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Two-Component Systems and Their Co-Option for Eukaryotic Signal Transduction

Review

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Two-component signaling pathways involve histidine kinases, response regulators, and sometimes histidine-containing phosphotransfer proteins. Prevalent in prokaryotes, these signaling elements have also been co-opted to meet the needs of signal transduction in eukaryotes such as fungi and plants. Here we consider the evolution of such regulatory systems, with a particular emphasis on the roles they play in signaling by the plant hormones cytokinin and ethylene, in phytochrome-mediated perception of light, and as integral components of the circadian clock.

Introduction

On October 22 1993 the two-component signaling system came to eukaryotes. In truth it had been there all along, but this was the date of the *Science* issue in which two papers appeared, one using a plant system and one a fungal system, both describing the discovery of histidine kinases. Chang *et al.* [1] reported the cloning of the gene for the ethylene receptor ETR1 from the plant *Arabidopsis thaliana*, and Ota and Varshavsky [2] the cloning of the *SLN1* gene from yeast, the product of which plays a critical role in the osmosensing pathway. Until this point there had been a nice sharp division between how scientists thought about signal transduction in prokaryotes compared to eukaryotes: prokaryotes made use of two-component systems that relied on phosphorylation at histidine and aspartate residues; in contrast, eukaryotes relied on kinases that phosphorylate at serine, threonine and tyrosine residues. Suddenly, it wasn't quite so simple.

The next substantial leap forward in our understanding of these signaling systems in eukaryotes came with the advent of technology for obtaining full genome sequences because, for the first time, we could assess the number and complexity of two-component signaling elements in an organism. The yeast *Saccharomyces cerevisiae* turned out to have a fairly limited repertoire, but the genome sequence of the model plant *A. thaliana* revealed genes for a wealth of histidine kinases, response regulators and phosphotransfer proteins [3,4]. The question in *A. thaliana* now became: what do each of these elements do? Which ones 'play' together in transducing a common signal? Continued genome sequencing of various species of bacteria, fungi, and plants has laid the groundwork for examining the evolutionary history of these elements, tracing the origins of the modern-day plant signaling elements backwards in time, identifying ancestral forms of these signaling pathways and, in doing so, gaining a new awareness of how they have been adopted and reconfigured to the needs of a multi-cellular eukaryote.

Here we will consider, first, the canonical two-component systems as defined through prokaryotic studies; second, the acquisition of these systems by eukaryotes; third, some of the permutations possible within a fungal system; and fourth, the major mechanisms and pathways of plants that make use of these elements.

Prokaryotes and Their Two-Component Signaling Systems

Two-component signaling systems regulate most aspects of a bacterial life, including responses to almost all changes in the environment of a bacterium [5]. Two-component systems regulate global responses to stresses, the switch from free-living to symbiotic or virulent life styles, and the switch from free-living to biofilm or surface growth [6,7]. Two-component systems control cell division as well as the decision whether to continue growing or to move to stationary phase or to sporulate [8]. Here, we describe some of the key features of two-component systems uncovered from the study of prokaryotes, highlighting how genome sequencing projects are clarifying the multiple possibilities for regulation inherent in the prokaryotic two-component system.

The Canonical Two-Component System of Prokaryotes

The canonical two-component system in bacteria is composed of a transmembrane sensor histidine kinase which regulates the activity of a single diffusible response regulator, usually a transcription regulator (Figure 1A) [5,9]. In the transmembrane sensor kinase, a variable amino-terminal input domain signals to a conserved carboxy-terminal histidine-kinase domain, catalyzing auto-phosphorylation, with a phosphoryl residue being transferred from ATP to a conserved histidine residue. This phosphoryl is then taken by an aspartate residue in the conserved receiver domain of the response regulator. Once phosphorylated the associated output domain of the response regulator is activated [5]. Eventually the signal is terminated by a loss of the phosphoryl as inorganic phosphate, either spontaneously or with the help of a phosphatase. Depending on the role of the response regulator in the cell, the activated response regulator can have a lifetime of a couple of seconds to weeks. An excellent special review issue of *Current Opinion in Microbiology* covers many of the following aspects about prokaryotic two-component systems in detail [10].

What are the differences between histidine–aspartate based systems and serine/threonine kinase based systems? As described above, the earlier assumption that prokaryotes have two-component systems and eukaryotes serine and threonine kinase-based systems was shown to be incorrect when plants and fungi were found to have both systems, but we now know that many bacterial species also have both sensory systems, sometimes interconnected [11,12]. Serine/threonine kinases create phosphoesters, while histidine kinases create phosphoramidate intermediates. The hydrolysis of phosphoramidates involves a significantly

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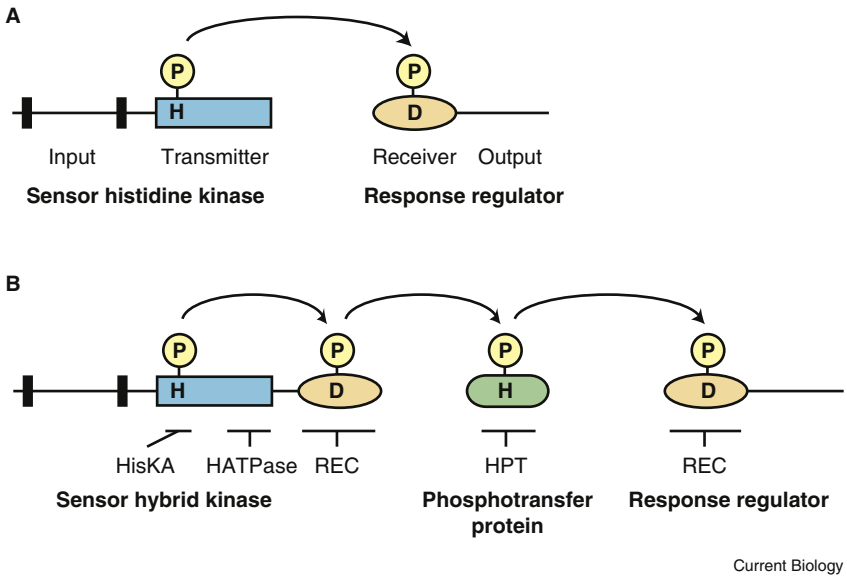

Figure 1. Elements of two-component signaling systems.

