Stable Isotope Metabolic Labeling-based Quantitative Phosphoproteomic Analysis of Arabidopsis Mutants Reveals Ethylene-regulated Time-dependent Phosphoproteins and Putative Substrates of Constitutive Triple Response 1 Kinase*

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Ethylene is an important plant hormone that regulates numerous cellular processes and stress responses. The mode of action of ethylene is both dose- and time-dependent. Protein phosphorylation plays a key role in ethylene signaling, which is mediated by the activities of ethylene receptors, constitutive triple response 1 (CTR1) kinase, and phosphatase. To address how ethylene alters the cellular protein phosphorylation profile in a time-dependent manner, differential and quantitative phosphoproteomics based on $^{15}$N stable isotope labeling in Arabidopsis was performed on both one-minute ethylene-treated Arabidopsis ethylene-overly-sensitive loss-of-function mutant rcn1-1, deficient in PP2A phosphatase activity, and a pair of long-term ethylene-treated wild-type and loss-of-function ethylene signaling ctr1-1 mutants, deficient in mitogen-activated kinase kinase activity. In total, 1079 phosphopeptides were identified, among which 44 were novel. Several one-minute ethylene-regulated phosphoproteins were found from the rcn1-1. Bioinformatic analysis of the rcn1-1 phosphoproteome predicted nine phosphoproteins as the putative substrates for PP2A phosphatase. In addition, from CTR1 kinase-enhanced phosphosites, we also found putative CTR1 kinase substrates including plastid transcriptionally active protein and calcium-sensing receptor. These regulatory proteins are phosphorylated in the presence of ethylene. Analysis of ethylene-regulated phosphosites using the group-based prediction system with a protein–protein interaction filter revealed a total of 14 kinase–substrate relationships that may function in both CTR1 kinase- and PP2A phosphatase-mediated phosphor-relay pathways. Finally, several ethylene-regulated post-translational modification network models have been built using molecular systems biology tools. It is proposed that ethylene regulates the phosphorylation of arginine/serine-rich splicing factor 41, plasma membrane intrinsic protein 2A, light harvesting chlorophyll A/B binding protein 1.1, and flowering bHLH 3 proteins in a dual-and-opposing fashion. Molecular & Cellular Proteomics 12: 10.1074/mcp.M113.031633, 3559–3582, 2013.

Ethylene is a volatile plant hormone that regulates versatile molecular and physiological processes in higher plants (1). The perception of this gaseous two-carbon hormone is achieved by a group of membrane-associated dimeric ethylene receptors that resemble bacterial two-component signaling systems and are composed of hybrid histidine (or aspartic acid) kinases, a histidine-containing phosphor-transfer domain, and response regulators (2). These receptors are made of two membrane-bound protein subunits cross-linked at the N-terminal region through two disulfide bonds (3). In Arabidopsis, there are five different ethylene receptor subunits: ethylene response 1, ethylene response 2, ethylene insensitive 4 (EIN4),1 ethylene response sensor 1, and ethylene repressor sensitive 4 (EIN4).1 ethylene response sensor 1, and ethylene re-
sponse sensor 2, each of which is encoded by an ethylene receptor gene of unique DNA sequence and structure (4). Ethylene gas binds to a pair of cross-linked transmembrane domains in a receptor with the help of an incorporated copper ion (5). The physical interaction of ethylene molecules with receptor complexes somehow induces inactivation of the negative regulation of another downstream signaling component, constitutive triple response 1 (CTR1) (AT5G03730) (6). This ethylene-signaling component has been perceived as a Raf-like Ser/Thr protein kinase, a putative mitogen-activated protein kinase kinase kinase (MAPKKK) (6, 7), and its primary function in the ethylene signal-transduction pathway has been defined as a negative regulator of ethylene responses according to molecular genetic studies (8). CTR1 physically interacts with both ethylene receptors (9, 10) and a downstream positive regulator of ethylene response, EIN2 (11, 12), and it directly inhibits the molecular function of EIN2 in ethylene signaling by phosphorylating EIN2 (12), which was identified as an endoplasmic reticulum (ER) membrane-localized natural resistance-associated macrophage protein homolog (13).

When ethylene binds to ethylene receptors, the negative regulation of ethylene signaling output from CTR1 is reduced, dephosphorylation of EIN2 occurs, and the C terminus of EIN2 is subsequently cleaved from the putative metal ion channel and translocated into the nucleus to initiate the activation of transcriptional cascades for most ethylene-responsive gene expression (11). The EIN2 C-terminus-activated EIN3 and ethylene insensitive 3-like 1 ethylene response transcription factors (14–16) consequently orchestrate combinatorial control over the transcriptional activities of a large number of ethylene response factor proteins (17) that lead to plant ethylene responses.

Given the established ethylene-signaling pathway, an emerging and pressing issue is how to deploy the current mechanistic paradigm of ethylene signaling to address diverse ethylene responses in plants (18, 19). The mode of ethylene action is dose-dependent in Arabidopsis (20, 21), and its response to ethylene is achieved over a wide range of concentrations (22). At lower concentrations, ethylene promotes both gravicurvature of inflorescence stem and elongation of etiolated seedlings of Arabidopsis (20, 23), whereas at higher concentrations it inhibits both gravicurvature and elongation of the etiolated seedlings (21, 23). In addition, plant responses to ethylene are also time-dependent (20, 23, 24). A short-term exposure to ethylene inhibits gravicurvature of inflorescence stem in Arabidopsis, whereas a long-term pre-treatment, regardless of the concentration of ethylene, stimulates gravicurvature (20, 24). Such a dual-and-opposing effect of ethylene on the Arabidopsis shoot gravitropic response was also found in the regulation of flowering (25). The sophisticated mode of action of ethylene is typified by the fact that it delays bolting and both the ethylene receptor mutant etr1-1 and the receptor-interacting signaling component mutant ctr1-1 exhibit similar delayed-bolting phenotypes (26, 27). Attempts to address the diverse and complex phenomena related to ethylene responses with a transcriptomics approach have confirmed the existence of a difference in gene expression profiles between ethylene-treated wild-type Arabidopsis and the constitutive ethylene response mutant ctr1-1 (11, 28, 29). Molecular biological analysis has successfully classified ethylene-regulated genes into early- and late-induction groups (30, 31). The integration of biochemical and cellular functions of each class of time-dependent gene products (i.e. either early or late ethylene-induced gene groups) should constitute separate yet overlapping molecular interaction matrices and networks to define diverse yet complex ethylene responses (32).

Moreover, post-translational modification (PTM) has recently emerged as one of the important mechanisms regulating the complex plant ethylene responses (19). One type of PTM involved in ethylene signaling is the ethylene-dependent and ubiquitin/26S proteasome-mediated protein degradation or stabilization of ethylene receptors and signaling components EIN2 and EIN3 (33–35). The other type of PTM-mediated ethylene signaling is interconversion between the phosphorylation and dephosphorylation statuses of signaling components catalyzed by both kinases and phosphatases (11, 12, 25, 33, 36, 37). Protein phosphor-relay has long been reported to participate in ethylene signaling events (2, 6, 38–40). Many ethylene signaling components, including ethylene receptor family proteins and their downstream regulator CTR1, have kinase activities (7, 34, 41, 42), and there are at least two MAPK cascades involved in the regulation of ethylene signaling and the ethylene-regulated phosphorylation of EIN3 (37, 43). Phosphorylation/dephosphorylation of the ethylene response factor 110 protein has been found to be EIN2-independent (25, 36). Thus, the diverse yet complex plant ethylene responses are actually consequences of the multifaceted and constant integration of ethylene signals mediated by both transcriptional activation cascades and PTM networks (i.e. protein phosphorylation/dephosphorylation, C-terminal cleavage, and ubiquitin/26S proteasome-mediated protein degradation).

