Phosphorylation Alters the Interaction of the Arabidopsis Phosphotransfer Protein AHP1 with Its Sensor Kinase ETR1

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Abstract
The ethylene receptor ethylene response 1 (ETR1) and the Arabidopsis histidine-containing phosphotransfer protein 1 (AHP1) form a tight complex in vitro. According to our current model ETR1 and AHP1 together with a response regulator form a phosphorelay system controlling the gene expression response to the plant hormone ethylene, similar to the two-component signaling in bacteria. The model implies that ETR1 functions as a sensor kinase and is autophosphorylated in the absence of ethylene. The phosphoryl group is then transferred onto a histidine at the canonical phosphorylation site in AHP1. For phosphoryl group transfer both binding partners need to form a tight complex. After ethylene binding the receptor is switched to the non-phosphorylated state. This switch is accompanied by a conformational change that decreases the affinity to the phosphorylated AHP1. To test this model we used fluorescence polarization and examined how the phosphorylation status of the proteins affects formation of the suggested ETR1–AHP1 signaling complex. We have employed various mutants of ETR1 and AHP1 mimicking permanent phosphorylation or preventing phosphorylation, respectively. Our results show that phosphorylation plays an important role in complex formation as affinity is dramatically reduced when the signaling partners are either both in their non-phosphorylated form or both in their phosphorylated form. On the other hand, affinity is greatly enhanced when either protein is in the phosphorylated state and the corresponding partner in its non-phosphorylated form. Our results indicate that interaction of ETR1 and AHP1 requires that ETR1 is a dimer, as in its functional state as receptor in planta.

Introduction
The plant hormone ethylene influences many processes in plant growth and development. Reverse genetics has identified a family of membrane-bound sensor kinases (ETR1, ERS1, ETR2, ERS2, EIN4) as receptors of the gaseous hormone [1–4]. Sequence homology of these receptors with sensor kinases in typical bacterial two-component signaling systems suggest that a histidine-aspartate phosphotransfer group transfer could be also employed in ethylene signaling [5]. In ETR1 representing the prototype of the ethylene receptor family, signal perception by the amino-terminal domain of the sensor kinase is proposed to control autophosphorylation of a conserved histidine in its catalytic transmitter domain. Then this phosphoryl group is supposed to be transferred to a conserved aspartate in the carboxyl-terminal receiver domain of the receptor. This is in contrast to typical bacterial two component systems which are characterized by a single histidine to aspartate phosphotransfer step between two individual proteins - a sensor kinase and a response regulator [6,7]. In ethylene signaling an additional phosphotransfer step is required to transfer the signal from the receiver domain of the membrane-localized sensor kinase ETR1 to the nuclear-localized response regulator proteins that control the cellular response to the plant hormone [5,8,9]. Connection between these two signaling modules is thought to be provided by histidine-containing phosphotransfer (HPt) proteins which transfer a phosphoryl group from the receiver domain of the receptor to the response regulator [10]. This type of multistep phosphorelay mechanism has been demonstrated unequivocally for signal transduction of the plant hormone cytokinin by the histidine kinases AHK2, AHK3, AHK4(CRE1) [11–14] and is also discussed for osmoregulation by the sensor kinase AHK1 [15].

The idea that a phosphorelay system is involved in ethylene signaling is further supported by yeast two-hybrid analyses which showed that the HPt proteins AHP1 and AHP3 can interact with the membrane extrinsic part (amino acids 321–721) of the ethylene receptor ETR1 but also with the A-type response regulator protein ARR4 [16]. Involvement of the B-type response regulator ARR2 in ethylene signaling was demonstrated by analyses of loss-of-function and over-expression lines as well as functional assays in protoplasts [17]. Furthermore, direct interaction of full-length ETR1 (amino acids 1–738) with the HPt protein AHP1 was recently demonstrated by fluorescence polarization studies providing quantitative analysis on the complex stability [18]. Although these experiments clearly emphasize that the receiver domain of the sensor kinase ETR1 forms a tight complex with the soluble cytoplasmic AHP1, they cannot answer whether phosphoryl-group transfer according to two-component signaling plays a functional role in ethylene signaling.

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To address this question, we have examined how the phosphorylation status of both, the sensor kinase and the HPi protein, affects formation of the ETR1–AHP1 signaling complex. Moreover, we have analyzed how binding of an ethylene agonist which is known to reduce phosphorylation activity of the ETR1 sensor kinase [19] controls complex formation. Our data presented here show that phosphorylation indeed plays an essential role in complex formation as affinity is dramatically reduced when both signaling partners are in their non-phosphorylated or phosphorylated form, respectively. On the other hand complex formation is promoted when either AHP1 or ETR1 are in their phosphorylated form and the corresponding binding partner is set to its non-phosphorylated status. Analysis of the effective molar volume of the AHP1–ETR1 signaling complex in our sensitive fluorescence assay revealed that complex formation involves a dimer of the sensor kinase as is the functional state of the ETR1 receptor in planta [20]. Consequently, detailed analysis of steady state fluorescence polarization data from recombinant proteins in a controlled in vitro system provide an excellent approach to understand some of the signaling processes involved in ethylene signaling in a quantitative context.