Histidine kinase domains are indicated by rectangles, receiver domains by ovals, histidine-containing phosphotransfer domains by rounded rectangles, and transmembrane domains by black bars. Sites of phosphorylation upon histidine (H) and aspartic acid (D) residues are indicated. (A) Simple two-component system that employs a histidine kinase and a response regulator. (B) Multi-step phosphorelay that employs a hybrid histidine kinase with both histidine kinase and receiver domains, a histidine-containing phosphotransfer protein, and a response regulator. Regions covered by consensus domains (HisKA, HATPase_c, HPT, and REC) are indicated.

higher free energy change than that of phosphoesters, and the equilibrium favors the unphosphorylated kinase at typical intracellular ATP:ADP levels [5]. Therefore, the flux through the two-component system is more important than the stoichiometry of the reaction. With a phosphoaspartate as the end product inducing conformational changes, this makes two-component systems phosphorylation-mediated switches, activating transcriptional regulators or enzyme activity.

Tens of thousands of histidine kinases and response regulators have now been identified in bacterial genomes [13–15]. A recent study [15] showed that 864 of 899 bacterial genomes encode two-component signaling components, with the systems only missing from a few obligate pathogens and endosymbionts with very small genomes. In general, the number of two-component systems reflects the genome size and complexity of the lifestyles of the bacterial species. The average number of two-component systems in bacteria is around 30, but *Myxococcus xanthus* has over 200 [16]. Despite extensive study over the past decade and the increasing number of two-component systems identified, it is still hard to predict what is sensed by the variable sensory domains of the sensor kinases. Specificities that presumably prevent cross-talk in species with large numbers of two-component systems are also only just beginning to be elucidated [17–22]. In addition, as more prokaryotic genomes have been sequenced, the great complexity and variation possible in these systems is becoming apparent. Initially it was assumed that most histidine kinases and response regulators were encoded next to each other in operons, making partners easy to identify. However, over 15% of the response regulator genes in bacteria and 50% of the archaeal response regulator genes are found alone [23]. While almost all bacteria have two-component systems, only about half the archaeal species have two-component systems, and most of those are in the Euryarchaeota. Phylogenetic analysis indicates that two-component systems originated in bacteria and were then acquired by archaebacteria through horizontal gene transfer, a finding consistent with the greater prevalence of two-component systems in bacteria [24].

The modular nature of the two components of the two-component system has resulted in a wide range of sensory and signaling architectures having evolved as different sensory protein folds have been added to the systems [25–27]. Such sensory components include HAMP, GAF, LOV, PAS, CACHE, PBP, and phytochrome domains, often in addition to periplasmic folds that bind specific substrates. Most sensor kinases have transmembrane domains, but in some cases the sensor domain may be cytoplasmic, and some sensory kinases are completely cytoplasmic, sensing cytoplasmic signals; for example, NtrB-related histidine kinases sense intracellular nitrogen dependent signals and regulate expression of nitrogen metabolic pathways in a wide range of species [28].

In line with the variable inputs, there are many different outputs from different response regulators, as suggested by the diversity of protein domains found in these two-component elements. Genome analysis has identified more than 30,000 non-redundant response regulator sequences and more than 200 structures of receiver domains have been solved [23,29]. Remarkably, the receiver domains are highly conserved, but can activate a wide range of output domains. As mentioned above, the most common output, activated by phosphorylation of the conserved response regulator aspartate domain, is a DNA-binding domain, regulating transcription of the target genes [30]. Nevertheless, a range of DNA-binding motifs have been identified associated with response regulator domains, from simple helix-turn-helix motifs to winged helix domains [30]. Many non-DNA binding output modules have also been identified that are activated by phosphorylation of the receiver domain [30]. These include methyl esterase, adenylate cyclases, diguanylate cyclases, serine/threonine kinases, phosphatases and cyclic di-GMP specific phosphodiesterases [23]. Other output domains include protein–protein interaction domains, involved, for instance, in regulating sigma factor activity, putative transmembrane transporter proteins fused to response regulator domains, and a number of metabolic, rather than regulatory, enzymes [31].

Given this range of inputs and outputs, but the structural similarity among the histidine kinase domains and the receiver domains, how is specificity achieved in signaling, particularly in some species where there may be up to several hundred different two-component systems operating in

any one cell? This is an area of active research at the moment [17–22]. Histidine kinase and response regulator proteins are often encoded next to each other on operons, and almost certainly have co-evolved [32]. However, as there are examples of both response regulators and histidine kinases being individually encoded on distinct genes, this being the norm in some species such as *Bdellovibrio*, it is not always immediately possible to identify partners or the mechanisms determining specificity [33]. Bioinformatic, statistical and structural studies suggest that the specificity tends to depend on as few as two or three amino acids in the interaction domains allowing phosphotransfer at the required rate for that specific two-component system [22]. Other sensor kinases will often, if in high enough copy number and in contact for long enough, carry out phosphotransfer, but the time course is not sufficient for physiologically relevant activation. Interestingly, genomic analysis in some bacterial species has identified pseudo-receiver domains with structures homologous to a conserved receiver, but lacking one or more of the conserved amino acids in the active site; how these function in the absence of phosphorylation is unclear [34].

