that drove such differential expansion in protein kinase families? Given what we understand now, it appears that the selection pressure on the ability to properly respond to changing environment is a major contributor [54]. In the case of RLK/Pelle family members, which play roles in recognition of non-self biotic factors, continuous selective pressure imposed by pathogens and symbionts potentially drove the rather drastic expansion in this family [34]. The expansion of the CDPK family, whose members are involved in calcium-modulated signalling, could conceivably be a consequence of adaptive evolution where a new CDPK duplicate allowed perception of a different calcium signal through variation in affinity for Ca^{2+} and/or interacting protein substrates [20]. For the other families (such as multiple TKL families, the WNK/NRBP and the NEK families) that have undergone moderate expansion, the reason for expansion remains unclear. One possibility is that many of these protein kinases were duplicated and retained owing to their roles in plant-specific processes. This notion is consistent with the plant-specific roles played by many plant protein kinases with animal/fungal orthologues. To test this hypothesis, comparative functional studies are required between early diverging plant species such as algae and bryophytes and flowering plants.

In addition to an adaptive explanation for the observation of significantly more kinases in plants, it is possible that some protein kinases have been retained owing to at least two non-adaptive reasons. First, some duplicate protein kinases may be retained simply owing to subfunctionalization where the functions of the ancestral kinase were partitioned among the duplicates [206]. In this situation, neither copy can be lost without clear phenotypic consequences. This possibility, at first glance, may run counter to the well-known finding in the plant research community that there is a substantial functional overlap between duplicate genes. There are a large number of plant studies demonstrating that loss-of-function in multiple paralogues is necessary to reveal mutant phenotypes in laboratory conditions. However, it is apparent that laboratory conditions are not as diverse as the natural environment. Thus, the absence of a phenotype with the loss-of-function of one paralogue cannot be regarded as evidence against subfunctionalization.

The second non-adaptive explanation is that some protein kinases may very well be on their way to becoming pseudogenes. There are several lines of evidence suggesting this may be the case. Note that species with recent whole genome duplications tend to have larger protein kinase repertoires. In a comparative study of *A. thaliana* and rice genomes, hundreds of protein kinase pseudogenes were found, although protein kinases as a whole tend to have proportionally less pseudogenes than other families [43]. Population genomic studies of *A. thaliana* have also shown that many members of the RLK/Pelle family, which experienced the most extensive expansion, have alleles with non-sense and/or frame-shift mutations expected to disrupt gene function [207]. Therefore, there may have been insufficient time after duplication to accumulate mutations that render some protein kinases completely non-functional.

Another potential indication that some duplicate kinases are becoming pseudogenes is the prevalence of pseudokinases in plants. Around 13 per cent of *A. thaliana* protein kinases were hypothesized to be pseudokinases with modifications in some residues critical for catalytic activity [208]. However, some predicted pseudokinases have been shown to have catalytic activity, and the numbers of pseudokinases may be over-estimated [209]. Among RLK/Pelle family members, the proportion of pseudokinases is even higher at approximately 20 per cent. In some cases, such pseudokinases have clear biological functions. One of the first examples is the STRUBBELIG kinase, which lacks enzymatic phospho-transfer activity but is essential for proper *A. thaliana* development [210]. Examples such as STRUBBELIG clearly demonstrate the importance of phosphorylation-independent mechanisms in plant signal transduction. However, a clear demonstration of loss-of-function phenotype is required to argue against the equally likely explanation that loss of kinase activity is due to relaxed or no selection on duplicates.

The relative importance of adaptive and non-adaptive explanations for protein kinase family expansion is unclear. But these discussions do highlight the challenge in elucidating plant protein kinase functions, given their exceptional numbers and similarities in all plant species. Comparative genomic studies examining molecular patterns of protein kinase evolution within and between species may provide some hints as to which protein kinases continue to experience strong purifying selection and are likely to remain functional.

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