Results
Analysis of the ETR1–AHP1 signaling complex in response to the phosphorylation status of ETR1 and AHP1
In previous studies we have established a sensitive fluorescence polarization assay to validate and to quantify the interaction of the HPi protein AHP1 with the ethylene receptor ETR1 [18]. To gain further insight into this interaction we have analyzed the impact of phosphorylation on complex formation. Substitution of the canonical phosphorylation sites in ETR1 or AHP1 is supposed to interrupt phosphoryl group transfer in the putative ETR1–AHP1 signaling complex providing that phosphorylation plays a functional role as in typical bacterial two-component phosphorelay modules [21]. In order to mimic permanent phosphorylation in the receiver domain of the receptor or in the HPi protein we have substituted the putative canonical phosphorylation sites aspartate-659 in ETR1 and histidine-79 in AHP1 by glutamate (ETR1-D659E, AHP1-H79E), respectively. Due to its negative charge and comparable surface volume glutamate acts as a suitable structural substitute for the phospho-aspartate [22] and phospho-histidine residues [23] that are formed in AHP1 and ETR1 upon phosphorylation (see Supplement Figure S1). Substitution by alanine represents the non-phosphorylated state of the proteins (ETR1-D659A, AHP1-H79A). Interaction of the mutant proteins was analyzed according to the protocol described for the wild type proteins [18]. In short, purified recombinant ETR1 (up to 20 μM final concentration) was mixed to 8 nM of a fusion of AHP1 to a green fluorescent protein (AHP1-GFP(S65T)) in a medium containing 50 mM Tris(hydroxymethyl)-aminomethan (Tris-HCl) pH 7.5, 100 mM potassium chloride, 0.1% (w/v) β-D-dodecylmaltoside and 0.002% (w/v) phenylmethylsulfonyl fluoride. For the fluorescent AHP1 either non-phosphorylatable mutant AHP1(H79A)-GFP(S65T) or mutant AHP1(H79E)-GFP(S65T) mimicking permanent phosphorylation were used. Dissociation constants of the mutant ETR1–AHP1 complexes were calculated from the binding curves by Equation 2. ETR1(D659A) and AHP1(H79E)-GFP(S65T) a Kd of 3.6 μM (ΔΔΔΔ), ETR1(D659E) and AHP1(H79A)-GFP(S65T) a Kd of 2.6 μM (△△△Δ) and ETR1(D659A) and AHP1(H79A)-GFP(S65T) a Kd of 15.4 μM (ΔΔΔΔ). The dashed grey curve (——) corresponds to the binding characteristics of wild type ETR1 and wild type AHP1 [18].

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Figure 1. Binding curves of phosphorylation mutants of the sensor kinase ETR1 and the histidine transfer protein AHP1. Purified recombinant ETR1(D659A) or ETR1(D659E) at concentrations from 0.04 μM to 20 μM were added to 8 nM fluorescent AHP1-GFP(S65T) in a medium consisting of 50 mM Tris-HCl pH 7.5, 100 mM potassium chloride, 0.1% (w/v) β-D-dodecylmaltoside and 0.002% (w/v) phenylmethylsulfonyl fluoride. For the fluorescent AHP1 either non-phosphorylatable mutant AHP1(H79A)-GFP(S65T) or mutant AHP1(H79E)-GFP(S65T) mimicking permanent phosphorylation were used. Dissociation constants of the mutant ETR1–AHP1 complexes were calculated from the binding curves by Equation 2. ETR1(D659A) and AHP1(H79E)-GFP(S65T) a Kd of 3.6 μM (ΔΔΔΔ), ETR1(D659E) and AHP1(H79A)-GFP(S65T) a Kd of 2.6 μM (△△△Δ) and ETR1(D659A) and AHP1(H79A)-GFP(S65T) a Kd of 15.4 μM (ΔΔΔΔ). The dashed grey curve (——) corresponds to the binding characteristics of wild type ETR1 and wild type AHP1 [18].

Analysis of the ETR1–AHP1 signaling complex in response to ethylene agonists
Fluorescence polarization measurements were also employed to address the question whether and to what extent the ETR1–AHP1 interaction is affected by the plant hormone ethylene - the natural trigger of the signaling cascade. As ethylene is a highly volatile compound potassium cyanide, a well-documented ethylene agonist that mimics ethylene action and responses in planta [24,25], and ethephon, a chemical that disintegrates into ethylene, phosphate and chloride, were applied