The key issue is, therefore, how the ethylene receptor–MAPKKK (including CTR1) complexes convert the binding of ethylene into a signal output in the form of kinase activities in a quantitative and substrate-specific manner (12, 22). The hypothesis that ethylene inactivates kinase activities of CTR1 has not been substantiated in vivo thus far. It is possible that the binding of ethylene to receptors may alter the substrate specificity of the putative CTR1 serine/threonine kinase. The
isolation of an enhanced ethylene response 1 (eer1-1) mutant further complicated the study of the already intriguing ethylene signal in Arabidopsis (44). The enhanced ethylene responsiveness observed in eer1-1 results from a loss-of-function mutation in the A regulatory subunit roots curl in naphthylphthalamic acid 1 (RCN1) (AT1G25490) of protein phosphatase 2A (PP2A) (45), which is one of the three subunits of PP2A in Arabidopsis. This phosphatase is a heterotrimeric serine/threonine phosphatase comprising a catalytic subunit C and two regulatory subunits A and B (46). The phosphatase activity of PP2A decreases significantly in the loss-of-function mutant rcn1-1 (or eer1-1) (47), yet it enhances both ethylene sensitivity and the amplitude of ethylene responses (45). The in vitro demonstration of a strong association of PP2A catalytic subunit PP2A-1C with the kinase domain of CTR1 suggests an interesting kinase activity output model involving both CTR1 serine/threonine kinase and PP2A serine/threonine phosphatase that may act on one or a group of common phosphotides antagonistically in diverse plant ethylene responses (25).

To address the complex and interesting ethylene signaling, a differential and quantitative phosphoproteomics has been employed in the present study. A SILIA-assisted PTM proteomic approach (48) (supplemental Fig. S1) is similar in principle to stable isotope labeling with amino acids in cell culture (49), which has been used frequently in the discovery and quantification of differentially regulated PTMs in various organisms in the past (50–56). The most important advantage of the SILIA approach is that the in vitro–derived variability resulting from each step of multi-dimensional protein identification technology peptide preparation (57) and mass spectrometry analysis is eliminated when protein samples from different treatments are labeled with either light or heavy nitrogen isotopes in parallel, mixed at the beginning step, different treatments are labeled with either light or heavy nitrogen isotopes in parallel, mixed at the beginning step, and thereby the metabolic labeling approach (48). The natural light nitrogen isotope-coded KNO3 (K14NO3), used as the sole nitrogen source in the medium, was substituted with the heavy nitrogen-coded K15NO3 (Cambridge Isotope Laboratories, Inc., Andover, MA) based on the metabolic labeling approach (48).

Both Col-0 and ctr1-1 Arabidopsis plants were treated with constant ethylene by being grown on medium containing 5 mM ACC (the immediate precursor of ethylene). In the one-minute ethylene treatment of rcn1-1 mutant, the growth medium was supplemented with 0.15 mM aminoxyacetic acid, an inhibitor of ACC synthase. One minute of ethylene treatment was applied to rcn1-1 mutant just before it was harvested. To do that, two groups of 3-week-old rcn1-1 plants, one labeled with 14N and the other with 15N, were placed in an airflow chamber for 5 h to reduce the endogenous ethylene level. Afterward, one of the two groups (either 14N- or 15N-labeled plants) was treated with 10 ppm ethylene for 1 min, and the other group of plants was retained in air for the same amount of time. Both groups of plants were harvested in liquid nitrogen and stored in a refrigerator at −140 °C. Approximately 1.5 to 1.7 g tissue were collected often from each treated or unique genotype plant group.

To label the plant proteins with the heavy nitrogen stable isotope 15N, the natural light nitrogen isotope-coded KNO3 (K14NO3), used as the sole nitrogen source in the medium, was substituted with the heavy nitrogen-coded K15NO3 (Cambridge Isotope Laboratories, Inc., Andover, MA) based on the metabolic labeling approach (48).

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**Isolation of 14N/15N-coded Total Cellular Proteins under Fully Denaturing Conditions**—14N- and 15N-labeled Arabidopsis tissues were ground separately with a −20 °C pre-cooled mortar and pestle in the presence of a urea extraction buffer containing 150 mM Tris-Cl (pH 7.6), 8 M urea, 0.5% SDS, 1.2% Triton X-100, 20 mM EDTA, 20 mM EGTA, 50 mM NaF, 1% glycerol 2-phosphate, 1 mM PMSF, 5 mM DTT, 0.5% phosphatase inhibitor mixture 2 (Sigma), an EDTA-free protease-inhibitor mixture (Complete™), 5 mM ascorbic acid, and 2% polyvinylpyrrolidone. The ratio of Arabidopsis frozen powder to urea extraction buffer was 1:3 (w/v). Plant cell debris were removed.
via centrifugation at a lower speed of relative centrifugal force of 10,000 × g for 1 h at 4 °C and then a higher speed at a relative centrifugal force of 110,000 × g for 2 h at 13 °C. The protein supernatant was then mixed with three volumes of a cold acetone/methanol (12:1 v/v) organic mix for urea-methanol protein precipitation. The protein pellet was rinsed with 15 volumes (v/v) of a cold acetone: methanol:H2O mix (12:1:1.4 v/v) to remove residual pigment and urea precipitates. The protein pellet was air-dried and resuspended in a one-third volume of resuspension buffer containing 50 mM Tris-HCl (pH 6.8), 8 mM urea, 5 mM DTT, 1% SDS, and 10 mM EDTA, and this was followed by another round of protein precipitation and resuspension. The cycling of protein precipitation and resuspension was repeated twice. The resulting total cellular protein was measured via protein DC assay (Bio-Rad) and calculated according to a bovine serum albumin (BSA) protein standard curve. With this method, 1 g of wild-type, ctr1-1, and rcn1-1 Arabidopsis routinely yield 5 to 15 mg of total cellular proteins. Both 15N- and 15N-coded proteins were stored at −80 °C for later use.

**SDS-PAGE Fractionation of Total Cellular Proteins and In-gel Protease Digestion**—In order to control the mixing ratio of proteins well and eliminate possible effects of the variable total cellular protein content (per gram of fresh weight) in the plants and the hand-mixing of frozen tissue powders, both 15N- and 15N-coded total cellular proteins were mixed together at a ratio of 1/q, where q is slightly greater than 1. The rationale was that metabolic labeling with heavy nitrogen often generates an incomplete incorporation, so that the 15N-coded monoisotopic peak is slightly shorter than the 14N-coded ones on average. To give the 15N-coded peptide ion precursor an equal chance of MS/MS acquisition, various values of q (1.0–1.2) were adopted in different biological replicates. A total of 10 mg of protein mixture was loaded onto a 190 mm × 180 mm preparative SDS-PAGE slab gel (10%). Four to five SDS-PAGE slab gels were often used in each biological experiment for the first-dimension separation. Each slab gel was immersed in a Coomassie Brilliant Blue staining buffer for 0.5 h with gentle shaking and destained in a buffer for 0.5 to 2 h. The whole slab gel was sliced evenly into five strips and further diced into (1 × 1.5 × 1.5) mm³ cubes. The gel cubes were washed several times with 50% CH₂CN/50 mM NH₄HCO₃ to remove residual Coomassie Brilliant Blue dye, dehydrated by 100% ACN, and flushed with compressed air until completely dry. Finally, the cubes were rehydrated using 10 mM DTT and alkylated using 55 mM iodoacetamide. Following two additional washes using 50% CH₂CN/50 mM NH₄HCO₃ and complete dehydration, the dried gel cubes were rehydrated with 30 ng/ml trypsin (25 mU NH₄HCO₃, pH 8.0) on ice for 0.5 h and then digested overnight at 37 °C. The digested peptides were then extracted by 1% formic acid in 50% ACN three times. The peptide mixture was then mixed with three volumes of a cold acetone/methanol/methacrylonitrile mixture (12:1:1.4 v/v) to remove residual pigment and urea precipitates. The protein pellet was air-dried and resuspended in a one-third volume of resuspension buffer containing 50 mM Tris-HCl (pH 6.8), 8 mM urea, 5 mM DTT, 1% SDS, and 10 mM EDTA, and this was followed by another round of protein precipitation and resuspension. The cycling of protein precipitation and resuspension was repeated twice. The resulting total cellular protein was measured via protein DC assay (Bio-Rad) and calculated according to a bovine serum albumin (BSA) protein standard curve. With this method, 1 g of wild-type, ctr1-1, and rcn1-1 Arabidopsis routinely yield 5 to 15 mg of total cellular proteins. Both 15N- and 15N-coded proteins were stored at −80 °C for later use.

