Engineering key components in a synthetic eukaryotic signal transduction pathway

Mauricio S Antunes1, Kevin J Morey1, Neera Tewari-Singh1,3, Tessa A Bowen1, J Jeff Smith2,4, Colleen T Webb1, Homme W Hellinga2 and June I Medford1,*

1 Department of Biology, Colorado State University, Fort Collins, CO, USA and 2 Department of Biochemistry, Duke University Medical Center, Durham, NC, USA
3 Present address: Department of Pharmaceutical Sciences, University of Colorado Denver, Aurora, CO 80045, USA
4 Present address: Precision BioSciences, 104 TW Alexander Drive, Building 7, PO Box 12292, Research Triangle Park, NC 27709, USA

* Corresponding author. Department of Biology, Colorado State University, 1878 Campus Delivery, Fort Collins, CO 80523-1878, USA.
Tel.: + 1 970 491 78 65; Fax: + 1 970 491 06 49; E-mail: June.Medford@colostate.edu

Received 7.11.08; accepted 16.4.09

Signal transduction underlies how living organisms detect and respond to stimuli. A goal of synthetic biology is to rewire natural signal transduction systems. Bacteria, yeast, and plants sense environmental aspects through conserved histidine kinase (HK) signal transduction systems. HK protein components are typically comprised of multiple, relatively modular, and conserved domains. Phosphate transfer between these components may exhibit considerable cross talk between the otherwise apparently linear pathways, thereby establishing networks that integrate multiple signals. We show that sequence conservation and cross talk can extend across kingdoms and can be exploited to produce a synthetic plant signal transduction system. In response to HK cross talk, heterologously expressed bacterial response regulators, PhoB and OmpR, translocate to the nucleus on HK activation. Using this discovery, combined with modification of PhoB (PhoB-VP64), we produced a key component of a eukaryotic synthetic signal transduction pathway. In response to exogenous cytokinin, PhoB-VP64 translocates to the nucleus, binds a synthetic PlantPho promoter, and activates gene expression. These results show that conserved-signaling components can be used across kingdoms and adapted to produce synthetic eukaryotic signal transduction pathways.

Molecular Systems Biology 5; Article number 270; published online 19 May 2009; doi:10.1038/msb.2009.28
Subject Categories: synthetic biology; plant biology
Keywords: PhoB; response regulator; signal transduction; synthetic biology

This is an open-access article distributed under the terms of the Creative Commons Attribution Licence, which permits distribution and reproduction in any medium, provided the original author and source are credited. Creation of derivative works is permitted but the resulting work may be distributed only under the same or similar licence to this one. This licence does not permit commercial exploitation without specific permission.

Introduction

Living organisms sense and respond to their environments using an array of signal transduction systems. Better understanding of natural signaling, as well as ‘rewiring’ systems to produce new biological functions and potential biotechnological applications, are goals of synthetic biology. Bacteria, fungi, and plants use histidine kinase (HK) or two-component systems to sense environmental factors, such as the presence of ligands, osmotic and oxidative conditions, or pathogenic factors (Stock et al, 2000; Mizuno, 2005; Nemecek et al, 2006). HK-based signal transduction systems exhibit relatively modular architecture built from a limited number of protein domains, with individual domains often conserved across pathways and species (Koretke et al, 2000; Stock et al, 2000; Ferreira and Kieber, 2005; Mizuno, 2005; Zhang and Shi, 2005). Information transfer in signal transduction systems may not be linear; components can exhibit cross talk to establish networks that integrate multiple signals (Hass et al, 2004; Laub and Goulian, 2007). Modular components and the cross talk between them are postulated to be crucial in the evolution of complex signal transduction pathways (Aharoni et al, 2005; Bhattacharyya et al, 2006). For example, new connectivities are thought to have evolved through the (re)arrangement of components in various combinations and compositions (Aharoni et al, 2005; Bhattacharyya et al, 2006).

In bacteria, fungi, and plants, extracellular stimuli bring about a conformational change in HK dimers located in an ‘input layer’ (Figure 1). This conformational change results in autophosphorylation of a His residue in the HK cytoplasmic domain. The resulting high-energy phosphate group serves as a signal, and is transferred successively between His and Asp residues among various protein components of a pathway. In bacteria, simple systems are found, in which two proteins...
are sufficient to sense stimuli and initiate transcriptional responses. In this arrangement, a transmembrane HK phosphorylates an intracellular response regulator (RR) protein that initiates gene transcription (Figure 1).

More complex, hybrid systems, which involve additional components, are also found in bacteria and in plants. In hybrid systems, the high-energy phosphate can cascade through three or more proteins in a ‘transmission layer’ before reaching the ‘response layer’ (Figure 1). For example, in plants, cytokinin sensing involves transmembrane HKs that first transfer a high-energy phosphate group intramolecularly from the autophosphorylated His to an Asp residue (Figure 1) (Kakimoto, 2003; Ferreira and Kieber, 2005). Subsequently, the phosphate group is transmitted to a His residue on a separate protein, histidine phosphotransferase (Hpt, or in Arabidopsis, AHPs). Phospho-AHPs either directly translocate to the nucleus or signal to cytoplasmically localized cytokinin response factors that also translocate to the nucleus. In the nucleus, both pathways result in transcriptional activation (Rashotte et al. 2006).

The added components and complexity used in hybrid systems, such as plant cytokinin perception, are hypothesized to enable greater ability to regulate input from stimuli, compared with the simpler systems (Appleby et al., 1996). However, the added complexity significantly complicates rational design of synthetic signal transduction pathways. Designing a synthetic signal transduction pathway in a complex eukaryotic system presents two additional challenges. First, the various signal transduction components are encoded by multigene families that are typically differentially regulated (Mason et al., 2005; Hutchison et al., 2006). Second, signals from environmental stimuli must be transferred not only to a cell’s interior, but also from the cytoplasm to the nucleus, providing means for sub-cellular regulation.