mutant AHP1-H79A where phosphorylation at the canonical phosphorylation site is abolished are also given in Figure 1. Compared to the wild type proteins which show a Kd = 1.4 μM [18], the dissociation constants obtained from these binding curves show a 10-fold increase (Kd = 16.3 μM for ETR1-D659E–AHP1-H79E and Kd = 15.4 μM for ETR1-D659A–AHP1-H79A). These results show that the interactions in the complex are much weaker when ETR1 and AHP1 are either both in their phosphorylated or both in their non-phosphorylated form. In contrast, dissociation constants obtained for ETR1–AHP1 when one of the binding partner mimics the phosphorylated form and the other one corresponds to the non-phosphorylated form, are almost the same as observed for the wild type (Kd = 2.6 μM for ETR1-D659E–AHP1-H79A and Kd = 3.6 μM for ETR1-D659A–AHP1-H79E). Taken together, these results clearly demonstrate that the interaction between AHP1 and the ethylene receptor ETR1 is modulated by the phosphorylation at the canonical phosphorylation sites of both proteins.
in these studies. Wild type ETR1 was pre-incubated with 0.1 mM copper chloride which is an essential cofactor for ethylene binding [26], before the receptor was added to a buffer containing wild type AHP1-GFP, 50 mM Tris-HCl pH 7.5, 100 mM potassium chloride, 0.1% (w/v) β-D-dodecylmaltoside and 0.002% PMSF. Figure 2 illustrates the effect of ethephon and cyanide on the ETR1-AHP1 complex formation. Dissociation constants found in the presence of these ethylene agonists are increased by a factor of 5–6 compared to the dissociation constant obtained in the absence of a ligand (Kₐ = 9.0 μM for ETR1−AHP1 with cyanide and Kₐ = 7.7 μM for ETR1−AHP1 with ethylene). Binding curves obtained in the presence of cyanide or ethephon, but in the absence of the essential copper cofactor correspond to those of wild type ETR1 and AHP1 obtained in the absence of ethylene agonists. Hence, our results imply that not only the phosphorylation state of receptor and HPt protein but also the binding of the natural ligand, or an agonist, triggers conformational changes that affect the interaction in the ETR1−AHP1 complex.

Determination of molecular volume and stoichiometry of the ETR1−AHP1 complex

In addition to detailed information on complex formation and complex stability steady-state fluorescence polarizations studies can also provide information on the molecular volume and the stoichiometry of a protein complex. Measurements of fluorescence polarization of 20 nM AHP1-GFP at different temperatures and/or viscosities of the medium are summarized in Figure 3 which shows a Perrin plot, a plot of the reciprocal fluorescence anisotropy versus the temperature/viscosity ratio of the medium. From the slope and the intercept of the plot the molar volume and of the HPt protein.

Measurements were done in the presence of 10% (w/v) glycerol. At these conditions an increase in the molecular volume of the GFP-fusion protein is observed at temperatures below 17 °C probably due to protein aggregation.

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Discussion

In the present work we have studied intermolecular interactions of a protein complex involved in ethylene signaling by steady state fluorescence polarization. We have demonstrated that phosphorylation at a canonical phosphorylation site of the HPt protein AHP1 reduces its affinity for a mutant of the membrane sensor ETR1 that mimics the phosphorylated form of the kinase by at least 10-fold. The same clear reduction in affinity was observed when non-phosphorylatable mutants of both binding partner were correlated time of complexes above 100 kDa we changed the fluorophor and employed 5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl chloride) as fluorescent reporter for the analysis of the apparent molecular weight of ETR1 and for the stoichiometry of the ETR1−AHP1 complex. Proteins were dansylated as described in Material and Methods and analyzed at different temperatures and viscosities according to the protocol described for AHP1-GFP.

From the Perrin plot which is shown in Figure 4 an apparent molecular weight of 42 kDa was obtained for the dansylated AHP1 (M₀ monomer 18 kDa), while the molecular size of dansylated ETR1 (M₀ monomer 85 kDa and M₀ dodecylmaltoside micelle 72 kDa [27]) was estimated to 280 kDa. Both data which were confirmed in three independent measurements indicate that both recombinant proteins, AHP1 and also ETR1 form homodimers at the conditions applied in the fluorescence polarization assay. Note that for AHP1, dimerization was already observed with the GFP-fusion. Analysis of the ETR1−AHP complex was done in the same medium as for the individual proteins at 0.2 μM AHP1 and 2 μM ETR1. From the Perrin plot an apparent molecular weight of approximately 350 kDa was obtained for the ETR1−AHP1 complex suggesting that the signaling complex contains a homodimer of each of the receptor and of the HPt protein.

![Figure 2](image)

**Figure 2.** Binding curves of the sensor kinase ETR1 and histidine transfer protein AHP1. Measurements were done in the presence of 100 μM ethephon (●) or 100 μM potassium cyanide (○). In a control experiment (△) the cofactor copper essential for ethylene binding was omitted. The dashed grey curve (—) represents a fit to these data according to Equation 2 corresponding to a dissociation constant of 1.4 μM [18]. Binding curves fitted to the experimental data obtained in the presence of ethephon (—) or in the presence of cyanide (—) correspond to dissociation constants of 7.7 μM and 9 μM, respectively.