Permutations of the Two-Component Signaling System in Prokaryotes

Multiple pathway architectures have evolved in prokaryotes using just the two signaling modules of the canonical two-component system (Figure 1A) [32,35]. The simplest is a linear pathway, with one histidine kinase regulating one response regulator, and this response regulator then regulating expression of one target operon. Other two-component systems, however, may have a single histidine kinase regulating one response regulator, but that response regulator may activate 30% of the bacterial genome; this occurs, for example, when switching to a dormant state, or from aerobic to anaerobic growth [36]. Alternatively, one histidine kinase might regulate multiple response regulators, or multiple histidine kinases might regulate one response regulator. The potential combinations are vast, and the apparent ability of prokaryotes to use hundreds of closely related sensory proteins simultaneously without cross-talk is fascinating [37,38].

More complex signaling pathways involving histidine kinases and response regulators have also evolved [15,35]. These multi-step phosphorelays make use of multiple phosphotransfers and often involve an additional domain called a histidine-containing phosphotransfer domain (Figure 1B). For example, following the initial autophosphorylation of the histidine kinase, the phosphoryl group may be transferred to the aspartate of a receiver domain, then to a histidine on a phosphotransfer domain, and finally, possibly after several more receiver and phosphotransfer domains, reaching the aspartate of an output response regulator. The two-component system domains found in the phosphorelay can be separate or fused, such that several two-component system domains are found on one hybrid protein, such hybrid proteins being strongly indicative of their participation in a phosphorelay. While 90% of the eukaryotic two-component systems use hybrid histidine kinases (with histidine kinase and receiver domains), only 20% of the characterized bacteria genomes encode hybrid kinases, and only 1% in archaea are hybrid [15]. There is evidence that some of these hybrid histidine kinases dephosphorylate by reversing the phosphotransfer [39].

One of the best understood phosphorelays is that involved in *Bacillus subtilis* sporulation [8]. Five sensor histidine kinases act as phosphodonors of the response regulator Spo0F, which phosphorylates the phosphotransfer protein Spo0B, which in turn phosphorylates the response regulator Spo0A, the transcriptional regulator of sporulation. The different sensor histidine kinases are all regulated by different proteins that ultimately respond to different environmental signals linked to cell division or nutrient levels, ensuring sporulation is only triggered when absolutely necessary. But in addition to this control at the kinase input level, a number of other proteins regulate the cascade at the Spo0F and Spo0A level, dephosphorylating the response regulators if growth conditions improve, and shifting the decision towards stationary phase rather than sporulation [8]. The advantage of these multi-step phosphorelays over the single step two-component system in other systems is unclear, but, as with sporulation, it may allow additional control, with regulators controlling the activity at different points in the relay, allowing more than one input into a final output. Alternatively, the relay may allow noise to be damped and this may prevent inappropriate outputs.

Acquisition of Two-Component Signaling Elements by Eukaryotes

Sequence analyses identify two-component signaling elements in a variety of eukaryotes [15]. As shown in Figure 2 and Table 1, in which the genomes of representative eukaryotes are examined, two-component signaling elements are present in the angiosperms *A. thaliana* and rice, in the green algae *Chlamydomonas*, in the diatom *Thalassiosira*, in the slime mold *Dictyostelium*, and in 18 out of the 19 species of fungi examined (missing only in the fungal relative *Encephalitozoon cuniculi*, which has a highly reduced genome), but are lacking in the metazoans. These two-component signaling elements are lineage specific and are therefore thought to have been acquired from lateral gene transfer occurring after the mitochondrial endosymbiosis that resulted in the last eukaryotic common ancestor [40]. The primary contribution of the mitochondrial endosymbiosis event is likely to have been pyruvate dehydrogenase kinase, a serine/threonine kinase related to the histidine kinases but now so highly diverged it is not recognized under typical search parameters for histidine kinases [41]. Sources of later lateral gene transfer events include endosymbiosis involving cyanobacterium-like ancestors that gave rise to plastids, host-parasite interactions, and bacterial phagocytosis [40].

The domains associated with eukaryotic two-component signaling elements point to conservation of some signaling mechanisms between prokaryotes and eukaryotes, as some of the domains associated with eukaryotic histidine kinases are also found in prokaryotes (Figure 2). These include the GAF domain of the light-sensing phytochromes found in fungi and plants as well as in the ethylene receptors of plants [42–44], and the CHASE domain of cytokinin receptors of plants [45,46]. The similar domain architecture of the phytochromes and ethylene receptors between prokaryotes and eukaryotes supports their bacterial origin.

But the domains associated with eukaryotic two-component signaling elements also point to adaptations made following co-option of the two-component system by eukaryotes (Figure 2). First, the genomes of the eukaryotic species code for hybrid histidine kinases as well as histidine-containing phosphotransfer proteins, consistent with

