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Engineering key components in a synthetic eukaryotic signal transduction pathway

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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.
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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

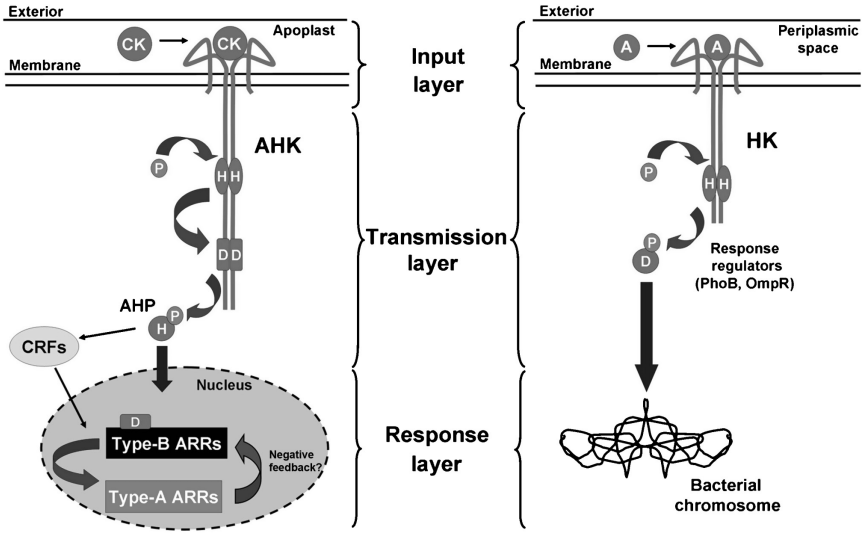

Figure 1 Comparison of HK signal transduction systems from plants and bacteria. Ligands bind to the extracellular domain of transmembrane HKs and activate a cytoplasmic kinase domain. A phospho-relay (His → Asp in bacteria or His → Asp → His → Asp in plants) transmits the signal to DNA. Both systems can be defined as perceiving an input stimulus (input layer), transmitting the signal (transmission layer), and bringing about a response (response layer), but use different numbers of components. In simple bacterial systems (right panel), two proteins (HK and RRs) function in three layers. In plants (left panel), cytokinin responses involve multiple components that are each encoded by multigene families in these three layers. AHK, arabidopsis histidine kinase; AHP, arabidopsis histidine phosphotransfer protein; CK, cytokinin; HK, histidine kinase; H, histidine residue; D, aspartate residue; P, phosphate group; CRF, cytokinin response factor; ARR, Arabidopsis response regulator; A, bacterial HK ligand.

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 intra-molecularly 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