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![Figure 3](image)

**Figure 3.** Perrin plot analysis of purified recombinant AHP1-GFP. Measurements were performed in a buffer containing 50 mM Tris-HCl pH 7.5, 100 mM potassium chloride, 0.1% (w/v) β-D-dodecylmaltoside and 0.002% (w/v) phenylmethylsulfonyl fluoride at temperatures between 10 °C and 30 °C and glycerol concentrations of 10% (w/v) (△) and 20% (w/v) (○). Closed circles (●) correspond to measurements at 0% (w/v) glycerol. At these conditions an increase in the molecular volume of the GFP-fusion protein is observed at temperatures below 17 °C probably due to protein aggregation.

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Fluorescence Assay for Phosphorelay Signaling

Figure 4. Perrin plot analysis of recombinant ETR1, AHP1 and ETR1–AHP1 complex. Purified proteins were dansylated and fluorescence anisotropy was recorded in 0.2 M potassium phosphate pH 8.0, 0.05% (w/v) β-D-dodecylmaltoside and 10 mM β-cyclodextrin at temperatures between 5°C and 25°C. Curves correspond to data obtained with 0.2 μM dansylated AHP1 (●), 0.2 μM dansylated ETR1 (○) and 2 μM ETR1 together with 0.2 μM dansylated AHP1 (▲). doi:10.1371/journal.pone.0024173.g004

used. The apparent dissociation constant for the interaction of either both phosphorylated or both non-phosphorylated proteins was approximately 15 μM which corresponds to the low dissociation constant of 17 μM obtained for the typical bacterial two component system CheA–CheW [28]. When the kinase or the transfer protein, respectively, mimics its phosphorylated form (ETR1-D659E or AHP1-H79E) and the corresponding binding partner its non-phosphorylatable form (ETR1-D659A or AHP1-H79A), the interaction is tight. These complexes show dissociation constants of 2–3 μM which are similar to those observed for the recombinant wild type proteins. This finding suggests that both proteins are partly phosphorylated when purified from the bacterial host. Alternatively, wild type ETR1 that is purified at denaturing conditions and refolded to its active conformation after purification [19] is present in its non-phosphorylated state, while the natively purified AHP1 is in its phosphorylated form. Nevertheless, a charged residue, i.e. either the phosphorelay phosphor domain or a genetically placed negatively charged glutamate which has been shown in previous studies to efficiently mimic the phosphorylated state of a protein kinase [29], seem to be necessary for tight interaction of the sensor kinase with its Hpt protein. The idea that phosphorylation at the canonical sites affects the stability of the sensor kinase–Hpt protein complex is further supported by yeast two hybrid studies on the hybrid-type histidine kinase ATHK1 [16]. When the putative phosphorylation site aspartate-1074 in the receiver domain of ATHK1 was substituted by glutamate and expressed in yeast, the mutant showed no interaction with AHP1 - an Hpt protein that was previously shown to interact with the wild type receiver domain of ATHK1 [16]. This result indicates that the phosphorylation state of the osmosensor is essential for the binding of AHP1. Yeast two-hybrid analysis did not resolve the phosphorylation state of the canonical phosphorylation site since it could not be discriminated whether D1074 is phosphorylated by crosstalk with other two component systems when expressed in yeast. Hence, it seems possible that only the non-phosphorylated receiver domains of ATHK1 can interact with Hpt domains, and that a phosphorylated receiver domain shows only weak interaction which is not resolved by yeast two hybrid analysis. The fact that phosphorylation in the receiver domain has a negative effect on the interaction with the non-phosphorylated Hpt protein seems to contradict the results obtained in this study. However, this apparent paradox might be resolved by the fact that in contrast to the osmosensor ATHK1, ethylene receptors are negative regulators which are in the absence of the plant hormone in their phosphorylated state [19]. In contrast to yeast two hybrid studies the assay presented in this study provides not only a quantitative read-out of the interaction, but also the opportunity to test the effect of the natural trigger ethylene or its agonists on the interaction of sensor kinase and Hpt protein. Measurements obtained in the presence of cyanide or ethephon - a chemical releasing ethylene in situ - indicate that the affinity of both binding partner in the ETR1–AHP1 complex is reduced by a factor of 5. These results suggest that a conformational change in the receiver domain triggered by the binding of the plant hormone is necessary in addition to the electrostatic requirements in the receiver domain to regulate tight interaction with the Hpt protein. Controls obtained in the absence of essential copper cofactor where binding of the plant hormone is prevented showed no effect of cyanide or ethephon on the ETR1–AHP1 interaction and support this analysis.