**LC-MS/MS Data Acquisition and Analysis**—Tandem mass spectrometry (MS/MS) analysis of phosphopeptides was performed using a nanoflow LC (Nano Acquity™, Waters) coupled to an electrospray ionization hybrid Q-TOF Premier tandem mass spectrometer (Waters). Mass spectra of peptides were acquired using the data-dependent acquisition mode of electrospray ionization Q-TOF. The computer program MassLynx (version 4.1, Waters) was used for data acquisition and instrument control. A 180 mm × 20 mm Symmetry C18 trap column and a 75 µm × 250 mm BEH130 C18 analytical column were used. The mass spectrometer was operated in positive ion mode with the following basic parameters: source temperature of 80 °C, capillary voltage of 2.4 kV, sample cone voltage of 35 V, and collision cell gas flow rate of 0.5 ml/min. The collision energy was variable during MS/MS scans according to the z and m/z. The exact settings followed the instructions from the MS machine manufacturer. The data-dependent analysis settings were as follows: 1 s MS, m/z 250–2500 and a maximum of 3 s MS/MS, m/z 50–2500 (continuum mode), 60 s dynamic exclusion. The most abundant +2, +3, or +4 charged ions whose intensity rose above 40 counts/s were subjected to the subsequent MS/MS scan survey.

**Identification of 14N/15N-coded Phosphopeptides**—The resulting MS/MS spectra of phosphopeptide samples were converted to peak list files by Proteinlynx (version 2.2.5) and searched against the Arabidopsis Information Resource Arabidopsis protein database, version 10 (TAIR10, December 2012 release, 35,386 proteins included) using the Mascot search engine (version 2.3, Matrix Science, London, UK). The search parameters were set such that up to one missed cleavage site was allowed. Mass tolerances were set as ±0.5 ppm for MS precursor ions and 0.2 Da for MS/MS fragment ions. Carbamidomethylation (C) was specified as the fixed modification, and phosphorylation (S, T, and Y) and oxidation (M) were allowed as the variable modifications. The quantitation method ‘‘15N metabolic’’ was included in order to identify heavy nitrogen-labeled peptides.

In order to establish a stringent filter for the peptide identification, the false discovery rate (FDR) of peptide identification was estimated using target-decoy searching (61). According to the recommendation of Blanco et al. (62), MS/MS spectra were searched against the target and decoy databases independently. Then the FDR was calculated as...
the number of decoy hits divided by the number of target peptide identifications (63, 64), each of which possesses a satisfactory E-value (i.e. expectation value) that is defined as a composite score for the quality of peptide identification offered by the Mascot search engine. In addition to the FDR estimated for the identification of either 14N- or 15N-coded peptides, the FDR for peptides discovered in pairs (i.e. both 14N- and 15N-coded identical peptides) were also calculated (supplemental Fig. S2).

Quantitative Analysis of 14N/15N-coded Phosphopeptides—Ion chromatograms of a ±150 ppm m/z window were extracted around the monoisotopics of both 14N and 15N isotopic envelopes. These ion chromatograms were integrated for calculation of the areas under the chromatographic peaks, in which the phosphopeptide MS/MS survey was performed. The isotopologue distributions of both 14N- and 15N-coded phosphopeptides were obtained based on the Yergey algorithm (65). The ratio of the whole isotopic envelope of 14N/15N-coded phosphopeptides was calculated using ion counts from the monoisotopic peak and its isotopologue distribution.

\[ \frac{M_{14}}{k_{14}} \frac{M_{15}}{k_{15}} \]  

(Eq. 1)

where \( M_{14} \) and \( M_{15} \) are the ion counts of the monoisotopic peaks of 14N- and 15N-coded phosphopeptides, respectively, and \( k_{14} \)% and \( k_{15} \)% are the percentages of monoisotops in the isotopic envelopes of 14N- and 15N-coded phosphopeptides, respectively. When calculating the isotopologue distribution of 15N-coded phosphopeptides, a 97.43% incorporation rate of 15N into light nitrogen-coded peptides was measured following a procedure described previously (48).

To further eliminate the random variation resulting from the protein mixing, the median value \( m \) of the log2 ratios of ion intensities of 14N/15N-coded phosphopeptide pairs was generated based on each experimental data set (either forward or reciprocal). The actual q of each protein-mixing pair was obtained from the equation \( q = \frac{1}{k} \log_2 \frac{M_{14}}{M_{15}} \). The log2 ratio of the ion intensity of each phosphopeptide pair was normalized against the median values (supplemental Fig. S3). Afterward, the average log2 ratio of the ion intensity of each phosphopeptide pair was calculated according to the normalized ratio of ion intensity of 14N/15N-coded phosphopeptides found from both forward and reciprocal experimental data sets. Only the phosphopeptides that were detected in both forward and reciprocal experiments and found in both 14N- and 15N-coded isoforms were included in the data set for quantification.

The up- or down-regulated phosphopeptides were obtained using the following method. First, the statistical significance of each selected phosphopeptide was determined via a two-tailed t test (unequal variance, \( p < 0.05 \)) (66). Next, the averaged log2 ratios of each experiment were fitted by a normal distribution, and values outside the mean ±2 S.D. of this distribution of phosphopeptides were set as threshold values having a significant change. Only the phosphopeptides showing a significant difference at both individual (i.e. the statistically significant ones from the t test) and population (those with log2 ratios located more than ±2 S.D. apart from the mean of the distribution) levels were considered as regulated phosphopeptides. The detection limit (10%) of the SILIA method (48) was used as an additional filter for significantly changed phosphopeptides.

Bioinformatic Analysis of Phosphorylation Site and Motif Construction—BLAST-based phosphorylation site and motif mining was performed according to a method described previously (38). The newly identified putative phosphoprotein regions with 55.5% or greater homology to the query phosphoprotein sequence were further aligned based on amino acid sequences using the alignment program ClustalW. For the construction of a phosphosite motif, several amino acids on both sides of a phosphosite were chosen from BLAST-searched putative phosphoprotein sequences and subjected to alignment using the substitution matrix pam30. A phosphorylation motif was constructed from the MS-derived authentic phosphosite and three or more of those ClustalW-identified putative phosphorylation sequences.

The in vitro Protein Kinase Assay—His-tagged synthetic peptides containing phosphorylation sites were made and used as substrates in Arabidopsis kinase assays according to a modified protocol (25, 38). To prepare the plant kinase extract, 20 mg of frozen plant tissues were ground in liquid nitrogen in the presence of 60 \( \mu \)l kinase extraction buffer containing 20 mM HEPES, pH 7.5, 50 mM NaCl, 50 mM KCl, 0.3% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM sodium molybdate, 1 mM glycerol-2-phosphate, 1 mM PMSF, and an EDTA-free protease inhibitor mixture (Complete™). The phosphatase inhibitors were excluded in the case of RCN1 phosphatase-specific substrate discovery (sodium pyrophosphate, sodium fluoride, sodium orthovanadate, sodium molybdate, and glycerol-2-phosphate) (67, 68). The kinase extract was centrifuged at for 1 min 4 °C and 14,000 \( \times \) g to remove the cell debris and further subjected to a spun column containing Ni-NTA agarose beads (Qiagen, Venlo, Netherlands) to remove non-specific histag-binding proteins present in the crude Arabidopsis protein extract. A flow-through fraction of 50 \( \mu \)l was mixed with 12.5 \( \mu \)l of kinase assay buffer consisting of 45% glycerol, 2.5 mM ATP, 50 mM MgCl₂, and 125 mg/ml BSA. The peptide substrates of 2 nmoles were added, and the kinase assay mixture was incubated at 30 °C for 30 min. The kinase reaction was terminated by the addition of 130 \( \mu \)l of denaturing buffer (8 M urea, 50 mM Na₂HPO₄, pH 8.0). Polyhistidine-tagged peptides were enriched using 15 \( \mu \)l of Ni-NTA agarose beads as described above. An on-bead trypsin digestion was performed at 37 °C for 4 h. Digested peptides were desalted by C18 ZipTips and reconstituted in 0.1% HCOOH for the LC-MS/MS analysis. As a control, the substrate peptides were also digested alone by trypsin and subjected to LC-MS/MS.