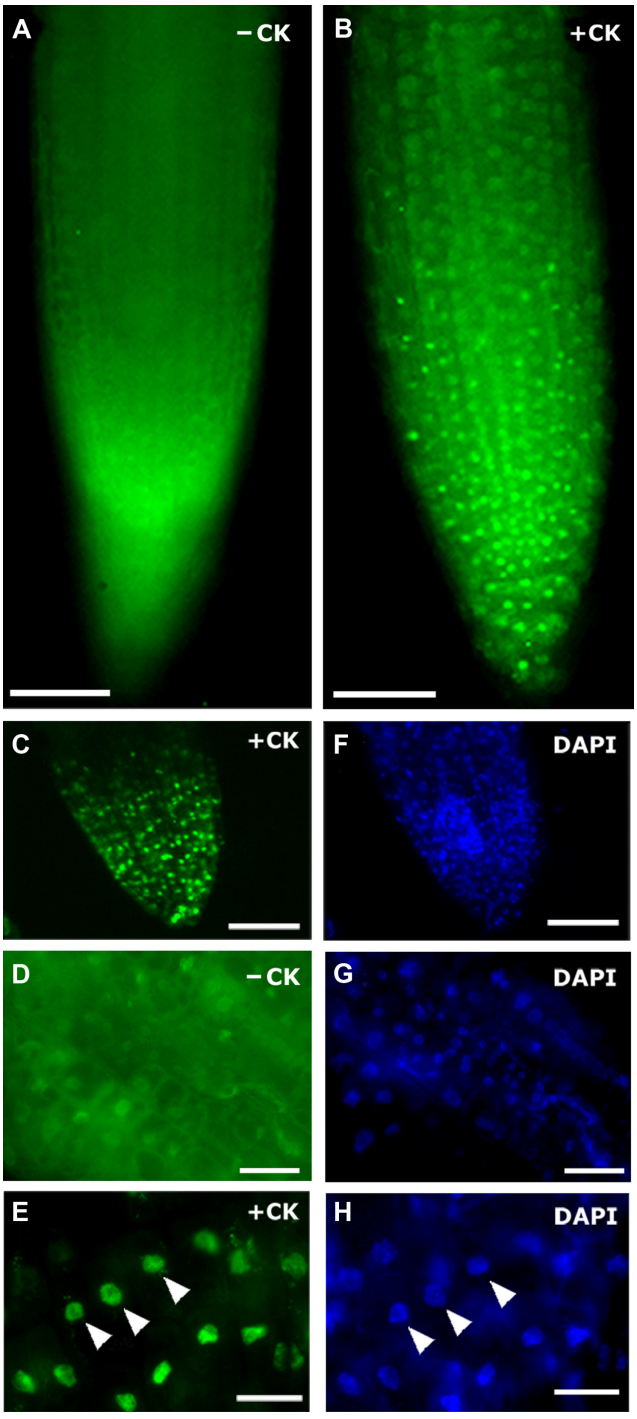

Figure 2 Bacterial RR PhoB translocates to plant nuclei in root cells in response to HK activation with exogenous cytokinin. (**A, B**) Cellular localization of PhoB-GFP in roots of transgenic Arabidopsis plants. (A) Before cytokinin treatment, PhoB-GFP fluorescence appears diffused and throughout the cells. (B) After exogenous 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).

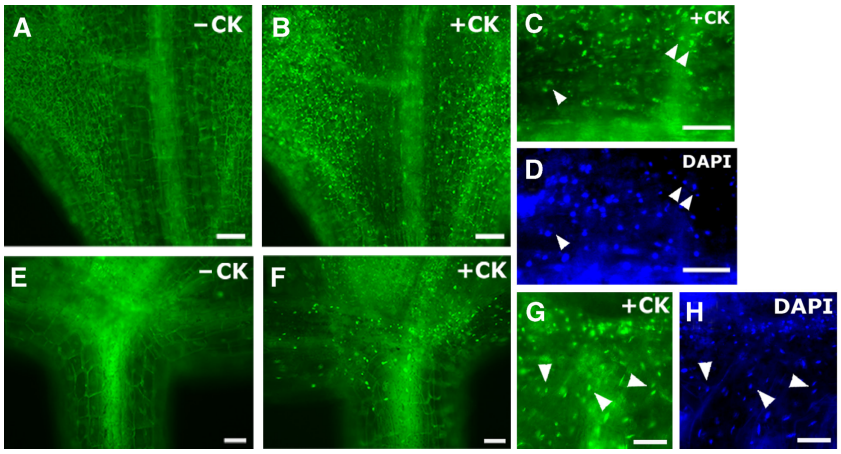

Figure 3 PhoB also translocates to plant nuclei in leaf and crown cells in response to HK activation with exogenous cytokinin. (**A–D**) Localization of PhoB-GFP in leaves. Leaf (A) before and (B) after exogenous cytokinin treatment. (C) Close-up view of leaf showing punctate PhoB-GFP. (D) DAPI staining of the same area showing that the punctate compartments are nuclei. (**E–H**) PhoB-GFP localization in the Arabidopsis crown, a stem-like region. Crown (E) before and (F) after cytokinin treatment. (G) Close-up view of (F) showing punctate GFP localization. (H) DAPI staining of area shown in (G), indicating that punctate GFP compartments are nuclei. Arrowheads point to nuclei. − CK, tissues before cytokinin treatment; + CK same tissue after cytokinin treatment; DAPI, tissues treated with DAPI to stain DNA. Scale bars, 50 μm in (A, B, E, F); scale bars, 10 μm in (C, D, 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