HK-based-signaling components are highly modular and conserved across different kingdoms (Koretke et al., 2000; Stock et al., 2000; Santos and Shiozaki, 2001; Ferreira and Kieber, 2005; Mizuno, 2005; Zhang and Shi, 2005). This high degree of sequence conservation has allowed functional assays to be developed for plant HKs and AHPs in bacteria and yeast (Inoue et al., 2001; Yamada et al., 2001; Reiser et al., 2003). Conservation and modularity can be further seen in an alignment of the receiver domain from the bacterial RRs, PhoB and OmpR, with the receiver domains of multiple plant HK components (Supplementary Figure S1). These plant components function in different parts of the HK response, for example membrane-localized HKs, and cytoplasmic and nuclear-localized Arabidopsis RRs. This suggests that these bacterial components might be able to interact with plant HK components. We tested this hypothesis by heterologous expression of PhoB and OmpR in Arabidopsis and found that these proteins are sensitive to phosphate signals from endogenous cytokinin-mediated HK-signaling components. We further found that these bacterial proteins translocate to the plant nucleus in response to this cytokinin signal. In Escherichia coli, phosphorylation of PhoB results in a conformational change in the protein that uncovers a DNA-binding domain, which has high affinity for a specific DNA sequence, the Pho box. Binding of phospho-PhoB to Pho boxes results in gene transcription (Blanco et al., 2002; Bachhawat et al., 2005). We exploited the cytokinin-dependent nuclear translocation and phospho-dependent DNA binding of PhoB, and added a eukaryotic transcriptional activation domain to produce a signal-dependent eukaryotic transcriptional response system. In response to an activated HK, PhoB-VP64 translocates to the nucleus, binds a synthetic PhoB-responsive plant promoter, and activates transcription of the
β-glucuronidase (GUS) reporter gene. These results show that conserved-signaling components can be used across kingdoms and adapted to provide key components of synthetic signal transduction pathways in eukaryotes.

Results

Nuclear translocation of bacterial RRs

The requirement for nuclear translocation of a phosphorylated carrier protein is a key difference between bacterial and plant HK-based signal transduction systems (Figure 1). As the first step in building a synthetic signal transduction pathway with a reduced number of components, such as those found in bacteria, we examined the cellular partition of bacterial transmission layer components in response to activation of a plant HK-signaling pathway. In plants, cytokinin binds to and activates transmembrane HKs, initiating an intracellular phospho-relay, in which transmission layer proteins (Hpts) translocate to the nucleus (Hutchison et al., 2006). To assess whether heterologously expressed RRs and Hpts respond to cytokinin in a similar way, we constructed C-terminal GFP fusions of the bacterial RRs: OmpR (Mizuno et al., 1982; Wurtzel et al., 1982), PhoB (Makino et al., 1986, 1989), RcsB (Chen et al., 2001), the putative Hpt YojN (Chen et al., 2001), and the yeast Hpt Ypd1 (Posas et al., 1996). In transient assays, we found that PhoB and OmpR appeared to show signal-dependent nuclear translocation in plant cells, whereas the responses of RcsB, YojN, and Ypd1 were equivocal (data not shown). We, therefore, focused our subsequent work on PhoB and OmpR.

Transgenic Arabidopsis plants were generated that constitutively expressed either PhoB-GFP or OmpR-GFP. Figure 2 shows epi-fluorescence images of PhoB-GFP in transgenic plants in the presence or absence of exogenously added cytokinin (for OmpR-GFP see Supplementary Figure S2). Control plants containing GFP alone exhibited a diffuse fluorescence pattern and showed no change in sub-cellular localization in response to cytokinin (data not shown). Before cytokinin addition, plants containing either PhoB-GFP or OmpR-GFP have fluorescence that is diffused and uniform in all tissues and within the cell’s cytoplasm and nucleus (Figure 2A and D; Supplementary Figure S2). After treatment with cytokinin, GFP fluorescence from the PhoB-GFP fusion is found in discrete punctate compartments (Figure 2B, C and E; for OmpR-GFP see Supplementary Figure S2). This pattern of cytokinin-dependent PhoB-GFP localization was observed in all cells, tissues, and developmental stages examined (Figures 2 and 3). To determine whether these punctate compartments correspond to nuclei, tissues were stained with the DNA dye DAPI (4’,6-diamidino-2-phenylindole) (Figures 2F–H, 3D and H). Compartmentalized GFP fluorescence co-localizes with the DAPI stain, indicating that PhoB-GFP translocates to the plant nucleus or accumulates at the nuclear membrane. OmpR-GFP had a similar, albeit weaker, response (Supplementary Figure S2). Cytokinin-dependent nuclear translocation of PhoB-GFP is observed with as little as 0.01 μM t-zeatin, although a more consistent and widespread nuclear localization is seen with 1 and 10 μM t-zeatin (Supplementary Table 1). We also investigated the time course for nuclear translocation of PhoB-GFP in root cells in response to HK activation with exogenous cytokinin. (A, B) Cellular localization of PhoB-GFP in roots of transgenic Arabidopsis plants. (A) Before cytokinin treatment, PhoB-GFP fluorescence appears diffused and throughout the cells. (B) After cytokinin treatment, the same root shows PhoB-GFP accumulation in sub-cellular compartments. (C–H) Detail views of roots (D, G) before and (C, E, F, H) after treatment with cytokinin showing that before cytokinin is applied, GFP fluorescence is diffused; after cytokinin exposure, the compartments in which PhoB-GFP accumulates (C, E) also stain with DAPI (F, H), indicating that they are nuclei (arrowheads). –CK, tissue before cytokinin treatment; +CK, tissue after cytokinin treatment; DAPI, tissues treated with DAPI to stain DNA. Scale bars, 50 μm in (A–C, F); scale bars, 10 μm in (D–E, G–H).
response to 1 μM t-zeatin. Some hint of nuclear translocation of the fusion protein is seen at our first time point, 30 min. The PhoB-GFP punctate pattern becomes more apparent after 1 and 2 h of incubation with cytokinin, whereas after 3 h, PhoB-GFP is mostly localized to nuclei (Supplementary Figure S3).