During isobaric tag for relative and absolute quantitation (iTRAQ) labeling, the trypsin-digested substrate peptide mixtures were extracted from the incubation tube using a GE loader tip (GE Healthcare). The resulting mixtures containing 0-, 1-, 5- and 15-min ethylene-treated kinase extracts were lyophilized and incubated with iTRAQ 4-plex labeling buffer (Applied Biosystems, Foster City, CA) that contained iTRAQ reagents providing a chemical linker of m/z 114, 115, 116, and 117 in MS acquisition, respectively, for 1 h at room temperature as previously described (25). The differentially iTRAQ-labeled peptide samples were combined, purified by Fe³⁺ IMAC and TiO₂ beads, desalted sequentially on C18 ZipTips, and resuspended in 0.1% TFA. MALDI-TOF/TOF analyses were performed with a Bruker AutoFlex III MALDI-TOF/TOF MS/Dionex device (Ultraflex, Bruker, Billerica, MA), and the spectra of peptides were collected. The sample was directly spotted onto the MALDI plate because one peptide was analyzed at a time and the phospho-isofrom was enriched. The TOF mode was activated for the precursor ion profiling. When a matching precursor of the target peptide/ phosphorylated peptide was found, the mass spectrometer was switched to LIFT-TOF/TOF mode to acquire the MS/MS spectrum. Finally, the iTRAQ reporter ions of 114, 115, 116, and 117 m/z were integrated. The ion intensity of the reporter ion of the 0-min ethylene-treated sample (m/z 114) was set as 100%. Consequently, changes in the relative phosphorylation level of the ethylene-treated sample at different time points were calculated by comparing the ion intensities of their reporter ions against that of the 0-min ethylene-treated sample. The experiment was repeated at least three times for each peptide.

Computational Prediction of the Kinases Associated to the Phosphorylation Sites—Previously, we developed a GPS software package for the prediction of kinase-specific phosphorylation sites (60). Because the plant kinases were not included in GPS 2.1 (60), here we
Ethylene-regulated Phosphoproteins and Substrates of CTR1

A  **Ethylene treatment time**

| Treatment | Plant Type |
|-----------|------------|
| Air       | rcn1-1     |
| Ethylene  | rcn1-1     |
| Ethylene  | wt         |
| Ethylene  | ctr1-1     |

**SILIA-based quantitative phosphoproteomic analysis**

**Short-term ethylene treatment**

**Long-term ethylene treatment**

**B** Control

**C** 14N

**D** 15N

**E** 14N

**F** 15N

** Fig. 1.** 15N stable isotope metabolic labeling and workflow of differential and quantitative PTM proteomics. A, the metabolic labeling-based quantitative PTM proteomic experimental design for the study of the time-dependent regulation of phosphoproteomes by a plant hormone, ethylene. The left-hand panel is a short-term (1 min) ethylene treatment of rcn1-1, and the right-hand panel is a long-term (3 weeks) ethylene treatment of both the wild type (wt) and ctr1-1. B, overall morphology of Col-0 plants grown in a naturally 14N-abundant growth medium. C, overall morphology of wild-type Arabidopsis plants grown in the naturally 14N-abundant medium (left) and the medium enriched with 15N isotope (right). D, overall morphology of ctr1-1 plants, the loss-of-function line of CTR1 protein, a Raf-like kinase, and a member of the MAPKKK family that is thought to be a negative regulator in the ethylene signaling pathway, grown in the naturally 14N-abundant medium (left) or a medium enriched with 15N isotope (right). E, the immediate precursor of ethylene biosynthesis, 1-aminoacyclopropane-1-carboxylic acid, was added to the growth media at a concentration of 5 μM for both the wild type (C) and ctr1-1 (D). F, overall morphology of 14N- (left) and 15N-labeled (right) rcn1-1, the loss-of-function line of the R CN1 gene, which encodes the A regulatory subunit of PP2A protein phosphatase, treated with 10 ppm ethylene.

**RESULTS**

**15N Stable Isotope Labeling and Ethylene Treatment of Arabidopsis**—The wild-type plant and two loss-of-function ethylene response mutants, ctr1-1 and rcn1-1 (or eer1-1), were selected for quantitative phosphoproteomics to study the phosphoproteomes of time-dependent ethylene responses in Arabidopsis. These plants were grown in either 14N or 15N stable isotope labeling medium (see “Experimental Procedures”). Because both wild-type and ethylene-response mutants are known to have internal ethylene biosynthesis (20, 24, 44, 76, 77), an ACC synthase inhibitor (aminoxyacetic acid) was added to rcn1-1 mutant growth media specifically to reduce the influence of endogenous ethylene. Morphologies of ethylene-treated wild-type Arabidopsis (ecotype Col-0), ctr1-1, and rcn1-1 are shown in Fig. 1. The long-term ethylene-treated forms of both Col-0 (Fig. 1C) and ctr1-1 (Fig. 1D) produce a uniform dwarf phenotype with smaller leaves (6) relative to those of untreated air-grown Arabidopsis (Fig. 1B). To confer a rapid ethylene response to Arabidopsis plants, the ethylene overly sensitive mutant rcn1-1 was also grown on the metabolic labeling medium and subjected to 1 min of ethylene gas fumigation. As a result, no visible morphological alteration was observed between 3-week-old rcn1-1 (Fig. 1E) and Col-0 (Fig. 1B). However, when a standard ethylene triple response assay was performed on rcn1-1, the hypocotyl length of the etiolated seedling of this mutant was reduced 14.9%, 10.2%, and 2.6% in the presence of 0.1, 0.5, and 1 ppm ethylene, respectively, as compared with that of Col-0 treated under the same conditions, which is consistent with the previous finding that rcn1-1 is more sensitive to a lower level of ethylene (44).

**15N isotope (right).** The immediate precursor of ethylene biosynthesis, 1-aminoacyclopropane-1-carboxylic acid, was added to the growth media at a concentration of 5 μM for both the wild type and ctr1-1 (D). E, overall morphology of 14N- (left) and 15N-labeled (right) rcn1-1, the loss-of-function line of the R CN1 gene, which encodes the A regulatory subunit of PP2A protein phosphatase, treated with 10 ppm ethylene.
To perform SILIA-based quantitative phosphoproteomic analysis of both the short-term ethylene-treated rcn1-1 plants and the long-term ethylene-treated Col-0 and ctr1-1, each pair of plant groups was grown on either a medium containing a natural abundance of nitrogen isotope (14N-coded salt) or a medium containing the heavy nitrogen isotope (15N-coded salt). To confirm that heavy nitrogen had no detectable effect on the growth of these plants, the biomass of each Arabidopsis genotype was measured under both light and heavy nitrogen growth conditions. As expected, no significant difference was found in biomass at the time of tissue harvesting between the plant groups (Fig. 1F), which concurs with the previously published conclusion (66).