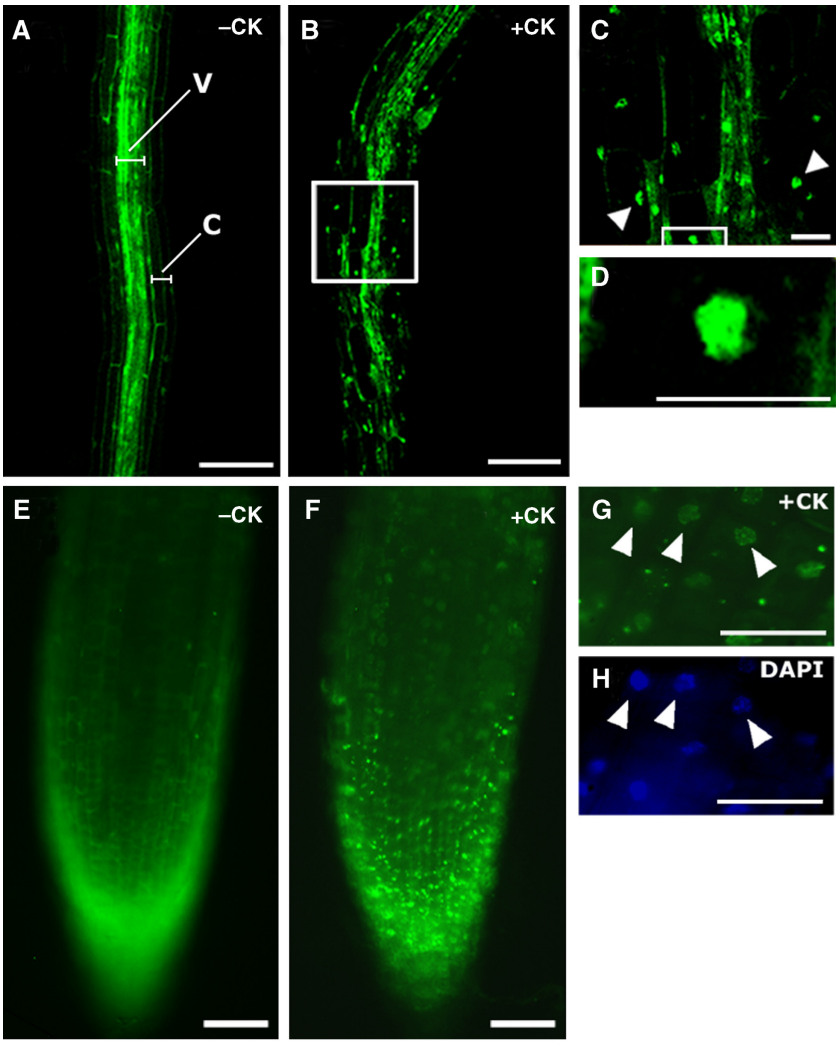

Figure 4 Analysis of signal-dependent nuclear translocation of PhoB. (**A**–**D**) Confocal microscope images of PhoB-GFP protein in roots (A) before and (B–D) after cytokinin treatment. (C) Detail view of the boxed area in (B) shows PhoB-GFP accumulation in nuclei. (D) Detail view of the area boxed in (C), showing a single nucleus with PhoB-GFP accumulation throughout. (**E**–**H**) Cellular localization of PhoB-GFP-GUS fusion protein in roots of transgenic Arabidopsis plants (E) before and (F–H) after cytokinin treatment showing compartmentalized accumulation. (G) Detail view of a root treated with cytokinin, showing compartments (arrowheads) that also stain with (H) DAPI, indicating that they are nuclei (arrowheads). − CK, plants before cytokinin treatment; + CK, same plant tissue after cytokinin treatment; DAPI, same tissues treated with DAPI to stain DNA. Scale bars, 50 μm in (A, B, E, F); scale bars, 10 μm in (C, D, G, H).