Confocal study
To determine whether the bacterial RRs actually move into the nucleus or accumulate at the nuclear membrane, we examined the fluorescence patterns in more detail using a confocal microscope. Without exogenous cytokinin treatment, fluorescence from PhoB-GFP is observed throughout all sub-cellular regions in the root cells. Densely cytoplasmic vascular cells show more intense fluorescence and some vaguely defined nuclei before the controlled HK activation (Figure 4A). After activation of endogenous HKs with cytokinin, Figure 4B–D shows that PhoB-GFP accumulated in the nucleus (for OmpR-GFP see Supplementary Figure S2). Nuclear accumulation was observed in all cells (e.g., both vascular and non-vascular) (Figure 4C), and sub-micron optical sections of the nucleus show uniform distribution of GFP fluorescence throughout (Figure 4D). These results indicate that the bacterial RRs enter the nucleus. Quantification of the cytokinin-stimulated changes in PhoB-GFP cellular localization (Supplementary Table 2) showed approximately four-fold greater accumulation of PhoB-GFP in nuclei of the root cortical cells after cytokinin treatment. Root vascular cells exhibited some nuclear localization before the exogenous cytokinin treatment (Figure 4A), consistent with the fact that those cells have higher levels of endogenous cytokinin than the adjacent cortical cells (Aloni et al., 2005, 2006). Nevertheless, nuclei of vascular cells also showed a quantitative (two-fold) increase in GFP fluorescence after cytokinin treatment (Supplementary Table 2). In contrast to PhoB-GFP, cytokinin-induced OmpR-GFP nuclear localization was weaker. Cytokinin-treated nuclei of cortical cells expressing OmpR-GFP had 1.3-fold greater GFP fluorescence, with a similar increase observed in vascular cells.

Diffusion cannot readily account for nuclear translocation
As both bacterial RRs are small (27 kDa), their signal-dependent nuclear translocation could result from diffusion combined with an enhanced affinity for DNA. We tested the contribution of each to the signal-dependent translocation. Both PhoB and OmpR bind specific bacterial DNA sequences in their phosphorylated form (Okamura et al., 2000; Blanco et al., 2002); no sequences with significant homology to PhoB- or OmpR-binding sites were identified in the Arabidopsis genome (data not shown). To test whether the signal-dependent movement involves diffusion, we constructed larger fusion proteins by adding the GUS (Jefferson et al., 1987)-coding region to the C-terminal end of the individual bacterial RR–GFP fusion proteins. The resulting proteins, PhoB-GFP-GUS and OmpR-GFP-GUS, have predicted molecular masses of 122 and 123 kDa, respectively. Transgenic plants that contained PhoB-GFP-GUS or OmpR-GFP-GUS show strong expression of the GUS reporter, confirming that the fusions produce functional protein (data not shown). We then examined the cellular localization of GFP fluorescence from PhoB-GFP-GUS in roots before and after cytokinin treatment to determine whether the bacterial RR’s nuclear translocation occurs by diffusion or by an active process (Figure 4E–H; for OmpR-GFP see Supplementary Figure S2). PhoB-GFP-GUS fusion proteins accumulate in punctate compartments after cytokinin treatment (Figure 4F and G), although to a lesser extent than the accumulation observed for PhoB-GFP (compare Figures 2B with 4F). DAPI staining confirmed that the compartments are nuclei (Figure 4H). We have also observed nuclear translocation of PhoB-VP64-GFP (molecular weight 59 kDa) in plants using input from a synthetic HK and...
computationally re-designed receptors (Antunes et al., in preparation). Taken together, these data show that the bacterial RRs, PhoB and OmpR, translocate into plant nuclei in a signal-dependent manner and that the movement is unlikely to result from diffusion.

The canonical bacterial phospho-accepting aspartate is required for efficient nuclear translocation

In bacteria, the high-energy phosphate signal is transmitted from a phosphorylated His on the HK to a conserved Asp residue on the RR (Walthers et al., 2003). We tested whether this conserved Asp in the bacterial RRs is required for signal-dependent nuclear translocation in planta by constructing alanine mutations of Asp53 in PhoB and Asp55 in OmpR. PhoBD53A-GFP and OmpRD55A-GFP were separately introduced into Arabidopsis plants. Figure 5 shows the response of PhoBD53A-GFP in roots of transgenic plants. Before exogenous cytokinin treatment, PhoBD53A-GFP is, in general, diffused throughout the root cells, with some GFP fluorescence seen in some nuclei (Figure 5A). After treatment with exogenous cytokinins, PhoBD53A-GFP generally did not exhibit a uniform pattern of nuclear localization (Figure 5B) that is typical for plants containing PhoB-GFP (for OmpRD55A-GFP see Supplementary Figure S2). We examined numerous roots from at least 10 independent transgenic lines and found that in the presence of an exogenous cytokinin signal, PhoBD53A-GFP shows highly variable nuclear translocation that appears sporadic in non-vascular cells (Figure 5C–F), not at all in leaves and mature roots, and variable in the plant crown. In root vascular tissues, PhoBD53A-GFP is in nuclei to some extent before cytokinin treatment and appears to increase after...
treatment with cytokinin. This pattern mirrors the nuclear accumulation observed for intact PhoB-GFP (see Discussion). For OmpR-GFP, signal-dependent nuclear translocation of the OmpRD55A-GFP mutant was not observed in non-vascular cells and tissues (Supplementary Figure S2). In vascular tissues, some nuclear localization was seen, but the reduced fluorescence made signal-dependent responses difficult to discern (Supplementary Figure S2). These results indicate that the phospho-accepting Asp used in bacteria is required for strong, efficient nuclear localization of PhoB and OmpR in non-vascular plant cells and tissues.