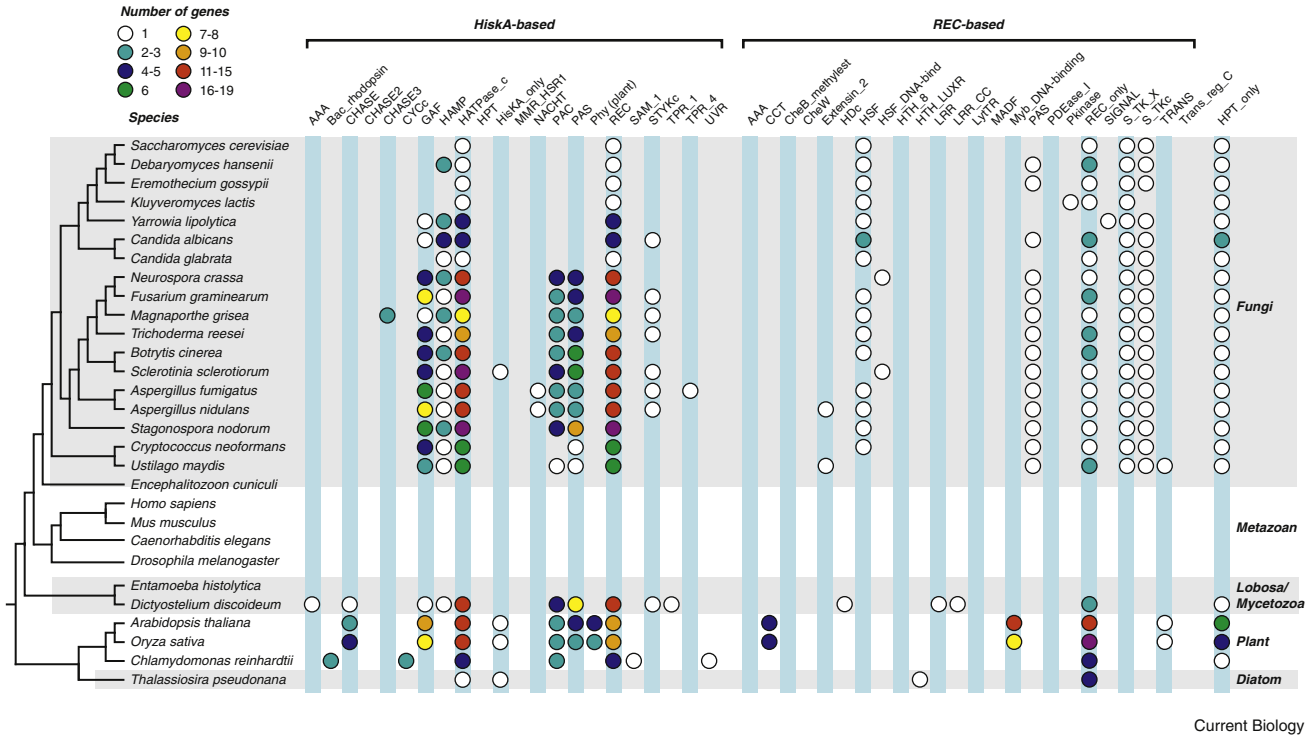

Figure 2. Domain composition and distribution of eukaryotic histidine kinase, phosphotransfer, and receiver domain containing proteins.

The names on top are the protein domains present in the same protein as histidine kinase (HisKA) or receiver (REC) domains. The species and their phylogenetic relationships are indicated on the left. The major taxonomic groups are labeled in alternating gray backgrounds. A circle indicates the presence of a particular domain combination in an organism, with the circle color indicating the number of genes with such combination. For identification of the two-component signaling elements, hidden Markov models and alignment seed sequences for histidine kinase catalytic domain (HATPase_c) and acceptor domain (HisKA), histidine-containing phosphotransfer domain (HPT), and response regulator receiver domain (REC) were obtained from Pfam [111], and these hidden Markov models were used to search against the genome protein sequences with HMMER [112]. Sequences were submitted to the SMART database to verify the presence of target domains and to annotate other domains in candidates [113].

the use of multi-step phosphorelays. The prevalence of such systems in eukaryotes may be due to the selective advantage conferred by the ability to transmit the signal across a greater distance, from membrane to nucleus, with the phospho-histidine being more stable than the phospho-aspartate linkage. Second, the finding that two-component signaling elements are also paired with eukaryotic-specific domains, such as the Myb domain paired with receivers [47], points to the recombining and shuffling that has occurred that is important for eukaryotic signaling.

The comparative numbers of two-component signaling elements in eukaryotes also deviates from what is typically found in prokaryotes (Figure 2) [40,42,48]. In prokaryotes, the genes encoding signaling elements for a particular phosphorelay are often found on the same operon, resulting in a one-to-one correspondence among the histidine kinases and response regulators. The eukaryotes deviate from such a one-to-one correspondence. The angiosperms have greater numbers of response regulators than of histidine kinases and phosphotransfer proteins, in large part due to lineage-specific expansion of the response regulators. In contrast, fungi have experienced greater levels of expansion in the histidine kinase families relative to the phosphotransfer protein and response regulator families. These differences imply potential differences in the complexity of two-component system inputs and outputs between plant and fungi.

Although eukaryotes contain sequences characteristic of two-component signaling elements, a number of the eukaryotic elements have diverged such that they now lack residues essential for activity (Table 1). Some of these diverged elements would be unable to participate in a canonical phosphorelay and therefore point to substantial changes that have occurred following the acquisition of two-component elements by eukaryotes. Diverged two-component signaling elements are present in plants and fungi but, in comparison to fungi, plants contain greater numbers of diverged elements and include families composed solely of diverged elements [43,49]. Given that the evolutionary distances between members of such diverged element families are large, many of these diverged elements have apparently persisted over millions to tens of millions of years of evolution. Thus, they are not simply remnants of pseudogenes. The role of these diverged elements is discussed in more detail below, in reference to the specific signaling systems of fungi and plants.

Two-Component Signaling Systems of Fungi

In fungi, two-component signaling systems have been implicated in such processes as osmosensing, oxidative stress response, cell-cycle control, red/far-red light responses, and the dimorphic switch from non-pathogenic to pathogenic states [3,42,43,50]. The number of two-component signaling elements varies considerably in the

Table 1. Number of conserved and diverged two-component system elements in eukaryotes.

	Histidine kinase		Response regulator		Phosphotransfer protein	
Species	Conserved	Diverged	Conserved	Diverged	Conserved	Diverged
Fungi						
SCE	1	0	2	1	1	0
DHA	1	0	3	1	1	0
EGO	1	0	2	1	1	0
KLA	1	0	2	1	1	0
YLI	3	1	1	2	1	0
CAL	5	0	5	1	2	0
CGL	1	0	2	1	1	0
NCR	11	0	2	1	1	0
FGR	16	0	3	1	1	0
MGR	8	0	2	1	1	0
TRE	10	0	3	1	1	0
BCI	13	2	3	2	1	0
SSC	12	4	2	1	1	0
AFU	12	1	2	1	1	0
ANI	14	1	3	1	1	0
SNO	19	0	2	1	1	0
CNE	6	0	3	0	1	0
UMA	6	0	5	0	1	0
Lobosa/Mycetozoa						
DDI	14	0	5	0	1	0
Plant						
ATH	8	9	23	9	5	1
OSA	8	7	28	8	2	3
CRE	5	0	4	0	1	0
Diatom						
TPS	1	0	6	0	0	0