In addition to quantitative analysis and evaluation of the effect of ethylene agonists on the interaction of the sensor kinase ETR1 and the Hpt protein AHP1 steady state fluorescence polarization studies also provide information on the stoichiometry of the signaling complex. Analysis of the polarization data according to Perrin (see Fig. 4) revealed that recombinant ETR1 and AHP1 are forming a tetrameric complex consisting of two homodimers at the conditions applied in the in vitro assay. Taking into account the theoretical molecular weight for AHP1 (18.3 kDa), AHP1-GFP (45.6 kDa) and ETR1 (84.9 kDa) the data determined from the individual proteins and the ETR1–AHP1 complex account for an AHP1 dimer (41±5 kDa for dansylated AHP1 and 89.6±1.4 kDa for AHP1-GFP), a detergent-solubilized ETR1-dimer (282±21 kDa) considering an average dodecylmaltoside micelle size of 72 kDa [27], and for an ETR1–AHP1 dimer (347±30 kDa) in a dodecylmaltoside micelle. Studies on ethylene receptors ETR1 and ERS1 of Arabidopsis indicate that they form disulfide-linked homodimers in planta [20,30]. Moreover, membrane recruitment studies demonstrated that all five members of the Arabidopsis ethylene receptor family form homomeric and heteromeric protein complexes at the ER in living plant cells [31]. Similarly, Hpt proteins might also form dimers in vivo as indicated by the dimerization of the Hpt protein Spo0B from Bacillus subtilis [32]. Other Hpt proteins such as the phosphorelay protein YPD1 [39], the AecB Hpt domain [34], and the CheA PI domain [35] are probably monomers in their functional form.

A homology model of AHP1 built on the crystal structure of the Hpt protein OsHPI from rice (PDB code 1YVI) which is shown in Figure 5 further supports our experimental data that AHP1 forms homodimers in solution. The Poisson-Boltzmann electrostatic potential which is displayed on the protein surface clearly indicates a negatively charged (Helix 3, residues D40-D65) and a positively charged arm (Helix 6, residues R114-K135). Formation of the AHP1 homodimer might occur via these two complementary electrostatic surfaces when two monomers interact in a back-to-back orientation αHelix3-βHelix6 and αHelix6-βHelix3). The catalytic histidine-79 in both monomers are well accessible in this putative complex as they are facing the opposite site of the interacting surfaces provided by Helixes 3 and 6 in both monomers.

Figure 5 further supports our experimental data that AHP1 forms homodimers in solution. The Poisson-Boltzmann electrostatic potential which is displayed on the protein surface clearly indicates a negatively charged (Helix 3, residues D40-D65) and a positively charged arm (Helix 6, residues R114-K135). Formation of the AHP1 homodimer might occur via these two complementary electrostatic surfaces when two monomers interact in a back-to-back orientation αHelix3-βHelix6 and αHelix6-βHelix3). The catalytic histidine-79 in both monomers are well accessible in this putative complex as they are facing the opposite site of the interacting surfaces provided by Helixes 3 and 6 in both monomers.
The affinity of both binding partners decreases in the affinity of the receptor and further signal transfer to nuclear response regulators. Thereby AHP1 would regain its non-phosphorylated state which binds with high affinity only to the phosphorylated, but not to the non-phosphorylated ETR1 sensor kinase (see high $K_d$ of alanine mutants of AHP1 and ETR1). Interference of AHP1 that has been phosphorylated by other plant two-component based systems (e.g., cytokinin-receptors AHK2, AHK3, AHK4/CRE1/WOL [36]) resulting in an obstruction of these pathways is suspended by the low affinity of the phosphorylated HPt protein with the phosphorylated form of the ETR1 receptor (see high dissociation constant of the ETR1-D659E–AHP1-H79E complex) occurring in the absence of ethylene.

In summary, our work emphasizes that individual steps in a signal transduction pathway have to be characterized separately in order to rationalize the mechanistic basis of phenotypes observed in molecular genetic studies.

### Materials and Methods

#### Materials

Chemicals and reagents were purchased from Serva (Heidelberg, Germany), AppliChem (Darmstadt, Germany), Glycon (Luckenwalde, Germany), VWR International (Geldenaaksaan, Belgium), BD (Le Pont de Clais, France) and Carl Roth (Karlsruhe, Germany) at analytical grade. Plasmids were derived from pET vectors from Merck/Novagen (Darmstadt, Germany). Oligonucleotides were from Sigma-Aldrich (Steinheim, Germany).

#### Methods

Cloning, expression, and purification. Site directed mutagenesis was carried out using the single-tube megaprimer PCR method as described in [37]. The plasmids pET16b_AtETR1 and pET21a_AtAHP1 used for mutagenesis were previously described [18]. Primers are listed in Table 1.

Plasmids encoding mutant AHP1 were transformed into *Escherichia coli* strain BL21 Gold (DE3) (Stratagene, La Jolla, USA). Proteins were expressed recombinantly and purified from the bacterial host by immobilized metal ion affinity chromatography under native conditions as described for wild type AHP1 [18]. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad, München, Germany) using bovine serum albumin as a standard. Plasmids coding for mutant ETR1 were transformed into *E. coli* strain C43 (DE3) [38]. Expression and purification was performed following the protocol described earlier [19]. Protein concentration of the purified ETR1 receptor was determined by the bicinchoninic acid assay (Perbio Science, Bonn, Germany) using bovine serum albumin as a standard.