Building key components of a synthetic eukaryotic signal transduction system

Eukaryotic adaptation of PhoB

In bacterial cells, phosphorylation of PhoB causes a protein conformational change that results in removal of the N-terminal receiver domain repression over the C-terminal effector domain (Okamura et al, 2000; Bachhawat et al, 2005). The 99 amino-acid effector domain binds to a 22-bp Pho box, organized into two 11-bp repeats (Blanco et al, 2002), and functions as a transcriptional activator. If these phosphorylation-dependent conformational changes and DNA-binding properties are conserved, PhoB could serve as a starting point to build a synthetic plant signal transduction network using conserved, heterologous components. The Pho effector domain activates transcription by recruiting the RNA Polymerase 70 factor in bacteria (Okamura et al, 2000). This transcriptional activation mechanism is unlikely to work in eukaryotes. We, therefore, engineered PhoB to function as a eukaryotic transcriptional activator by retaining the DNA-binding domain and fusing four copies of the eukaryotic transcription activator VP16 (Triezenberg et al, 1988) to the C-terminus of PhoB. Plant expression of the PhoB-VP64 fusion protein was directed by the strong, constitutive FMV promoter (Sanger et al, 1990).

Design of a synthetic PhoB-responsive promoter

PhoB-regulated genes in bacteria have multiple Pho boxes in their promoter regions (Blanco et al, 2002). Eukaryotic promoters typically also have multiple-binding sites for transcription factors. Hence, we designed a synthetic PlantPho promoter (Figure 6A) using four copies of the Pho box (Blanco et al, 2002) upstream of a minimal plant promoter (−46 CaMV35S). BLAST searches of the Pho box, as well as the synthetic PlantPho promoter sequence, against the Arabidopsis genome (The Arabidopsis Information Resource, www.tair.org) showed no homologous genomic sequences and, therefore, the PlantPho promoter is unlikely to be recognized by endogenous plant transcription factors.

We tested whether activation of the PlantPho promoter requires PhoB-VP64, the signal transmission/transcriptional activation protein, by producing transgenic lines containing only PlantPho::GUS. GUS activity measured before and after treatments with exogenous cytokinin to activate endogenous HK components showed no significant differences in GUS activity with or without exogenous cytokinin (t-test, n=36, t=2.73, P=0.92) (Supplementary Figure S4). Therefore, the PlantPho promoter does not respond to cytokinin in the absence of PhoB-VP64.

Function of the synthetic PhoB-VP64 → PlantPho system in plants

Homozygous transgenic Arabidopsis lines containing both elements of the synthetic signal transduction system (PhoB-VP64 and PlantPho promoter) were tested for response to HK
activation with exogenous cytokinin (t-zeatin). Cytokinin-independent GUS induction was observed in transgenic plants with the synthetic components (Figure 6B). Moreover, the response is dose dependent with more cytokinin producing increased GUS activity. The response did, however, show significant variability. To confirm that the observed induction correlates with the cytokinin signal, we statistically analyzed our data with linear regression. A highly significant relationship was observed between cytokinin dose and GUS activity ($n=119$, $F=37.99$, $P=1.02 \times 10^{-8}$, $R^2=0.24$) (Figure 6C). In addition, other cytokinins that activate the HK signal pathway, such as kinetin and BAP (Yamada et al, 2001; Spichal et al, 2004), also activate the PlantPho promoter, producing GUS induction levels similar to those obtained with t-zeatin (Supplementary Figure S5).

To determine whether transcriptional activation depends on phospho-relay through PhoB, transgenic Arabidopsis plants were constructed containing PlantPho::GUS and PhoB-VP64, in which the phospho-accepting Asp53 was mutated to alanine. Eight independent transgenic lines were analyzed for cytokinin-dependent activation of the PlantPho promoter (Supplementary Figure S6). Five of the eight lines showed no difference in GUS activity with or without exposure to exogenous cytokinin. Three independent transgenic lines showed variable patterns of induction and/or repression in progeny from the individual lines. Statistical analyses of the eight transgenic lines indicate that the PhoBD53A mutation largely prevents cytokinin-induced GUS activity (Supplementary Table 3).

Discussion

Synthetic signal transduction systems will allow us to better understand the behavior of endogenous systems and produce new types of biological sensing and responses. Earlier work toward this end used modular components from endogenous signal transduction systems to change the input–output connectivity in yeast cells (Zarrinpar et al, 2003; Dueber et al, 2004), and rational changes in protein specificity were used to rewire a bacterial two-component signal transduction system (Skerker et al, 2008). In higher organisms, the complexity of signal transduction processes presents a considerable challenge to design synthetic systems. The signal transduction process can be viewed as three connected functional layers: input → transmission → response (Figure 1). However, eukaryotic signal transduction systems are not linear; each layer has multiple proteins that are themselves often composed of multiple functional domains and typically encoded by multigene families.

As these complex signal transduction systems are thought to have arisen from new combinations of protein domains (Bhattacharyya et al, 2006), we tested whether conserved modular domains from highly evolved bacterial systems could retain functionality in a eukaryotic system. The requirement for nuclear translocation of a phosphorylated carrier protein is a key difference between bacteria and plant HK signal transduction systems. We discovered that PhoB-GFP and OmpR-GFP can translocate to the plant cell nucleus in response to a cytokinin-induced HK signal. We used this discovery, detailed knowledge about phospho-PhoB’s affinity for DNA, and known DNA-binding sites to re-design the bacterial RR for eukaryotic function. A eukaryotic transcriptional activator was added to the C-terminal end of PhoB and a signal-receptive transcriptional promoter designed for plant function. The synthetic PhoB-VP64 → PlantPho::GUS system responded to cytokinin-mediated HK activation and expressed the GUS reporter.