Identification of two-component elements is described in the legend to Figure 2. Functionality of the histidine kinases is based on the presence of a phosphorylatable His in the HisKa domain, as well as conservation of two out of three of the conserved amino acids in each of the G1 and G2 boxes of the HATPase_c domain. Functionality of the phosphotransfer protein and response regulator sequences is based on the presence of a phosphorylatable His or Asp, respectively. Species abbreviations are generated by taking the first character of the genus name and the first two characters of the species names as found in Figure 2. Species without any of the two-component system genes are not shown.

fungi, yeasts such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* having relatively few but filamentous fungi such as *Neurospora crassa* and *Aspergillus nidulans* having significantly larger numbers, 11 different families of histidine kinases having been identified in the filamentous fungi (Figure 2, Table 1) [42]. As mentioned, the prevalence of hybrid kinases and phosphotransfer proteins is consistent with the fungi making use of a multi-step phosphorelay. Interestingly, even though some fungi have many histidine kinases, allowing for multiple inputs into the phosphorelay, they typically have limited numbers of phosphotransfer proteins and response regulators, suggesting that fungi may integrate multiple input signals into a limited set of outputs [42].

Some fungi also contained diverged sequences (Table 1), in which two-component-like elements lack residues considered essential for canonical activity. Several generalities can be made about the diverged fungal sequences. First, where a fungal species has a diverged histidine kinase, it also tends to contain a conserved histidine kinase within the same gene family. Thus, rather than resulting in the lack of a two-component signaling pathway, the diverged histidine kinases may serve to modulate signaling by an authentic two-component signaling pathway. Second, no diverged phosphotransfer proteins were detected in fungi. Third, in 11 of the 18 cases in which a diverged receiver domain was found in a response regulator, the aspartate was substituted with glutamate, which may serve to mimic the activated phosphorylated form of the receiver. In such cases, alternative methods of regulation would be necessary and, based on what is known from fungi and plants, these may involve serine/threonine phosphorylation and targeted degradation.

Among the histidine kinases found in fungi are red/far-red light-sensing phytochromes similar to those found in bacteria and plants [42,43]. Interestingly, the fungal phytochromes are more similar to those found in non-photosynthetic bacteria than to those found in photosynthetic cyanobacteria and plants, as they contain diagnostic sequences for attachment of a biliverdin chromophore rather than a bilin chromophore [42,51,52]. Thus, the evolutionary event resulting in acquisition of the fungal phytochromes is likely to be independent from that which resulted in acquisition of phytochromes by plants. The fungal phytochrome from *Aspergillus nidulins* has been characterized in most detail, where it functions in control of red-light-dependent asexual sporulation [52]. Biochemical analysis of the phytochrome from *A. nidulins* demonstrates that it is a functional histidine kinase that autophosphorylates in response to red-light stimulation and that, being a hybrid kinase, is likely to transmit its signal through a multi-step phosphorelay [53]. The fungal phytochromes illustrate how a bacterial histidine kinase can be directly adapted to mediate a eukaryotic function, but other fungal two-component elements point to how these signaling domains can be mixed and matched with novel domains not usually seen in bacterial systems. Perhaps most interesting along these lines is the presence of both histidine kinases and response regulators fused to domains derived from serine/threonine kinases, suggestive of cross-talk between two different types of phosphorylation pathway (Figure 2) [42].

Although the yeast *Saccharomyces cerevisiae* has relatively few two-component signaling elements, these have been extensively studied and some interesting adaptations to eukaryotic signaling can be discerned. The histidine kinase Sln1 acts through a multi-step phosphorelay incorporating the phosphotransfer protein Ypd1 to regulate two response regulators, Ssk1 and Skn7, under conditions of low osmolarity [3,54,55]. The response regulator Ssk1 interfaces with the MEK kinases that initiate the HOG1 MAP-kinase pathway for control of the osmotic stress response; the phosphorylated form of Ssk1 is rapidly degraded and is unable to activate the MEK kinases, but the unphosphorylated form of Ssk1, present under conditions of high osmolarity, interacts with and activates the MEK kinases [56]. Thus, here we have a typical two-component system interfacing with a quintessential eurkaryotic phosphorylation cascade. The second response regulator, Skn7, is a transcription factor and its Sln1-dependent phosphorylation may serve to activate hypo-osmotic response genes [57]. However, Skn7 has also been adapted for the control of oxidative-stress response genes in a manner that is Sln1-independent. In this case, Skn7 need not be phosphorylated on the conserved aspartate, but is instead the target of an oxidant-dependent serine/threonine kinase; phosphorylation serves to stabilize the interaction of Skn7 p with the Yap1 transcription factor, and together they regulate expression of oxidative stress response genes [58]. This last result points to how a response regulator-like protein can perform a eukaryotic role independent of its role in a two-component pathway.