Dansylation of ETR1 and AHP1. Purified proteins were concentrated in a buffer containing 0.2 M potassium phosphate, pH 8.0, and 0.05% (w/v) β-D-dodecylmaltoside. The protein solutions, 0.05 mg/ml (2.8 μM) AHP1 and 1 mg/ml (11.8 μM) ETR1, respectively, were incubated at a ratio of 1:10 with 5% (v/v) of a freshly prepared 20-fold solution of dansyl chloride in dimethylformamide. The protein/dansyl chloride solution was incubated for 1 hour in the dark at room temperature. Final labeling ratio was determined spectrophotometrically using a Beckman-Coulter DU800 spectrophotometer assuming a molar extinction coefficient of 4500 M$^{-1}$ cm$^{-1}$ at 340 nm [39]. Typical ratio of protein-dansyl labeling was 1:4.

Fluorescence polarization assay. Fluorescence polarization measurements were performed in a LSS5 Luminescence Spectrophotometer (Perkin Elmer, Rodgau-Jügesheim, Germany).
For all experiments a SUPRASIL quartz 4×4 mm macro/semi-microcuvette (Perkin Elmer) with stirrer was used. Fluorescence polarization was calculated from parallel and perpendicular fluorescence intensities according to the following equation,

$$P = \frac{I_{VV} - GF \cdot I_{VH}}{I_{VV} + GF \cdot I_{VH}}$$

where GF is the grating factor. Determination of the dissociation constant ($K_d$) and fraction of bound protein ($f_B$) was done by the equation,

$$f_B = \frac{P - P_{min}}{\Delta P} = \frac{F}{K_d + F}$$

where $P$ is the measured polarization, $\Delta P = P_{max} - P_{min}$ is the difference between polarization before and after addition of the ETR1 receptor [40]. F corresponds to the concentration of free ETR1. The binding curve was obtained by plotting the normalized fluorescence polarization $f_B$ versus the ETR1 concentration. Dissociation constants were determined from this curve using the program GraFit (Erithacus Software, Horley, Surrey, U.K.) by a fit of the experimental data to a model assuming a single binding site in the interacting partners.

**Analysis of molecular weight and stoichiometry of the AHP1–ETR1 complex.** The molecular weight of AHP1, ETR1 and the AHP1–ETR1 complex was determined using the Perkin Elmer LS55 Spectrophotometer equipped with a Biokinetics Accessory, a PTP-1 Fluorescence Peltier System and a PCB1500 Water Peltier System (Perkin Elmer) by measuring anisotropy at different viscosities and temperatures of the medium. The following equation was used for calculating the anisotropy,

$$r = \frac{I_{VV} - GF \cdot I_{VH}}{I_{VV} + 2GF \cdot I_{VH}}$$

The molecular weight ($M_w$) of the analyzed proteins was calculated according to

$$M_w = \frac{\tau R}{r_0(\bar{v} + h) \text{slope}}$$

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**Figure 6. Schematic model of ETR1–AHP1 signaling.** In the absence of ethylene autophosphorylation of the sensor kinase ETR1 ensures low affinity of the receptor to phosphorylated Hpt proteins. On the other hand, non-phosphorylated AHP1 binds to the phosphorylated ETR1 receptor with high affinity enabling phosphoryl group transfer to the Hpt protein. After ethylene binding the receptor is switched to the non-phosphorylated state. This switch is accompanied by a conformational change that decreases the affinity to the phosphorylated AHP1. The transfer protein, AHP1-P is released and can move to the nucleus for further signal transfer onto response regulator proteins causing gene response. Binding of non-phosphorylated AHP1 to the activated form of the receptor is prevented by the conformational change caused in the receiver domain of the receptor by the binding of the plant hormone.

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**Table 1. Oligonucleotides used for mutagenesis.**

| Name       | Sequence                      |
|------------|-------------------------------|
| S’ AHP1(H79A) | GAT CCC CAT GTT GCT CAA       |
| S’ AHP1(H79E) | GAT CCC CAT GTT GAG CAA       |
| T7 Promotor primer | TAA TAC GAC TCA CTA TAG GG     |
| T7 Terminator primer | GCT AGT TAT TGC TCA GGC G     |
| S’ ETR1(D659E) | GTG TCC ATG GAG GTG TGC ATG   |
| S’ ETR1(D659A) | AAA GTG GTC TTC ATG GCC GTG TGC ATG |

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respectively. Limiting anisotropy $r_0$ and slope were obtained from a plot of the reciprocal fluorescence anisotropy versus the temperature/viscosity ratio of the medium according to Perrin. An excitation wavelength of 489 nm and an emission wavelength of 510 nm were used for GFP. Slit width was set to 8.5 nm. For dansylated proteins the excitation wavelength was fixed to 333 nm and emission wavelength to 513 nm with a slit width of 5 nm.