The signal-dependent nuclear translocation of bacterial RR seems remarkable because bacteria do not have a nuclear compartment. To our knowledge, this is the first example in plants of proteins from non-pathogenic bacteria showing signal-dependent nuclear translocation. Although some Avr proteins from plant pathogenic bacteria localize to plant cell
nuclei, these proteins have been shown to contain nuclear localization signal (NLS) sequences (Kjemtrup et al., 2000). The effector domain of PhoB contains an arginine–lysine-rich region that may act as a cryptic NLS with phosphorylation-dependent ‘uncovering’ of the DNA-binding domain. However, mutations in this region did not alter the cellular partition of PhoB-GFP in the presence or absence of cytokinin (data not shown). Therefore, PhoB does not appear to have a canonical NLS sequence. Although a complete mechanistic interpretation for this signal-dependent nuclear translocation phenomenon awaits further experimentation, our work reveals aspects about the process. PhoB-GFP and OmpR-GFP fusions accumulate in the nuclei in a signal-dependent manner not consistent with diffusion. Although it may not be possible to establish an absolute size limit, small proteins <20–40 kDa are capable of nuclear diffusion, whereas larger proteins require transport through selectivity filters provided by phenylalanine-glycine (FG) repeats in proteins of the nuclear pore complex (Sun et al., 2008). Our bacterial RR-GFP fusions are ~55 kDa, suggesting that they cannot diffuse into the nucleus. In addition, after cytokinin treatment, we observed nuclear accumulation. As the Arabidopsis genome has no homology to PhoB’s DNA-binding sequence, the signal-dependent nuclear accumulation cannot be explained by diffusion combined with DNA affinity. Collectively, these data suggest that some type(s) of transport mechanism(s) is involved (Figure 4E–H; Supplementary Figure S2).

In non-vascular cells, the nuclear translocation largely required the signal-receptive Asp residue for both PhoB and OmpR (Figures 2 and 5; Supplementary Figure S2), implying that some aspect of the phospho-protein is required for efficient nuclear transport. One possibility is suggested from the conformation change that PhoB undergoes with phosphorylation in bacteria (Ellision and McCleary, 2000; Bachhawat et al., 2005). If this or a similar conformation change takes place in planta, the receiver domain of PhoB becomes more exposed. As PhoB’s receiver domain has homology to plant receiver domains, plant machinery could recognize and transport the phosphorylated PhoB to the nucleus. In response to exogenous cytokinins, cortical cells showed variable and sporadic nuclear localization of the mutant PhoBDSSA-GFP, and vascular cells accumulated PhoBDSSA-GFP to some extent (Figure 5C–F). These observations suggest that there could be various inefficient means by which PhoB is translocated to the nucleus, or that PhoB can be phosphorylated at other residues in plants.

In bacteria, PhoB is known to undergo a conformational change with phosphorylation that significantly increases affinity of this protein for its target DNA sequence, the Pho box (Blanco et al., 2002; Bachhawat et al., 2005). We engineered our eukaryotic PhoB-responsive promoter with four Pho boxes located upstream of a minimal transcriptional promoter (~46 CaMv35S) (Beneyt et al., 1989). We chose four Pho-binding sites based on other plant-inducible transcription systems that use prokaryotic DNA-binding proteins (Padidam, 2003; Moore et al., 2006). Experimentally determining the optimal number of Pho boxes in the PlantPho promoter may lead to an improved PlantPho system.

By combining PhoB-VP64 with the PlantPho promoter, we constructed a synthetic eukaryotic signal transduction system (PlantPho system). Activation of endogenous plant HKs with increasing concentrations of the cytokinin t-zeatin resulted in a near linear increase in GUS activity (Figure 6B and C). The PlantPho system showed high un-induced GUS levels with variability at each cytokinin level tested (Figure 6B and C). This may result from activation of the synthetic system by endogenous cytokinin along with accumulation of the highly stable GUS in the 2-week-old plants assayed. Also, because vascular tissues are highly sensitive to cytokinin (Moritz and Sundberg, 1996; Brugiere et al., 2003; Aloni et al., 2005; Hutchison et al., 2006; Kuroha et al., 2006), and entire plants were assayed, the vascular tissues could have high GUS levels even without induction. Consistent with this hypothesis, we observed that both PhoB-GFP and OmpR-GFP accumulated in the nuclei of vascular cells before exogenous cytokinin application (Figure 4; Supplementary Figure S2). As vascular cells already have some nuclear-localized PhoB before cytokinin application, a signal-dependent increase would be difficult to see in these cells. Our system depends on promiscuous cross talk (Supplementary Figure S7) and does not create a privileged signal transduction system, in which one input produces one specific response. As such, in addition to endogenous cytokinins, cross talk from other plant HK systems, such as ethylene (Grefen and Harter, 2004), could also contribute to the high background in GUS activity.

Here, we show that synthetic eukaryotic systems can be produced by using conserved components from prokaryotic systems, taking advantage of the cross talk from conserved bacterial HK systems. Remarkably, this heterologous cross talk is so highly conserved that plant two-component signal transduction components can function in bacteria (Suzuki et al., 2001; Spichal et al., 2004; Romanov et al., 2005) and bacterial components in plants (this study). It is tempting to speculate that cross talk coupled with horizontal gene transfer is a conserved mechanism by which new signal transduction systems evolve. In this model, nascent systems are initially promiscuous and later become more specialized, not unlike the theory of new enzyme function (Kraut et al., 2003). On one hand, the ability to establish new connectivities from bacteria in a higher eukaryote is remarkable. It will be interesting to determine whether such adaptation of other conserved signal transduction components and/or components from other highly evolved systems can function in other eukaryotic systems. The Pho system itself would likely function in yeast, which has conserved HK components, whereas mammalian cells may require a better understanding of the nuclear translocation process. On the other hand, it is also equally clear that the system is far from optimal. The possibility of experimentally controlling signal transduction systems provides a useful tool for plant and other biological studies, as it provides a means to control input and response. This approach, along with a simple readout system (Antunes et al., 2006), may also allow us to develop plant sentinels that can detect chemical threats and pollutants (Looger et al., 2003).