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. PhoB^{D53A}-GFP and OmpR^{D55A}-GFP were separately introduced into Arabidopsis plants. Figure 5 shows the response of PhoB^{D53A}-GFP in roots of transgenic plants. Before exogenous cytokinin treatment, PhoB^{D53A}-GFP is, in general, diffused throughout the root cells, with some GFP fluorescence seen in some nuclei (Figure 5A). After treatment with exogenous cytokinins, PhoB^{D53A}-GFP generally did not exhibit a uniform pattern of nuclear localization (Figure 5B) that is typical for plants containing PhoB-GFP (for OmpR^{D55A}-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, PhoB^{D53A}-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, PhoB^{D53A}-GFP is in nuclei to some extent before cytokinin treatment and appears to increase after

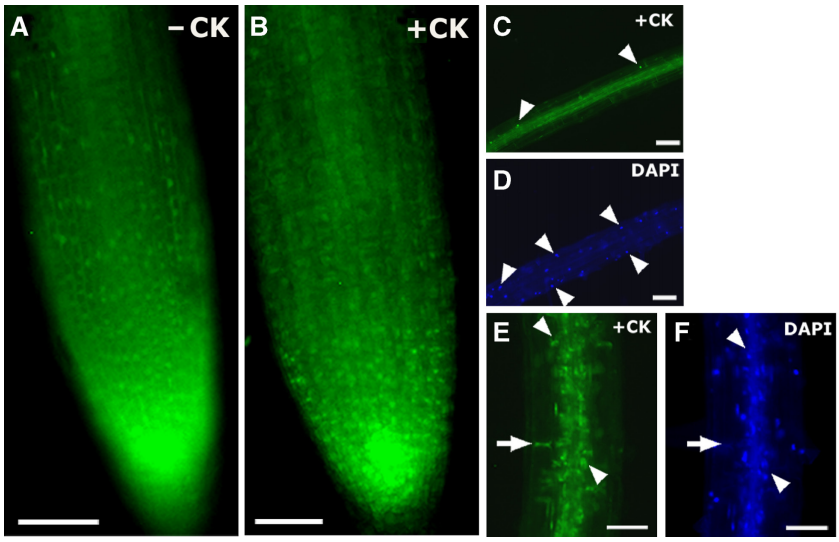

Figure 5 Cellular localization of mutagenized PhoB^{D53A}-GFP in roots of transgenic Arabidopsis plants. (**A**) Fluorescence from PhoB^{D53A}-GFP is diffused in an untreated root. (**B**) The same root showing PhoB^{D53A}-GFP localization after cytokinin treatment. (**C**, **D**) Detailed view of a root showing that nuclear localization of PhoB^{D53A}-GFP is variable and sporadic (arrowheads point to nuclei). (**E**, **F**) Detail view of another root showing that PhoB^{D53A}-GFP accumulates at the base of cortical cells (arrows). Some nuclear localization of PhoB^{D53A}-GFP can be seen in the root vascular tissue (arrowheads). −CK, tissues before cytokinin treatment; + CK same tissue after cytokinin treatment; DAPI, tissues treated with DAPI to stain DNA. Scale bars, 50 μm in (A, B); scale bars, 10 μm in (C–F).