LC-MS/MS-based Phosphopeptide Profiling of Ethylene-treated Wild Type and Mutants—For quantitative phosphoproteomics analysis on both ethylene-treated wild-type Arabidopsis and ethylene-response mutants, the total cellular proteins were extracted separately from two groups of differentially labeled plant tissues (supplemental Fig. S1). Both 14N- and 15N-labeled proteins were mixed together at a chosen ratio depending on the actual heavy nitrogen labeling efficiency (see “Experimental Procedures”). Four biological replicates (both forward and reciprocal) were performed for both rcn1-1 and ctr1-1 experiments. The mixture of 14N-/15N-coded proteins was then analyzed according to a modified protocol combining gel electrophoresis with LC-MS/MS (38). By this approach, both isotope-coded proteins were processed into five fractions of trypsin-digested peptides derived from five strips of gels and further fractionated via strong cation exchange liquid chromatography. At the end, a total of 40 subfractions of peptides (5 peptide mixtures × 8 LC fractions) were collected and subjected to phosphopeptide enrichment using both Fe3+–IMAC and TiO2 beads in a sequential order (supplemental Fig. S1). The resulting 40 phosphopeptide mixtures were analyzed using a nano-LC electrospray ionization Q-TOF mass spectrometer. As a result, 67,576 peptides were identified by the Mascot search engine. The expectation value was defined as a composite score for peptide identification were determined to be 0.275 and 1.65, respectively (see “Experimental Procedures”; supplemental Table S2). These chemically synthesized peptides were randomly selected from the set of unique phosphopeptides and validated with an in vitro kinase assay of 27 nonphosphorylated synthetic peptides linked with a polyhistidine tag (supplemental Table S2). These chemically synthesized peptides were randomly selected from the set of unique phosphopeptides and used as substrates in in vitro plant kinase assays according to an established protocol (25, 38). As a result, 16 His-tagged peptides were phosphorylated on the expected phosphosites (supplemental Table S2). Thus, 60% of randomly selected MS-identified phosphopeptides were further confirmed by the in vitro plant kinase assay.

To perform a bioinformatics-based differential phosphoproteomic analysis between the newly acquired unique phosphopeptides and those deposited in the PhosPhAt 4.0 database, we selected 627 repetitively detected phosphopeptides (i.e. those identified at least twice; supplemental Table S1) from the 1079 unique phosphopeptides and compared them with those in the PhosPhAt 4.0 database, in which a relatively stringent filter was defined to select the novel phosphopeptides detected. Considering the possible ambiguity in the
Fig. 2. Bioinformatic analysis of phosphopeptides from wild-type Arabidopsis and its ethylene response mutants. A, classification of the phosphopeptides based on the phosphosites identified in which. S, T, and Y represent phospho-Ser, phospho-Thr, and phospho-Tyr in a phosphopeptide, respectively. SS, TT, SY, and ST stand for doubly phosphorylated sites in a phosphopeptide, respectively. STY represents a triply phosphorylated phosphopeptide at three separate sites. The number of phosphopeptides in each class is labeled at the top of each bar. B, the phosphosites identified from Arabidopsis of different genotypes. The phosphosites found in all three genotypes, two genotypes, and the genotype-specific is displayed in cyan, white, and lime colored bars, respectively. The numbers of genotype-specific phosphosites are
ethylation of phosphorylation sites based on MS/MS spectra, all phosphopeptides containing phosphosites deposited in PhosPhAt 4.0 were subtracted regardless of the actual position of Ser, Thr, or Tyr phosphorylation on the peptide. Afterward, the remaining phosphopeptides were examined based on the same criteria used in the PhosPhAt website, and those phosphopeptides collected after the release of PhosPhAt 4.0 were also manually removed. As a result, a total of 44 phosphopeptides were defined as novel phosphopeptides from this work (Table I). Among these novel phosphopeptides, there were 33 rcn1-1-specific phosphopeptides (supplemental Table S3). This is a relatively larger portion of novel phosphopeptides found from rcn1-1, and it might be the result of a deficiency in dephosphorylation activities of PP2A in rcn1-1.

Some of them probably serve as direct phosphoprotein substrates for Arabidopsis PP2A phosphatase, because some rcn1-1-specific phosphoproteins share a significant homology with those PP2A phosphatase substrates found from human cells (see supplemental Table S3 and the section below) (79).

To determine the unique molecular and cellular functions and the subcellular locations of these newly identified phosphoproteins, gene ontology categories of phosphoproteins identified from the wild type (151 phosphoproteins), ctr1-1 (198 phosphoproteins), and rcn1-1 (639 phosphoproteins) were compared. The background for the comparison was the known phosphoproteome (2348 phosphoproteins) of light-grown Arabidopsis (~2 weeks old) deposited in the PhosPhAt 4.0 database (Figs. 2F–2H). The log2 change of each category presented in Figs. 2F–2H was calculated from the following equation:

\[
R_i = \log_2 \left( \frac{n_i}{N} \right)
\]

where \(n_i\) and \(N\) represent the numbers of phosphoproteins belong to the \(i\)th category in a genotype-classified and Arabidopsis phosphoproteomes, respectively, and \(N\) and \(N\) are the total numbers of phosphoproteins in the genotype-classified and Arabidopsis phosphoproteomes, respectively. Comparative analysis of the phosphoproteins from three different genotypes and that of the light-grown Arabidopsis phosphoprotein data set in PhosPhAt 4.0 revealed several interesting findings (Figs. 2F–2H). Both ethylene-treated wild-type and ctr1-1 plants had relatively greater numbers of phosphoproteins in the categories of structure molecule activity (2.30- and 1.98-fold, respectively), nucleic acid (2.14- and 2.06-fold, respectively), protein binding (1.78- and 1.74-fold, respectively), and DNA and RNA binding (1.40- and 1.35-fold, respectively) (Fig. 2F) yet had reduced numbers of phosphoproteins in hydrolase (–1.39- and –1.28-fold, respectively), kinase (–1.92- and –1.60-fold, respectively), and transferase activities (–2.65- and –1.99-fold, respectively) (Fig. 2F). In contrast, phosphoproteins from rcn1-1 enriched in kinase (1.64-fold) and transferase (1.25-fold) and had fewer phosphoproteins in the functions of nucleic acid binding (1.07-fold) than the ethylene-treated wild type (2.14-fold) and ctr1-1 (2.06-fold). Other than that, in all three genotypes of Arabidopsis, phosphoproteins functioning in electron transport or energy pathways (1.81- to 3.76-fold; Fig. 2G) and ribosome localization (1.82- to 2.69-fold; Fig. 2H) or cytosol localization (1.79- to 2.48-fold; Fig. 2H) were much more abundant, and those phosphoproteins relatively less represented were in the categories of DNA or RNA metabolism (–1.40- to –1.91-fold; Fig. 2G) and ER (–1.94- to –4.81-fold; Fig. 2H). The fact that the proportion of proteins functioning in nucleic acid binding increased in both the wild type (2.14-fold) and ctr1-1 mutant (2.06-fold) but not in rcn1-1 mutant (1.07-fold) (Fig. 2F) is quite consistent with the fact that the proportion of phosphoproteins in both transcription and DNA-dependent categories is 1.5-fold in wild type, 1.2-fold in ctr1-1, and 1.1-fold in rcn1-1 (Fig. 2G). A more interesting finding is that the dramatic increase in the categories of kinase activity and transferase activity occurred in rcn1-1 (1.64- and 1.25-fold, respectively) (Fig. 2F); this is consistent with the fact that the phosphoproteins from rcn1-1 are also overrepresented in signal transduction.