Materials and methods

DNA constructs

GFP fusion constructs including the mutated PhoBDSSA-GFP and OmpR155AS-GFP fusions were assembled in the binary vector pCB302-3
The PlantPho system (FMV::Pho-B-VP64 and PlantPho::GUS) was assembled in the pCAMBIA2300 binary vector. Oligonucleotide primers were synthesized by IDT (Coralville, IA). GFP fusions were initially made in a modified pGFP vector (TAIR CD3-326). The 5' end of smGFP was modified using primers (5'-TTCGCAACGAGCATATGCTAAGTGT-3' and 5'-ATTCTAGACTCTTATTGATAGTTCATC-3') to introduce an Ndel site (underlined). This site was used to make C-terminal smGFP fusions. All PCR reactions were performed using a High Fidelity polymerase (Roche Diagnostics, Indianapolis, IN). The resulting product was used to replace the original smGFP gene in pGFP. A lower primer removed the stop codon from PhoB and added a six amino-acid (2 × Gly-Gly-Ser) repeat linker. Primer set: upper, 5'-TAGAGGATCCATGGCGAGACGTATTC TTGTT-3' and lower, 5'-TTTACTCATATGAGATCTCCGAGATCCTCCAAAA GCGGTT-3'. The resulting PhoB product was fused to the modified smGFP. OmpR-GFP fusions were prepared using a similar cloning strategy as described above for PhoB-GFP. For plant transformation, the GFP fusions were cloned downstream of a CaMV35S promoter in the binary vector pCB302-3.

To assemble the synthetic signal transduction component, we made a translational fusion of PhoB-coding region to four copies of the transcriptional activator VP16, producing Pho-B-VP64. The Nos terminator sequence was added and the resulting Pho-B-VP64-nos fragment was sub-cloned into pCAMBIA2300 containing the FMV promoter. The synthetic PlantPho promoter (Figure 6A) was synthesized by BlueHeron Biotechnology (Bothell, WA) and fused to a GUS gene and Nos terminator in pBluescript. PlantPho::GUS::nos was then sub-cloned into p3500-FMV::Pho-B-VP64-4. A transcription block (Padidam and Cao, 2001) was placed between the two genes to prevent read through.

Site-directed mutagenesis

Asp residues at position 53 in PhoB and position 55 in OmpR were mutagenized to Ala using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Plant materials and transformation

Arabidopsis thaliana, ecotype Columbia (Col-0), grown under a 16-h light/8-h dark cycle, 25 ± 2 °C, photon density flux of ~100 µEm-2 s-1 was used for experiments. Plants were transformed with Agrobacterium GV3001 harboring the plasmids described above following standard procedures (Clough and Bent, 1998). The T0 seeds were sterilized and plated on MS media supplemented with 50 mg l-1 kanamycin sulfate (Sigma-Aldrich, St. Louis, MO) for selection of the pCAMBIA 2300 T-DNA, or 5 mg l-1 Glufosinate ammonium (BASTA) (Crescent Chemical Islandia, NY) for selection of the pCB302-3 T-DNA.

Fluorometric GUS assays

Fourteen-day-old plants or plant tissue containing the T-DNAs described above were incubated for 16 h in water (control), or water and t-zeatin. Total protein extraction and fluorometric measurements of GUS activity were performed on a DynaQuant 200 fluorometer (Hoefer Inc, San Francisco, CA), according to the methods described earlier (Gallagher, 1992). The 4-methylumbellifereone (4-MU) was used as a standard. GUS activity was normalized to the total protein content of samples and expressed as nmols 4-MU mg-1 protein h-1. Total protein content of samples was measured with the Bradford reagent (Bio-Rad Laboratories, Hercules, CA).

Statistical analyses

Statistical analyses were performed using JMP software, v. 6.0.3 (SAS Institute, Cary, NC). A t-test was used to analyze GUS activity resulting from induction of the PlantPho promoter alone. For the log-log regression, the dependent variable was the log (measured GUS activity (nmols 4-MU mg-1 protein h-1)), and the independent variable was log (t-zeatin concentrations +1) treated as a fixed effect. We used a log-log transformation to meet the assumption of normally distributed residuals and added one to the cytokinin concentrations to account for zero values. All the assumptions of parametric statistics were tested and met after transformation. For statistical analysis of the mutant PhoBD53A PlantPho system, because data were not normally distributed, non-parametric tests were used. Wilcoxon signed-rank tests were used to determine whether the difference between GUS activity in induced and non-induced tissues (paired data) were significantly different from zero. Bonferroni correction was used to account for potentially spurious significant results as a result of multiple tests of the T1-lines.

Observation of GFP expression

Nuclear translocation of the GFP-tagged proteins (and GFP control) was observed either under a Nikon Diaphot fluorescence microscope, or a Carl Zeiss LSM 510 META confocal microscope, as described by Morey et al., 2009. Tissues were also stained with 1 ng µl-1 DAPI (Sigma-Aldrich, St Louis, MO) for 10 min.

Supplementary information

Supplementary information is available at the Molecular Systems Biology website (www.nature.com/msb).

Acknowledgements

We thank Dr Eric Eisenstadt for support and insight, Dr ASN Reddy for the GFP clone, and Dr Michael Tamkun for help with confocal microscopy. We also gratefully acknowledge support of the US Defense Advanced Research Projects Agency and the US Office of Naval Research. MSA conducted experiments and wrote the manuscript, KJM conducted experiments and wrote the manuscript, NTS conducted experiments, TAB conducted experiments, JJS provided bacterial clones, conceived experiments, and wrote the paper, CTW designed statistical analyses and reviewed the data, HWH provided clones, conceived experiments, and wrote the manuscript, and JIM conceived the experiments and wrote the manuscript.