Supporting Information

Figure S1 Structural alignment of glutamate with phospho-aspartate (left panel) and phospho-histidine (right panel) from known protein structures. Structural information for phospho-aspartate was obtained from the response regulator protein from Burkholderia pseudomallei (pdb-code 3RQI) and from the receiver domain of the transcriptional regulatory protein FixJ from Sinorhizobium meliloti (pdb-code 1D3W). Coordinates for phospho-histidine were taken from Nucleoside Diphosphate Kinase from Dicyostelium discoideum (pdb-code 1NSP) and from E. coli Phosphoglycerate Mutase (pdb-code 1E5O). Phospho-amino acids and glyceraldehydes were aligned in PyMol. Carbon atoms of glutamate are colored in red, while carbon atoms extracted from the pdb coordinates are shown in white. Nitrogen and oxygen are drawn in blue and red for all residues. Phosphor atoms are colored orange. (TIF)

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Author Contributions

Conceived and designed the experiments: BS GG. Performed the experiments: BS. Analyzed the data: BS GG. Contributed reagents/materials/analysis tools: BS GG. Wrote the paper: BS GG.

References

1. Bleecker AB (1999) Ethylene perception and signaling: an evolutionary perspective. Trends Plant Sci 4: 269–274.
2. Bleecker AB, Kende H (2000) Ethylene: a gaseous signal molecule in plants. Annu Rev Cell Dev Biol 16: 1–18.
3. Stepanova AN, Ecker JR (2000) Ethylene signaling: from mutants to molecules.Curr Opin Plant Biol 3: 353–360.
4. Chang C, Stadler R (2001) Ethylene hormone receptor action in Arabidopsis. Bioessays 23: 619–627.
5. Huang J, Chen HC, Shen J (2002) Two-component signal transduction pathways in Arabidopsis. Plant Physiol 129: 500–515.
6. Stock JB, Ninau AJ, Stock AM (1989) Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol Rev 33: 450–490.
7. Stock AM, Robinson VL, Goudreau PN (2000) Two-component signal transduction. Annu Rev Biochem 69: 183–215.
8. Lehrmann J, Harter K (2002) Plant two-component signaling systems and the role of response regulators. Plant Physiol 128: 363–369.
9. D’Agostino IB, Kieber JJ (1999) Phosphorylase signal transduction: the emerging family of plant response regulators. Trends Biochem Sci 24: 432–436.
10. Suzuki T, Sakurai K, Imamura A, Nakamura A, Ueguchi, et al. (2000) Compilation and characterization of histidine-containing phosphotransmitter implicated in His-to-Asp phosphorelay in plants: AHP signal transducers of Arabidopsis thaliana. Biochim Biophys Acta 1486: 2486–2489.
11. Suzuki T, Misawa K, Ishikawa K, Yamada H, Aiba H, et al. (2001) The Arabidopsis sensor His-kinase, AHP4, can respond to cytokinins. Plant Cell Physiol 42: 107–113.
12. Inoue T, Hashigui M, Hashimoto Y, Seki M, Kobayashi M, et al. (2001) Identification of CRE1 as a cytokinin receptor from Arabidopsis. Nature 409: 1060–1063.
13. Ueguchi C, Sato S, Kato T, Tabata S (2001) The AHP4 gene involved in the cytokinin-signaling pathway as a direct receptor molecule in Arabidopsis thaliana. Plant Cell Physiol 42: 751–755.
14. Dourtay H, Mehnert N, Burké L, Schmulling T, Heyl A (2006) Analysis of protein interactions within the cytokinin-signaling pathway of Arabidopsis thaliana. FEBS J 273: 4631–4644.
15. Urao T, Yukahoshi K, Satoh K, Yamaguchi-Shinozaki K, Seki M, et al. (1999) A transmembrane-type histidine kinase in Arabidopsis thaliana as an osmosensor. Plant Cell 11: 1743–1754.
16. Urao T, Miyata S, Yamaguchi-Shinozaki K, Shinozaki K (2000) Possible His to Asp phosphorylation signaling in an Arabidopsis two-component system. FEBS Lett 476: 227–232.
17. Hass C, Lehrmann J, Albrecht V, Sweere U, Hummel F, et al. (2004) The response regulator 2 mediates ethylene signaling and hormone signal integration in Arabidopsis. EMBO J 23: 3290–3292.
18. Schärer B, Voet-van Vormizele J, Harter K, Groth G (2000) Ethylene signaling: identification of a putative ETR1-AHP1 phosphorelay complex by fluorescence spectroscopy. Anal Biochem 377: 72–76.
19. Voet van Vormizele J, Groth G (2000) Ethylene controls autoprophosphorylation of the histidine kinase domain in ethylene receptor ETR1. Mol Plant 3: 380–387.
20. Schaller GE, Ladd AN, Lanahan MB, Spanbauer JM, Bleecker AB (1995) The ethylene response mediator ETR1 from Arabidopsis forms a disulfide-linked dimer. J Biol Chem 270: 12526–12830.
21. Mattison K, Kenney LJ (2002) Phosphorylation Alters the Interaction of the Response Regulator OmpR with Its Sensor Kinase EnvZ. J Biol Chem 277: 11143–11148.