**Identification of Phosphosites in Short-term Ethylene-treated rcn1-1 and Long-term Ethylene-treated Wild-type Arabidopsis**—The effects of ethylene on plants have been reported as time dependent (20, 23, 24). To elucidate the molecular basis underlying such a time-dependent ethylene response, SILIA-based quantitative phosphoproteomic analysis was first performed on rcn1-1 to investigate the rapid change in phosphorylation caused by a short-term (1 min) ethylene treatment, because rcn1-1 has been reported to be overly ethylene sensitive (44) and may protect some phosphosites from dephosphorylation because of deficiency in PP2A phosphatase activity. Out of 627 repetitively identified nonredundant phosphopeptides (supplemental Table S1), 595 phosphopeptides were detected in rcn1-1, and 138 phosphopeptides were selected from these for quantitative phosphoproteomic analysis. The criteria for selecting these measurable phosphopeptides were as follows: (1) they were indicated at the top of the bars. C–E, classification of phosphorylation sites in four general amino acid sequence categories classified according to kinase specificity. The phosphorylation sites detected in this work (C), from PhosPhAt 4.0 (D), the overlapped of these two sets of data (E) were analyzed and classified. F–H, Gene Ontology analysis of the phosphoproteins identified from this work in three genotypes. F, molecular functions. G, biological processes. H, cellular components. In each category, the values presented are log2 ratios of percentages of phosphoproteins identified from three genotypes divided by the percentage of phosphoproteins of the whole phosphoproteome (see “Experimental Procedures” for details). Only parts of the bars of category 16 for the wild type and ctr1-1 are shown in panel H, because these two bars become infinitely negative (i.e., no protein detected in this category).
| Accession number | Phosphopeptide | Motif category | Protein | Subcellular location |
|-----------------|----------------|----------------|---------|----------------------|
| Hydrolase activity | AT1G22620 | 443ASQLSHANTAREpSLR | Rx[pS] | Suppressor of actin 1 (ATSAC1) | Golgi apparatus, nucleus |
| | AT1G23240 | 1oxMSHQpTVALASK | | Caleosin-related family protein | Extracellular region |
| Kinase activity | AT3G13530, AT3G07980 | 874,877ISLSANRT | Rx[pS] | MAP3K epsilon protein kinase 1/2 (MAP3KE1/E2) | Cytosol, plasma membrane, vacuole |
| | AT1G23240 | 1oxMSHQpTVALASK | | Forms aploid and binucleate cells 1A (FAB1A) | Cytoplasm |
| Nucleic acid binding | AT1G51140 | 276RPPLAHHM | [pT]xD/E | RAD3-like DNA-binding helicase protein | Mitochondrion, nucleus |
| | AT1G58380, AT1G58983, AT1G59359, AT1G58684 | 270,270,270,270AV | [pS]P | Ribosomal protein S5 family protein | Cytosol, intracellular, membrane, nucleolus, ribosome |
| | AT1G79950 | 106KSLGSFspTR | | Flowering bHLH 3 (FBH3) | Nucleus |
| | AT2G20280 | 106KSLGSFspTR | | Flowering bHLH 3 (FBH3) | Nucleus |
| | AT3G07030 | 7VAKPKPE | | XW6 ribosomal protein S5 family protein | Cell wall, chloroplast, intracellular, membrane, nucleus, plasma membrane, ribosome |
| Other binding | AT2G05100, AT2G05070, AT3G27690 | 40,40,41pTVKSTPQSMYGPD | | Photosystem II light harvesting complex gene 2.1/2.2/2.3 (LHCB2.1/2.2/2.3) | Chloroplast, membrane, vacuolar membrane |
| | AT3G01500, AT5G14740 | 142,137EKYETNPALYGELAKGQ | [pS]P | Carbonic anhydrase 1/2 (CA1/CA2) | Chloroplast, cytoplasm, cytosol |
| | AT3G15290 | 285EATQKPSPRL | [pS]P | 3-hydroxyacyl-CoA dehydrogenase family protein | Cytoplasm, peroxisome |
| | AT5G66190 | 164LVYpTNDGGENK | | Ferrodoxin-NADP(+) oxidoreductase 1 (FNR1) | Chloroplast |
| Structural molecule activity | AT3G60770, AT4G00100 | 20RS | [pS]P | Ribosomal protein S13/S13A (RPS13/RPS13A) | Cell wall, chloroplast, cytosol, intracellular, membrane, nucleolus, ribosome |
| Accession number | Phosphopeptidea | Motif category | Protein | Subcellular location |
|------------------|-----------------|----------------|---------|---------------------|
| **Transferase activity** |
| AT1G26850 | 111DpS\text{ox}MIYR | [pS]P | S-adenosyl-l-methionine-dependent methytransferase superfamily protein | Golgi apparatus, membrane |
| AT2G13360 | 3\text{p}N\text{p}S\text{p}APEKALT | [pS]P | Alanine:glyoxylate aminotransferase (AGT) | Chloroplast, membrane, peroxisome, plasma membrane |
| **Transporter activity** |
| AT1G75220 | 19RPFIHTGpSWYR | [pS]P | ERD6-like 6 (ERDL6) | Golgi apparatus, membrane, plant-type vacuole membrane, plasma membrane, vacuolar membrane |
| AT1G62020, AT2G21390 | 358,358RPFG | [pS]P | Coatomer, \( \alpha \) subunit | Cytosol, intracellular, membrane, plasma membrane |
| AT4G33530 | 203pS\text{ox}GLKPVPSPELER | [pS]P | K\textsuperscript{+} uptake permease 5 (KUP5) | Membrane, plasma membrane, vacuolar membrane |
| **Unknown functions** |
| AT1G11110 | 162EpTFNFAEK | LiSH and RanBPM domain-containing protein | Nucleus |
| AT1G20890 | 4\text{p}ETLPLKSgpSR | Unknown protein | Nucleus |
| AT1G35220 | 308LIDPSVHLHKGpSPR | [pS]P | Vacuolar protein sorting-associated protein 26 | Nucleus |
| AT1G52100 | 249ApS\text{ox}GKVTGSKFK | [pS]P | Mannose-binding lectin superfamily protein | Membrane |
| AT1G70100 | 9\text{p}NaSpSRFPQKNR | Rx[pS] | Unknown protein | Nucleus |
| AT1G70180 | 249D\text{p}SSPPRDAGR | Rx[pS]P | Sterile \( \alpha \) motif (SAM) domain-containing protein | Nucleus |
| AT1G70180 | 182pAGSFRPRpSR | Rx[pS]P | Sterile \( \alpha \) motif (SAM) domain-containing protein | Nucleus |
| AT3G50750, AT1G78700 | 33,25AIAAKIFpTGLR | [pS]P | BES1/BZR1 homolog 1/4 (BEH1/BEH4) | Nucleus, vacuole |
| AT2G30105 | 165LpSFGSMQK | Leucine-rich repeat | Nucleus |
| AT2G46180 | 319KLP\text{p}pTEDLSR | [pS]xX/E | Golgin candidate 4 (GC4) | Golgi apparatus |
| AT3G08780 | 19G\text{p}H\text{p}SFpSPK | [pS]P | Unknown protein | Nucleus |
| AT3G58440 | 538LF\text{p}VDEpS\text{p}QLQK | [pS]P | TRAF-like superfamily protein | Cytoplasm |
| AT3G60380 | 412K\text{p}EFFpSKK | [pS]xX/E | Unknown protein | Nucleus |
| AT4G21930 | 96H\text{p}VP\text{p}S\text{p}VKP\text{p}DWSK | Protein of unknown function, DUF584 | Cellular component |
| AT4G37210 | 478KPALEFSKD\text{pp}ADGNpSS | Tetraicopeptide repeat (TPR)-like superfamily protein | Nucleus |
| AT5G04550 | 300\text{ox}MG\text{p}pTPERPSISSVKK | [pT]P | Protein of unknown function (DUF668) | Nucleus |
| AT5G18590 | 19KVQ\text{p}LSDVQGYpSPLR | [pS]P | Galactose oxidase/kelch repeat superfamily protein | Cytosol, nucleus |
| AT5G63190 | 3SHTLF\text{p}AL\text{p}NpSPTGGK | [pS]P | MA3 domain-containing protein | Nucleus |

*a* The phosphorylated amino acids (Ser, Thr, or Tyr) are denoted by *p*, and oxidized Met is denoted by *ox.*
detected in both forward and reciprocal experiments, (2) they were found in both $^{14}$N- and $^{15}$N-coded phosphopeptide isoforms, and (3) they were detected at least three times. Lastly, the light ($^{14}$N-coded) and heavy ($^{15}$N-coded) phosphopeptide isotopic envelopes were measured only after the MS spectrum of each phosphopeptide was verified by means of manual spectrum inspection and all MS spectra of overlapping noise ion(s) had been removed. As a result, 138 nonredundant phosphopeptides were selected for quantification, each of which was supported by 2 to 27 pairs of MS ion peaks.