Conflict of interest

MSA, KJM, JJS, HWH and JIM are inventors on a pending patent using aspects of this system.

References

Aharoni A, Gaidukov L, Khersonsky O, McQ Gould S, Roodveldt C, Tawfik DS (2005) The ‘evolvability’ of promiscuous protein functions. Nat Genet 37: 73–76
Aloni R, Aloni E, Langhans M, Ullrich CI (2006) Role of cytokinin and auxin in shaping root architecture: regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. Ann Bot (Lond) 97: 883–893
Aloni R, Langhans M, Aloni E, Dreieicher E, Ullrich CI (2005) Root-synthesized cytokinin in Arabidopsis is distributed in the shoot by the transpiration stream. J Exp Bot 56: 1535–1544
Antunes MS, Ha S-B, Tewari-Singh N, Morey KJ, Troika AM, Kugrens P, Deyholos M, Medford II (2006) A synthetic de-greening circuit provides a reporting system that is remotely detectable and has a re-set capacity. Plant Biotechnol J 4: 605–622
Appleby JL, Parkinson JS, Bourret RB (1996) Signal transduction via the multi-step phosphoryl play: not necessarily a road less traveled. Cell 86: 845–848
Bachhawat P, Swapan GV, Montelione GT, Stock AM (2005) Mechanism of activation for transcription factor PhoB suggested
by different modes of dimerization in the inactive and active states. Structure 13: 1353–1363

Benfey PN, Ren L, Chua N-H (1989) The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. EMBO J 8: 2195–2202

Bhattacharyya RP, Remenyi A, Yeh BJ, Lim WA (2006) Domains, motifs, and scaffolds: the role of modular interactions in the evolution and wiring of cell signaling circuits. Annu Rev Biochem 75: 655–680

Blanco AG, Sola M, Gomis-Ruth FX, Coll M (2002) Tandem DNA recognition by PhoB, a two-component signal transduction transcriptional activator. Structure (Camb) 10: 701–713

Brugiere N, Jiao S, Hantke S, Reesler JA, Niu X, Jones RJ, Habben JE (2003) Cytokinin oxidase gene expression in maize is localized to the vasculature, and is induced by cytokinins, abscisic acid, and abiotic stress. Plant Physiol 132: 1228–1240

Chen MH, Takeda S, Yamada H, Ishii Y, Yamashino T, Mizuno T (2001) Characterization of the RcsC→Yn→RcsB phosphorelay signaling pathway involved in capsular synthesis in Escherichia coli. Biosci Biotechnol Biochem 65: 2364–2367

Chen YF, Etheridge N, Schaller GE (2005) Ethylene signaling. Ann Bot (Lond) 95: 901–915

Clough SJ, Bent AF (1998) Floral dip: a simplified method forAgrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735–743

Doebele RE, Yeh BJ, Bhattacharyya RP, Lim WA (2004) Rewiring cell signaling: the logic and plasticity of eukaryotic protein circuitry. Curr Opin Struct Biol 14: 690–699

Ellison DW, McLeary WR (2000) The unphosphorylated receiver domain of PhoB silences the activity of its output domain. J Bacteriol 182: 6592–6597

Ferreira FJ, Kieber JJ (2005) Cytokinin signaling. Curr Opin Plant Biol 8: 518–525

Gallagher SR (1992) GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression. San Diego: Academic Press

Grefen C, Harter K (2004) Plant two-component systems: principles, functions, complexity and cross talk. Planta 219: 733–742

Guo H, Ecker JR (2004) The ethylene signaling pathway: new insights. Curr Opin Plant Biol 7: 40–49

Hass C, Lohrmann J, Albrecht V, Sweere U, Hummel F, You SD, Hwang RJ, Kavanagh TA, Bevan MW (1987) GUS fusions: an approach to gene expression analysis in transgenic plants. Plant J 2: 637–645

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901–3907

Kakimoto T (2003) Perception and signal transduction of cytokinins. Annu Rev Plant Biol 54: 605–627

Kjemtrup S, Nimchuk Z, Dangl JL (2000) Effector proteins of phytopathogenic bacteria: bifunctional signals in virulence and host recognition. Curr Opin Microbiol 3: 73–78

Koretké KK, Lupas AN, Warren PV, Rosenberg M, Brown JR (2000) Evolution of two-component signal transduction. Mol Biol Evol 17: 1956–1970

Kraut DA, Carroll KS, Herschlag D (2003) Challenges in enzyme mechanism and energetics. Annu Rev Biochem 72: 517–571

Kuroha T, Ueguchi C, Sakakibara H, Satoh S (2006) Cytokinin receptors are required for normal development of auxin-transporting vascular tissues in the hypocotyl but not in adventitious roots. Plant Cell Physiol 47: 234–243

Laub MT, Goulian M (2007) Specificity in two-component signal transduction pathways. Annu Rev Genet 41: 121–145

Looger LL, Dwyer MA, Smith JJ, Hellenga HW (2003) Computational design of receptor and sensor proteins with novel functions. Nature 423: 185–190

Mahonen AP, Bishopp A, Higuchi M, Nieminen KM, Kinoshita K, Tormakangas K, Ikeda Y, Oka A, Kakimoto T, Helariutta Y (2006) Cytokinin signaling and its inhibitor AHP6 regulate cell fate during vascular development. Science 312: 94–98

Makino K, Shinagawa H, Amemura M, Kawamoto T, Yamada M, Nakata A (1989) Signal transduction in the phosphate regulon of Escherichia coli involves phosphotransfer between PhoR and PhoB proteins. J Mol Biol 210: 551–559

Makino K, Shinagawa H, Amemura M, Nakata A (1986) Nucleotide sequence of the phoB gene, the positive regulatory gene for the phosphate regulon of Escherichia coli K-12. J Mol Biol 190: 37–44