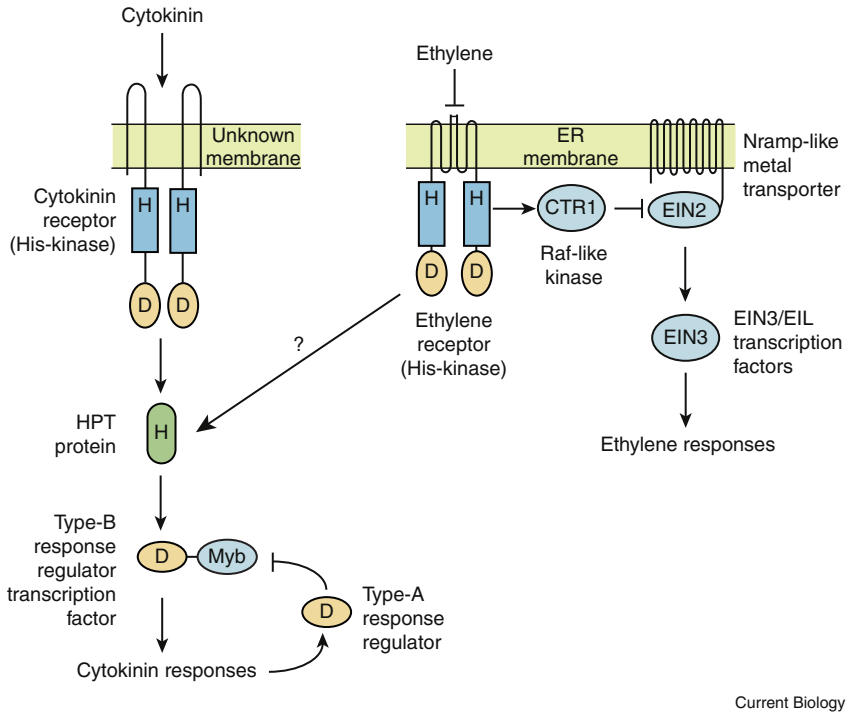

Figure 3. Comparison of cytokinin and ethylene signaling pathways of *A. thaliana*.

Cytokinin signaling makes use of a multi-step phosphorelay in which cytokinin receptors, phosphotransfer proteins (HPT), and type-B response regulators function as a positive regulatory circuit to relay the cytokinin signal from membrane to nucleus. Type-B response regulators act as transcription factors, one target being genes encoding type-A response regulators. The type-A response regulators feed back to inhibit their own transcription and may also mediate other cytokinin responses. Ethylene signaling involves positive and negative regulatory elements, including the Raf-like kinase CTR1, the transmembrane protein EIN2, and the EIN3/EIL family of transcription factors. Some ethylene receptors are functional histidine kinases and could potentially cross-talk with the cytokinin signaling pathway.

Two-Component Signaling in Plants

The dicot *A. thaliana* has eight histidine kinases, 23 response regulators, and five phosphotransfer proteins that contain the conserved residues required for enzymatic activity (Figure 2, Table 1) [4]. The histidine kinases contain several different types of input domain, such as those for the plant hormones cytokinin and ethylene, pointing toward their role in mediating signal transduction in multiple pathways. In addition, the response regulators fall into three major classes, the type-A, type-B, and type-C response regulators. The type-B response regulators contain long carboxy-terminal extensions with Myb-like DNA-binding domains and, like many of the prokaryotic response regulators, function as transcription factors [59,60]. The type-A and type-C response regulators contain only short extensions beyond their receiver domains, but can be clearly differentiated based on phylogenetic analysis [4]. The monocot rice has a similar complement of two-component signaling elements to that found in *A. thaliana* [61–64]. These angiosperms thus contain all the elements necessary for a multi-step phosphorelay. Moreover, genetic analysis in *A. thaliana* has clearly implicated such canonical two-component signaling elements in mediating signal transduction by the plant hormones cytokinin and ethylene [1,4,65,66].

Plants also contain a substantial number of diverged signaling elements, related to two-component elements but lacking key residues required for activity (Table 1). Like fungi, the angiosperms have families that contain both conserved and diverged sequences (for example, the ethylene receptors) [67]. But unlike fungi, angiosperms also contain families solely composed of diverged elements. Emblematic of these are the phytochromes, which are related to histidine kinases and function in the perception of red/far-red light [43], and the CCT family of pseudo-response regulators, which function in the circadian clock [68,69].

Cytokinin Signaling in Plants: A Multi-Step Phosphorelay

The majority of *bona fide* signaling elements from plants play a central role in cytokinin signaling, with histidine kinases, phosphotransfer proteins, and response regulators all participating in cytokinin signal transduction (Figure 3) [70]. Cytokinins are adenine derivatives that play a variety of roles in plant growth and development, notably in the control of cell division, for which cytokinins are named, and in the control of greening and the retardation of senescence. Genetic analysis in *A. thaliana* has demonstrated that the three histidine kinases containing CHASE domains act as cytokinin receptors, with binding of cytokinin to the receptors serving to activate their histidine kinase activity [66,71–73]. Genetic analysis has also shown that all five phosphotransfer genes as well as five of the eleven type-B response regulator genes from *A. thaliana* function downstream of the receptors in the initial pathway for cytokinin signal transduction [74–76]. The type-B response regulators are transcription factors responsible for mediating the primary transcriptional response to cytokinin [59,77]. Thus, cytokinin signaling incorporates and requires a multi-step phosphorelay, which serves to transmit the cytokinin signal from membrane to nucleus in three steps. But the role of two-component genes in cytokinin signaling does not end here, as expression of the genes for the type-A response regulators is regulated by cytokinin and their expression serves to negatively regulate the cytokinin signaling pathway [78].

All the *bona fide* phosphotransfer proteins and response regulators for which a function has been uncovered play roles in cytokinin signaling. Some may play additional roles in other pathways as well — for example, genetic evidence indicates that the phosphotransfer proteins also function downstream of another histidine kinase CKI1 in the control of female gametophyte development and vegetative growth [74]. But there is as yet no example of a novel pathway making use of a phosphotransfer protein or response regulator where that same signaling element that does not also function in cytokinin signaling. It remains to be seen if those response regulators for which no function has yet been assigned will turn out to play lesser or more specific roles in

cytokinin signaling or have an independent function altogether.