Because light and heavy isoforms of a phosphopeptide produce distinct isoform envelopes, the ratios of twin isoform envelopes of each peptide were calculated based on the extracted ion chromatograms of monoisotopic peaks and the isotopologue distribution (48). For all nonredundant phosphopeptides quantified, each pair of MS ion peaks yielded a ratio, and a two-tailed $t$ test ($p < 0.05$) was performed on the measurement of each nonredundant phosphopeptide to determine the statistical significance. In the $rcn1-1$ experiment, 17 out of 138 phosphopeptides that were quantified according to this method showed a statistically significant difference between 1-min ethylene-treated and air-treated samples (supplemental Table S4). The quantified phosphopeptides were further fitted using a normal distribution. Those phosphopeptides whose average variance was more than the mean ± 2 S.D. outside of the normal distribution were considered as significantly altered phosphopeptides (Figs. 3A–3C; supplemental Figs. S4A and S4B). As a result, three ethylene-enhanced and three ethylene-suppressed phosphopeptides were found from the 1-min ethylene-treated $rcn1-1$ mutant (Table II, supplemental Figs. S5 and S6).

Interestingly, the ethylene-induced alteration in phosphorylation level can be found on proteins ranging from plasma-membrane-bound leucine-rich repeat transmembrane protein kinase to nucleus-localized RNA-binding protein arginine/serine-rich splicing factor 41 (ATRSP41, AT5G52040). The phosphorylation level of $^{327}$GLIDTAGHHPpTV derived from $^{14}$N-ATPase 1 (AHA1, AT2G18960) was rapidly induced by ethylene. This protein is crucial in many processes such as nutrient uptake, cell elongation, pH homeostasis, stomatal movement, and salt tolerance (80). The phosphorylation of the conserved T948 residue at the C terminus modulates the enzymatic activity of proton pumps by either inducing or inhibiting association with 14–3-3 proteins (81). Thus, the altered phosphorylation level that we found may suggest a signaling pathway from ethylene to the inhibition of cell elongation via AHA1. Moreover, the phosphorylation level of high mobility group B2/3 (HMGB2/3, AT1G20693/AT1G20696) is up-regulated by ethylene within 1 min of ethylene induction. Previous research showed that both hydrophobic properties and negative charge determine the binding of proteins from this group to DNA (82, 83). In addition, protein sequence alignment shows that both hydrophobic and negatively charged residue surrounding the phosphorylation site are alternately allocated, and phosphorylated serine can contribute one extra negatively charged side chain and mimic aspartic acid (D) in DNA binding. Therefore, the conformational change resulting from the phosphorylation of serine on HMG proteins may result in enhanced stability and DNA-binding specificity for these proteins. Another interesting feature of HMBG proteins is that the phosphorylated sequence may possess a combinational code for functional modulation (84). Some of these combinations of PTMs will lead to higher residence in transcriptionally inactive heterochromatin.

To further confirm the significantly changed phosphorylation levels found via quantitative phosphoproteomic analysis on $rcn1-1$ phosphoproteins, additional validations were performed in which a slightly ethylene-enhanced phosphopeptide, $^{79,76}$SLpSDSEKAPYVAK (derived from HMGB2/ B3), and a slightly ethylene-suppressed phosphopeptide, $^{270}$GEpSPPPPYEKR (derived from ATRSP41), were selected for time course measurements of dynamic change in response to ethylene treatment. To that end, these two His-tagged and nonphosphorylated synthetic peptides were subjected to the $in vitro$ plant kinase assay as described (25, 38). As a result, both S81 of HMGB2 (S78 of HMGB3) and S274 of ATRSP41 were phosphorylated as expected by $rcn1-1$ kinase(s) (Figs. 4A–4D). To confirm such time-dependent phosphorylation on these phosphosites occurring within first 15 min of ethylene treatment, iTRAQ-based quantitative phosphoproteomics was employed to measure the phosphorylation levels of these two sites over a short period of time. Four groups of adult $rcn1-1$ Arabidopsis plants were treated with ethylene for 0, 1, 5, and 15 min, respectively, before being harvested. The plant kinases were then extracted and mixed with differentially isobaric tag-labeled synthetic peptides (25).

As shown in Figs. 4E and 4F, the phosphorylation of S81 of HMBG2 (or S78 of HMGB3) and S274 of ATRSP41 was indeed up- and down-regulated, respectively, by a few minutes of ethylene treatment in $rcn1-1$. The phosphorylation level of the HMBG site changed to 172.3% ± 17.3%, 183.7% ± 30.9%, and 149.4% ± 11.5%, respectively, at 1, 5, and 15 min of ethylene treatment (supplemental Fig. S7). In contrast, the phosphorylation of S274 of ATRSP41 decreased to 99.4% ± 8.6%, 82.6% ± 3.1%, and 44.7% ± 7.6%, respectively, at 1, 5, and 15 min of ethylene treatment (supplemental Fig. S7). These phosphorylation changes are presented as a function of ethylene treatment time for both ethylene early responsive phosphopeptides (Figs. 4E and 4F). The trend of altered phosphorylation of these phosphosites measured by iTRAQ within 5 to 15 min is the same as that detected via SILIA-based quantitative phosphoproteomics at 1 min of induction. Thus, it is concluded that metabolic labeling-based quantitative phosphoproteomics is more suitable for identifying phosphorylation alterations occurring within 1 min of induction, whereas iTRAQ may be suitable for the multiplex quantitation of phosphopeptides collected from multiple time points simultaneously. Our
results also showed that ethylene-triggered phosphorylation alteration quickly occurs on both plasma-membrane-bound and nucleus-localized nucleotide-binding proteins within a few minutes of induction.

The CTR1 gene has been defined as a negative regulator of ethylene response in Arabidopsis according to molecular genetic results because the recessive and loss-of-function mutant ctr1-1 exhibits constitutive ethylene-treated phenotypes.

**Fig. 3.** SIIIA-based quantitative and differential phosphoproteomic analysis of ethylene-treated Arabidopsis wild type and ethylene response mutants. A and D, quantitative analysis of 1-min ethylene-regulated phosphoproteome in rcn1-1 mutant, which lost the functional A regulatory subunit RCN1 of PP2A protein phosphatase (A), and a long-term ethylene-regulated phosphoproteome in both the wild type (wt) and ctr1-1, loss-of-function of CTR1 protein, which encodes a Raf-like kinase of the MAPKKK family (D). Each dot represents the average ratio of the peptide amount of a certain phosphopeptide identified from either the ethylene-treated to air control rcn1-1 (A) or the wild type to ctr1-1 (D). The phosphopeptides of statistical significance defined via t test are highlighted in red. The dashed lines delineate the mean ± 2 S.D. of the distribution. B and E, histograms of phosphopeptides from A and D, respectively, fitted using a normal distribution (red curve). The dashed lines demarcate the mean ± 2 S.D. of the distribution of the log2 ratio of phosphopeptide ion intensities. C and F, MS spectra of 1-min ethylene-up-regulated, -independent, and -down-regulated phosphopeptides in rcn1-1 and the MS spectra of long-term ethylene-treated and CTR1-up-regulated, -independent, and -down-regulated phosphopeptides. “Forward” and “reciprocal” refer to the order of mixing of 15N-coded proteins with 14N-coded protein samples (see “Experimental Procedures”). E and A, the isotopologues of an identical phosphopeptide ion derived from ethylene-treated and air-treated rcn1-1 protein samples, respectively (C). “W” and “C” indicate isotopologues of an identical phosphopeptide ion derived from wild-type and ctr1-1 mutant plant protein samples, respectively (F). “M/z” represents the ratio of the ion mass to the charge of each phosphopeptide. p marks the amino acid type at a phosphosite.
Ethylene-regulated Phosphoproteins and Substrates of CTR1