Mason MG, Mathews DE, Argyros DA, Maxwell BB, Kieber JJ, Alonso JM, Ecker JR, Schaller GE (2005) Multiple type-B response regulators mediate cytokinin signal transduction in Arabidopsis. Plant Cell 17: 3007–3018

Mizuno T (2005) Two-component phosphorelay signal transduction systems in plants: from hormone responses to circadian rhythms. Biosci Biotechnol Biochem 69: 2263–2276

Moore I, Samalova M, Kurup S (2006) Transactivated and chemically inducible gene expression in plants. Plant J 45: 651–683

Moye KJ, Tewari-Singh N, Smith JJ, Medford JJ (2009) Signal-dependent nuclear shuttling of Arabidopsis histidine phosphotransferases shows cell, tissue and developmental regulation (in preparation)

Moritz T, Sundberg B (1996) Endogenous cytokinins in the vascular cambial region of Pinus sylvestris during activity and dormancy. Physiologia Plantarum 98: 693–698

Nemecek JC, Wuthrich M, Klein BS (2006) Global control of dimorphism and virulence in fungi. Science 312: 583–588

Okamura H, Hanaoka S, Nagadoi A, Makino K, Nishimura Y (2000) Structural comparison of the PhoB and OmpR DNA-binding/ transactivation domains and the arrangement of PhoB molecules on the phosphate box. J Mol Biol 295: 1225–1236

Padidam M (2003) Chemically regulated gene expression in plants. Curr Opin Plant Biol 6: 169–177

Padidam M, Cao Y (2001) Elimination of transcriptional interference between tandem genes in plant cells. Biotechniques 31: 328–330, 332–334

Posas F, Wurgler-Murphy SM, Maeda T, Witten EA, Thai TC, Saito H (1996) Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 ‘two-component’ osmosensor. Cell 86: 865–875

Rashotte AM, Mason MG, Hutchison CE, Ferreira FJ, Schaller GE, Kieber JJ (2006) A subset of Arabidopsis AP2 transcription factors mediate cytokinin responses in concert with a two-component pathway. Proc Natl Acad Sci USA 103: 11081–11085

Reiser V, Raitt DC, Saito H (2003) Yeast osmosensor Sln1 and plant cytokinin receptor Cre1 respond to changes in turgor pressure. J Cell Biol 161: 1035–1040

Romanov GA, Spichal L, Lomin SN, Strnad M, Schmulling T (2005) A live cell horseradish-binding assay on transgenic bacteria expressing a eukaryotic receptor protein. Anal Biochem 347: 129–134

Sanger M, Daubert S, Goodman RM (1990) Characteristics of a strong promoter from figwort mosaic virus: comparison with the analogous 35S promoter from cauliflower mosaic virus and the regulated mannopine synthase promoter. Plant Mol Biol 14: 433–443

Santos JL, Shiozaki K (2001) Fungal histidine kinases. Sci STKE 2001: RE1
Skerker JM, Perchuk BS, Siryaporn A, Lubin EA, Ashenberg O, Goulian M, Laub MT (2008) Rewiring the specificity of two-component signal transduction systems. *Cell* 133: 1043–1054

Spichal L, Rakova NY, Riefler M, Mizuno T, Romanov GA, Strnad M, Schmulling T (2004) Two cytokinin receptors of *Arabidopsis thaliana*, CRE1/AHK4 and AHK3, differ in their ligand specificity in a bacterial assay. *Plant Cell Physiol* 45: 1299–1305

Stock AM, Robinson VL, Goudreau PN (2000) Two-component signal transduction. *Annu Rev Biochem* 69: 183–215

Sun C, Yang W, Tu LC, Musser SM (2008) Single-molecule measurements of importin alpha/cargo complex dissociation at the nuclear pore. *Proc Natl Acad Sci USA* 105: 8613–8618

Suzuki T, Miwa K, Ishikawa K, Yamada H, Aiba H, Mizuno T (2001) The Arabidopsis sensor His-kinase, AHK4, can respond to cytokinins. *Plant Cell Physiol* 42: 107–113

Triezenberg SJ, Kingsbury RC, McKnight SL (1988) Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. *Genes Dev* 2: 718–729

Urao T, Yakubov B, Satoh R, Yamaguchi-Shinozaki K, Seki M, Hirayama T, Shinozaki K (1999) A transmembrane hybrid-type histidine kinase in Arabidopsis functions as an osmosensor. *Plant Cell* 11: 1743–1754

Walthers D, Tran VK, Kenney LJ (2003) Interdomain linkers of homologous response regulators determine their mechanism of action. *J Bacteriol* 185: 317–324

Wolanin PM, Thomason PA, Stock JB (2002) Histidine protein kinases: key signal transducers outside the animal kingdom. *Genome Biol* 3: REVIEWS3013

Wurtzel ET, Chou MY, Inouye M (1982) Osmoregulation of gene expression. I. DNA sequence of the ompR gene of the ompB operon of *Escherichia coli* and characterization of its gene product. *J Biol Chem* 257: 13685–13691

Xiang C, Han P, Lutziger I, Wang K, Oliver DJ (1999) A mini binary vector series for plant transformation. *Plant Mol Biol* 40: 711–717

Yamada H, Suzuki T, Terada K, Takei K, Ishikawa K, Miwa K, Yamashino T, Mizuno T (2001) The Arabidopsis AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. *Plant Cell Physiol* 42: 1017–1023

Zarrinpar A, Park SH, Lim WA (2003) Optimization of specificity in a cellular protein interaction network by negative selection. *Nature* 426: 676–680

Zhang W, Shi L (2005) Distribution and evolution of multiple-step phosphorelay in prokaryotes: lateral domain recruitment involved in the formation of hybrid-type histidine kinases. *Microbiology* 151: 2159–2173

Molecular Systems Biology is an open-access journal published by European Molecular Biology Organization and Nature Publishing Group.

This article is licensed under a Creative Commons Attribution-Noncommercial-Share Alike 3.0 Licence.