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 OmpR^{D55A}-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 PhoB effector domain activates transcription by recruiting the RNA Polymerase σ70 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

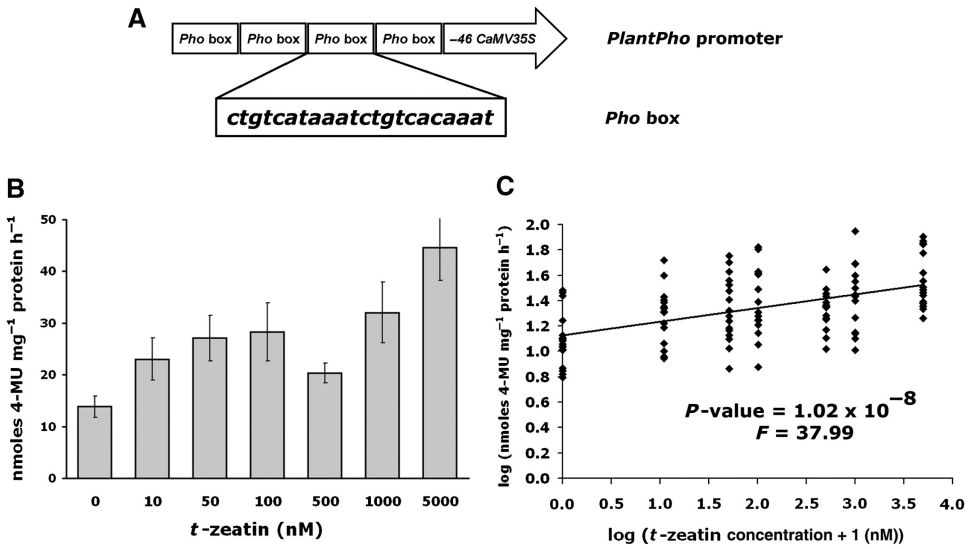

Figure 6 Design and function of the synthetic eukaryotic signal transduction system. (**A**) Diagram of *PlantPho* promoter, showing four *Pho* boxes fused to a minimal plant promoter, the −46 region of the *CaMV35S* promoter, with the nucleotide sequence of one *Pho* box indicated below. (**B**) Average GUS activity (nmoles 4-MU mg^{-1} protein h^{-1}) in transgenic plants, containing the PlantPho system as a function of cytokinin (*t*-zeatin) concentration. Error bars indicate ± one standard error. (**C**) Linear increase in GUS activity (nmoles 4-MU mg^{-1} protein h^{-1}) with *t*-zeatin concentration. 4-MU, 4-methylumbelliferone.

activation with exogenous cytokinin (*t*-zeatin). Cytokinin-dependent 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 × 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 PhoB^{D53A} 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 nucleus 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 $\sim$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 (Ellison 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 PhoB^{D53A}-GFP, and vascular cells accumulated PhoB^{D53A}-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*) (Benfey *et al*, 1989). We chose four PhoB-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; Mahonen *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 nucleus 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 *PhoB^{D53A}-GFP* and *OmpR^{D55A}-GFP* fusions were assembled in the binary vector pCB302-3

(Xiang *et al*, 1999). The PlantPho system (*FMV*::*PhoB-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 psmGFP vector (TAIR CD3-326). The 5′ end of *smGFP* was modified using primers (5′-TCTC GGATCCAAGGAGATATACATATGAGT-3′ and 5′-ATTCGAGCTCTTATTT GTATAGTTCATC-3′) to introduce an *Nde*I 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 psmGFP. 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 TGGT-3′ and lower, 5′-TTTACTCATATGAGATCCTCCAGATCCTCCAAA GCGGGT-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 *PhoB-VP64*. The *Nos* terminator sequence was added and the resulting *PhoB-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 *p2300-FMV*::*Pho-VP64-nos*. 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 μE m^{-2} s was used for experiments. Plants were transformed with *Agrobacterium* GV3011 harboring the plasmids described above following standard procedures (Clough and Bent, 1998). The T_0 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-methylumbelliferone (4-MU) was used as a standard. GUS activity was normalized to the total protein content of samples and expressed as nmoles 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 linear regression, the dependent variable was the log (measured GUS activity (nmoles 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 $PhoB^{D53A}$ 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 T_1-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).

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Conflict of interest

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

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