22. Horák J, Greifen C, Berendzen KW, Hahn A, Stierhof YD, et al. (2008) The Arabidopsis thaliana response regulator ARK2 is a putative AHP phospho-histidine phosphatase expressed in the chalaza of developing seeds. BMC Plant Biol 8: 77–94.
23. Huffine ME, Scholtz JM (1996) Energetic implications for protein phosphorylation. Conformational stability: HP variants that mimic phosphorylated forms. J Biol Chem 271: 20896–20899.
24. Solomos T, Laties GG (1974) Similarities between the Actions of Ethylene and Cyanide in Initiating the Climacteric and Ripening of Avocados. Plant Physiol 54: 506–511.
25. Sider EC (1977) Ethylene activity of some κ-receptor compounds. Tob Sci 21: 43–45.
26. Rodriguez F, Esch JJ, Hall AE, Binder BM, Schaller GE, et al. (1999) A copper cofactor for the ethylene receptor ETR1 from Arabidopsis. Science 283: 996–998.
27. Sprod P, Brünger AT (2005) Refractive index-based determination of detergent concentration and its application to the study of membrane proteins. Protein Sci 14: 2207–2211.
28. Greger JA, Dahlquist FW (1991) Signal transduction in bacteria: CheW forms a reversible complex with the protein kinase CheA. Proc Natl Acad Sci U S A 88: 750–754.
29. Tournavit S, Pietro ES, Terjung S, Schaeffer T, Wegehingel S, et al. (2009) Reversible phosphorylation as a molecular switch to regulate plasma membrane targeting of acylated SH1 domain proteins. Traffic 10: 1047–1060.
30. Hall AE, Endfelder J, Schaller GE, Sider EC, Bleecker AB (2000) Ethylene perception by the ERS1 protein in Arabidopsis. Plant Physiol 123: 1449–1458.
31. Greifen C, Stadle K, Ritzka C, Ohrlík P, Harter K, et al. (2008) Subcellular localization and in vivo interactions of the Arabidopsis thaliana ethylene receptor family members. Mol Plant 1: 308–320.
32. Varughese KL, Madhusudan Zhou XZ, Whiteley JM, Hoch JA (1998) Formation of a novel four-helix bundle and molecular recognition sites by dimerization of a response regulator phospho-transferase. Mol Cell 2: 405–493.
33. Xu Q, West AH (1999) Conservation of structure and function among histidine-containing phosphotransfer (HP) domains as revealed by the crystal structure of Ypd1. J Mol Biol 292: 1039–1050.
34. Kato M, Mizuno T, Shimizu T, Hokushima T (1997) Insights into multistep phosphorelay from the crystal structure of the C-terminal HPdomain of ArcB Cell 80: 717–723.
35. Zhou H, Lowry DF, Swanson RV, Simon MI, Dahlquist FW (1995) NMR studies of the phosphotransfer domain of the histidine kinase CheA from Escherichia coli: assignments, secondary structure, general fold, and backbone dynamics. Biochemistry 34: 18585–18597.
36. Hwang I, Shiner J (2001) Two-component circuitry in Arabidopsis cyanokinin signal transduction. Nature 413: 383–389.
37. Ke SH, Madison EL (1997) Rapid and efficient site-directed mutagenesis by single-tube ‘megaprimer’ PCR method. Nucleic Acids Res 25: 3371–3372.
38. Mirowski B, Walker JE (1996) Over-production of proteins in Escherichia coli: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. J Mol Biol 260: 209–298.
39. Hsieh WT, Matthews KS (1985) Lactose repressor protein modified with dansyl chloride: activity effects and fluorescence properties. Biochemistry 24: 3043–3049.
40. Park SH, Raines RT (2004) Fluorescence polarization assay to quantify protein-protein interactions. Methods Mol Biol 261: 161–166. Review.
41. Lakowicz JR Principles of Fluorescence Spectroscopy 3rd ed., Springer, Berlin. pp 367.
42. Swaminathan R, Hoang CP, Verkman AS (1997) Photobleaching recovery and anisotropy decay of green fluorescent protein GFP-S65T in solution and cell: cytoplasmic viscosity probed by green fluorescent protein translational and rotational diffusion. Biophys J 72: 1900–1907.
43. Takeda K, Yoshih I, Yamamoto K (1991) Changes of fluorescence lifetime and rotational correlation time of bovine serum albumin labeled with 1-dimethylaminonaphthalene-5-sulfonyl chloride in guanidine and thermal denaturations. J Protein Chem 10: 17–23.
44. Sali A, Blundell TL (1993) Comparative protein modelling by satisfaction of spatial restraints. J Mol Biol 234: 779–815.
45. Kuntal BK, Aparaj P, Reddanna P (2010) EasyModeller: A graphical interface to MODELLER. BMC Res Notes 3: 226–230.
46. Dolinsky TJ, Nielsen JE, McCammon JA, Baker NA (2004) PDB2PQR: an automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. Nucleic Acids Res 32: W663–W667.
47. Baker NA, Sept D, Joseph S, Holm MJ, McCammon JA (2001) Electrostatics of nanosystems: application to microtubules and the ribosome. Proc Natl Acad Sci USA 98: 10037–10041.
48. DeLano WL The PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, CA, USA.