Although cytokinin signaling functions through a multi-step phosphorelay in angiosperms, evolutionary evidence suggests that the basic two-component system existed within the plant lineage prior to their acquisition for cytokinin signaling [48]. In particular, phosphotransfer proteins and type-B response regulators are found in unicellular and multicellular algae and basal land plants (*Physcomitrealla patens* and *Selaginella moellendorffii*), as well as being found in the higher plants. In contrast, histidine kinases with the cytokinin-binding CHASE domain are lacking in the algae examined and do not appear until the advent of land plants. Interestingly, the type-A response regulators also first appear in the land plants, suggesting that the ability to negatively regulate the cytokinin-signaling pathway was acquired at about the same time the intact pathway came into existence. It is not clear how land plants acquired the CHASE domain that became adapted to cytokinin binding, but a viral vector has been suggested as one possibility because the genome of the virus *Ectocarpus siliculosus* encodes a CHASE-domain histidine kinase and this virus infects brown algae [48]. It should be noted that CHASE domains are an ancient motif with a number of functions and are found in prokaryotes as well as the non-plant eukaryote *Dictyostelium discoideum*. Alternatively, it is also possible that another algal lineage, as yet not sequenced, does contain a histidine kinase with a CHASE domain and it is this lineage that gave rise to the land plants. Thus, it is of particular interest to determine if a histidine kinase with a CHASE domain exists in charophyte green algae, which is the closest sister group to the land plant lineage.

Some non-canonical regulatory mechanisms have also begun to appear in the higher plant cytokinin signaling pathway. Angiosperms, besides having *bona fide* phosphotransfer proteins, also have diverged phosphotransfer proteins (pseudo phosphotransfer proteins) lacking the critical phosphorylated histidine (Table 1). The one pseudo phosphotransfer protein of *A. thaliana* serves to negatively regulate the cytokinin signaling pathway, potentially by competing with the *bona fide* phosphotransfer proteins in the initial phosphorelay [79]. Perhaps even more interesting is the finding that rice, but not *A. thaliana*, contains a CHASE domain-containing receptor linked to a serine/threonine kinase domain rather than a histidine kinase domain [80]. The appearance of this new type of cytokinin receptor is thus of recent occurrence, having apparently arisen since the divergence of monocots and dicots, and suggests that cytokinin signaling may have begun to co-opt elements of the more typical eukaryotic signaling system involving serine/threonine phosphorylation.

Ethylene Signaling in Plants: A Chimerical Signaling System

Like cytokinins, ethylene is a plant hormone that regulates many aspects of plant growth and development, best known for its ability to stimulate senescence [44]. The ethylene receptors of higher plants are related to histidine kinases (Figure 3) and in all likelihood were acquired through endosymbiosis because similar proteins are found in a variety of bacteria. One of the best characterized examples is in the cyanobacterium *Synechocystis*, which is thought to share a common ancestor with the cyanobacterium that gave rise to the plant chloroplast. The slr1212 protein of *Synechocystis*, like the plant ethylene receptors, contains ethylene-binding, GAF, and histidine-kinase domains [81]. Significantly, slr1212 binds ethylene, demonstrating that it can indeed function as an ethylene receptor, although its actual biological function in *Synechocystis* is not yet known. The slr1212 protein could conceivably be involved in sensing of another molecule (for example, some other hydrocarbon), with a fortuitous ability to bind ethylene then being exploited during the evolution of plants. Nevertheless, on the same operon with slr1212 is a response regulator (slr1213) pointing to the use of a simple two-component signaling system by this ethylene receptor-like histidine kinase [82].

Ethylene receptors are present in moss, which shares a common ancestor with the flowering plants about 400 million years ago [83]. Seven ethylene receptor-like proteins are encoded in the genome of the moss *Physcomitrella patens*, and each contains an ethylene-binding domain, GAF domain, histidine-kinase domain, and receiver domain [84]. Previous work has demonstrated that *P. patens* can bind ethylene, although no clear role for ethylene in moss growth has yet been demonstrated [85]. All seven ethylene receptors of *P. patens* are predicted to be enzymatically active as histidine kinases, although one member of the family lacks the phosphorylatable His; however, as in the case with some of the diverged fungal histidine kinases, such a diverged family member could potentially *trans*-phosphorylate another family member.

Considerably more divergence among ethylene receptors becomes apparent when we look at angiosperms. The ethylene receptors can be divided into two subfamilies based on phylogenetic analysis and some shared structural features [44,67,86,87]. One subfamily has histidine kinase activity and members of this subfamily clade with some of the moss ethylene receptors [84,88]. But members in the second subfamily have considerably diverged, *in vitro* analysis indicating that these members may have acquired serine/threonine kinase activity as they diverged [89]. Furthermore, genetic analysis indicates that the major downstream players in ethylene signaling are not two-component signaling elements but instead a grab-bag containing a Ser/Thr kinase CTR1, a transmembrane protein EIN2, and some transcription factors (Figure 3) [44,90]. Why, then, have some of the ethylene receptors retained histidine kinase activity? Evidence to date suggests that histidine kinase activity plays a minor and modulating role in output from the receptors [91,92]. It may allow for cross-talk with the multi-step phosphorelay, potentially influencing cytokinin signaling, and/or affect interactions of the receptors with members of the ethylene receptor signaling complex (for example, EIN2) [93].

In spite of the fact that their primary means of regulating downstream signal transduction no longer relies on histidine kinase activity, the ethylene receptors still retain biochemical features found in many histidine kinases. First, they function as obligate dimers, each receptor homo-dimer containing a single ethylene binding site [81]. Second, they appear to interact with each other to form higher order clusters, such clustering also having been observed in the histidine-kinase-linked chemoreceptors of bacteria, where it serves as a means to amplify the incoming signal [94,95].

Phytochrome Signaling in Plants: Histidine Kinases Gone AWOL

Phytochromes are red/far-red light receptors related to histidine kinases and are found in bacteria, fungi, and plants,

consistent with a prokaryotic origin for this photoreceptor [43]. But whereas the bacterial and fungal members of the family appear to function as typical histidine kinases, the phytochromes of higher plants are highly diverged and lack many of the residues required for histidine kinase activity. In fact, signal output no longer requires the histidine kinase domain because a truncated phytochrome lacking this domain is sufficient for signaling if targeted to the nucleus [96]. Thus, in plants, a critical aspect of light-mediated signal transduction is the re-localization of phytochromes from cytosol to nucleus, where the phytochromes are then thought to modulate transcription, inducing degradation of some transcription factors and potentially activating others [43].

What then is the role of the histidine kinase-like domain? Some plant phytochromes now possess serine/threonine kinase activity associated with the diverged domain, and this kinase activity is light-regulated [97]. The plant phytochromes are obligate dimers, unlike the reversible dimers formed by bacterial phytochromes and, in response to red-light stimulation, the phytochromes trans-phosphorylate each other as well as phosphorylate additional substrates [43]. Phytochrome auto-phosphorylation regulates the protein stability and protein–protein interactions of phyA, the unphosphorylated form having increased protein stability and interacting more strongly with downstream signaling partners [98–101]. In addition, phosphorylation is hypothesized to play a role in the phytochrome re-localization from cytosol to nucleus, potentially causing a cytosolic anchoring protein to dissociate and/or resulting in the exposure of a nuclear localization signal [43].

The histidine-kinase domain of phytochromes may also serve an additional role, allowing for cross-talk with the two-component signaling system of plants. Type-A response regulators modulate seedling sensitivity to red light, and one of these response regulators (ARR4) has been found to interact with and modulate activity of phyB [78,102]. Thus, although a histidine–aspartate phospho-transfer may not occur, the physical interactions of a two-component system may still be perpetuated and made use of in phytochrome signaling.

The Circadian Clock of Plants: Pseudo-Response Regulators as Gears

Pseudo-response regulators in plants contain complete receiver domains but are missing essential residues required for activity [49]. In particular, the aspartate that serves as a site for phosphorylation is missing, in many cases being replaced by a glutamate residue that may mimic the phosphorylated form. Plants contain a family of pseudo-response regulators that contain a distinctive and plant-specific CCT-motif in their carboxy-terminal extensions. These pseudo-response regulators are involved in the regulation of circadian rhythms, functioning as components in the regulatory feedback loops of the clock [68,69]. Four of the *A. thaliana* clock pseudo-response regulators are recruited to promoters, although they are not known to bind DNA directly, with one family member (PRR1/TOC1) serving as a positive regulator of gene expression and other family members (PRR5, PRR7, and PRR9) serving as negative regulators. Phylogenetic analysis indicates that the basic clock system involving pseudo-response regulators is conserved throughout angiosperms and predates the divergence of monocots and dicots [103].

Lacking the conserved aspartate, the pseudo-response regulators can no longer function as phospho-recipients in a two-component pathway. Instead, the replacement of aspartate with glutamate suggests that they are already in an 'active' conformation. How then are they regulated? Perhaps not surprisingly, their regulation appears to involve an interplay between serine/threonine phosphorylation and protein stability, hallmarks of eukaryotic regulatory mechanisms. Pseudo-response regulator protein stability is post-translationally regulated and, moreover, is differentially phosphorylated during the circadian cycle [104–108]. Phosphorylation of several pseudo-response regulators enhances their interaction with an F-box protein, providing a direct link between phosphorylation status and turnover [108]. In addition, phosphoryation can promote interactions among the pseudo-response regulator family members themselves [108].

Conclusions

The incorporation of two-component systems into the signal transduction pathways of plants, fungi, diatoms, and slime molds represents a significant difference in the signaling systems between these organisms and the metazoans. It is likely that the two-component system originally arose in bacteria, and was later appropriated by eukaryotes from bacterial endosymbionts or other forms of horizontal gene transfer. Although the basic mechanism of the histidine–aspartate phosphorelay has been preserved for some eukaryotic signaling pathways, it has also been specialized in multiple ways to the needs of eukaryotes.

First, perhaps because of the larger size of the eukaryotic cell, a multi-step phosphorelay rather than a simple two-component system appears the predominant form in eukaryotes. With a multi-step phosphorelay, the phosphate is carried as the more stable phospho-histidine by the phosphotransfer protein from plasma membrane to nucleus for transcriptional regulation. Movement of the phosphotransfer protein to the nucleus is, however, not driven by a change in phosphorylation status. Rather, it appears that the phosphotransfer proteins are in constant flux between cytosol and nucleus, whether phosphorylated or not, and this may allow for interactions with targets at multiple locations [109,110]. Second, some domains in eukaryotic two component systems are not found in bacteria, indicating eukaryotic-specific adaptations. Third, even among signaling elements containing the same domain organization, varying degrees of lineage-specific expansion can be seen among eukaryotic two component system genes. This is particularly apparent among the response regulators of rice and *A. thaliana* and is a potential means by which different signal outputs and multiple entry points of regulation can be obtained from the two-component signaling system in angiosperms. Finally, in many cases eukaryotic two-component signaling elements have lost their ability to participate directly in a phosphorelay, even when their prokaryotic ancestors appear to function in canonical histidine–aspartate phosphorelays. These diverged elements thus represent another source of innovations involving the two-component system.

The divergence of the eukaryotic two-component signaling elements is of particular interest. In some cases these diverged elements may perform a regulatory role, potentially to inhibit signaling through the pathway (for example, inhibition of cytokinin signaling by the pseudo-phosphotransfer protein APHP1/AHP6), but in other

cases — for example, phytochromes — the proteins may function by a histidine/aspartate-independent mechanism. Throughout the history of land plant evolution we seem to be observing multiple instances of evolutionary adaptation involving two-component signaling elements. In particular, it appears that these adaptations involved an increasing prevalence of diverging elements, so that they lack histidine–aspartate kinase activities, acquire serine/threonine kinase activity, and/or function in concert with serine/threonine signaling systems. But even when the elements have lost their histidine–aspartate enzymatic activity, they may still employ structural features found in two-component signaling elements as a means to facilitate signaling — for example, dimeric receptor structure and clustering of ethylene receptors, or interaction between phytochromes and type-A response regulators. Based on these examples, the evolutionary trajectory of the plant two-component elements frequently seems to be in a move away from the original enzymatic activity and the incorporation of these same elements within the serine/threonine-phosphorylation framework prevalent in eukaryotic systems.

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