| Phosphopeptide affected by 1-min ethylene treatment in rcn1-1 | Phosphopeptide | Motif category | Protein | Subcellular location |
|-------------------------------------------------------------|----------------|----------------|----------|----------------------|
| Accession number | Fold-change | p value | ETHylene enhanced | 1.15 | 3.1E-02 | AT2G02070 | Intracellular, nucleus | Arabidopsis thaliana | Arabidopsis thaliana |
|                  | 1.14 | 3.1E-02 | AT2G18960 | Intracellular, nucleus | Arabidopsis thaliana | Arabidopsis thaliana |
|                  | 1.18 | 7.4E-03 | AT1G07650 | Intracellular, nucleus | Arabidopsis thaliana | Arabidopsis thaliana |
|                  | 1.12 | 9.0E-03 | AT1G70730 | Intracellular, nucleus | Arabidopsis thaliana | Arabidopsis thaliana |
|                  | 1.12 | 7.6E-04 | AT5G52040 | Intracellular, nucleus | Arabidopsis thaliana | Arabidopsis thaliana |

The two values in bold are those selected for in vitro phosphorylation assays. The phosphorylated amino acids (Ser, Thr, or Tyr) are marked by c. (equivalent to a long-term ethylene treatment) in the absence of external ethylene gas in both seedling and adult plant stages (6). This gene encodes a homolog of the Raf-like family of protein kinases and a member of the plant MAPKKK family (6). This MAPKKK enzyme interacts with the ethylene receptor complex. Such an interaction may be involved in the conversion of CTR1 kinase into a dimeric and active conformation (9, 10). Ethylene is therefore postulated to inactivate the kinase activity of CTR1 and dissociate it from ethylene receptor complexes, leading to the plant ethylene response. To determine whether CTR1 MAPKKK still has protein phosphorylation activities under ethylene treatment and whether there are other direct and indirect protein substrates (in addition to EIN2) for CTR1 kinase, SILIA-based quantitative and differential phosphoproteomics were performed simultaneously on both wild-type and ctr1-1 plants under long-term ethylene treatment. In this experimental design, the sole difference between ethylene-treated wild-type plants and ctr1-1 is the CTR1 kinase. In the wild-type Arabidopsis, CTR1 kinase is still physically present in plant cells under ethylene treatment, but CTR1’s kinase activity is unknown. In contrast, in the ctr1-1 mutant, CTR1 (MAPKKK) kinase activity is completely lost. Differential and quantitative phosphoproteomic analyses of these plants grown under long-term ethylene treatment revealed 70 nonredundant measurable phosphopeptides, and each of them was supported by 2 to 21 pairs of MS ion peaks. Out of the quantified 70 phosphopeptides, there was a statistically significant difference between Col-0 and ctr1-1 samples for 17 (supplemental Table S5). All phosphopeptides quantified were fitted using a normal distribution. The mean ± 2 S.D. of the normal distribution was used for the selection of CTR1-regulated phosphopeptides (Figs. 3D–3F; supplemental Figs. S4C and S4D). Eventually, it was found that the phosphorylation levels of six phosphopeptides were indeed up-regulated and those of two phosphopeptides were down-regulated by the CTR1 gene under constant ethylene exposure (Table III, supplemental Figs. S8 and S9).

Because both wild-type Arabidopsis and ctr1-1 were treated with a higher dose of ethylene (10 ppm), the differential phosphoproteome subtraction between Col-0 and ctr1-1 is believed to have subtracted the phosphorylation differences resulting from receptor histidine and serine/threonine kinase activities and other unknown kinase activities and to have provided informative results on the putative direct or indirect substrates for CTR1 MAPKKK under the influence of constant ethylene exposure. In particular, using the loss-of-function mutant ctr1-1 as a genetic background, the long-term ethylene-treated wild-type Arabidopsis accumulates in parallel phosphorylation at the T451 site of plastid transcriptionally active 16 (PTAC16, AT3G46780) protein, suggesting that the CTR1 kinase in the wild-type plant may use PTAC16 as a putative (either direct or indirect) downstream substrate under ethylene treatment. This result implies that CTR1 protein may be active under ethylene treatment, or at least par-
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Fig. 4. Validation of phosphosites using in vitro plant kinase assay. A and B, the MS/MS spectra of a synthetic peptide SLSDSEKAPYVAK and its phosphorylated isofom at the S3 position, respectively. C and D, the MS/MS spectra of another synthetic peptide, GERSRPSPYFYER, and its phosphorylated isofom at the S5 position, respectively. The gray letters in brackets denote the residues digested from the synthetic peptide. E and F, the time course of ethylene-regulated in vitro kinase activities of rcn1-1. The x-axis indicates the duration (in minutes) of the ethylene treatment. The phosphorylation level of phosphopeptides marked along the y-axis was determined according to the iTRAQ method (38), and the relative phosphorylation levels were calculated versus the intensity of a reporter ion from 0-min ethylene-treated samples (see “Experimental Procedures” for details). The deviation at each time point represents the average of three to five individual experimental results. The synthetic substrate peptides used for iTRAQ-based relative phosphorylation quantification were SLSDSEKAPYVAK (E) derived from high mobility group B2/3 protein (AT1G20693/AT1G20696) and GERSRPSPYFYER (F) derived from arginine/serine-rich splicing factor 41 (AT5G52040).

was determined according to the iTRAQ method (38), and the relative phosphorylation levels were calculated versus the intensity of a reporter ion from 0-min ethylene-treated samples (see “Experimental Procedures” for details). The deviation at each time point represents the average of three to five individual experimental results. The synthetic substrate peptides used for iTRAQ-based relative phosphorylation quantification were SLSDSEKAPYVAK (E) derived from high mobility group B2/3 protein (AT1G20693/AT1G20696) and GERSRPSPYFYER (F) derived from arginine/serine-rich splicing factor 41 (AT5G52040).
### TABLE III

CTR1 gene-regulated phosphopeptides

| Accession number | Fold-change | p value* | Phosphopeptide* | Motif category | Protein | Subcellular location |
|------------------|-------------|----------|----------------|----------------|---------|---------------------|
| AT3G46780        | 1.77        | 4.06E-03 | 444VQVAPTVR    |                | Plastid transcriptionally active 16 (PTAC16) | Chloroplast, membrane |
| AT5G23060        | 1.73        | 3.05E-10 | 345STGKFLPSSD  |                | Calcium sensing receptor (CaS) | Chloroplast, mitochondrion |
| AT1G29920, AT1G29910, AT1G29930, AT2G34430, AT2G34420 | 1.71  | 2.05E-04 | 44444444444GSPSPWYGSDRKV | [pS]P | Light harvesting chlorophyll A/B binding protein 1.1/1.2/1.3/1.4/1.5 (LHCB1.1/1.2/1.3/1.4/1.5) | Chloroplast, membrane, mitochondrion |
| AT1G654500       | 1.61        | 2.04E-02 | 113KSAPSFHTLDELER |                | Glutaredoxin family protein | Nucleus |
| AT1G29920, AT1G29910, AT1G29930, AT2G34430, AT2G34420 | 1.60  | 3.62E-03 | 44444444444GSPSPWYGSDRKV | [pS]D/E | Light harvesting chlorophyll A/B binding protein 1.1/1.2/1.3/1.4/1.5 (LHCB1.1/1.2/1.3/1.4/1.5) | Chloroplast, membrane, mitochondrion |
| AT2G426500, AT3G14940, AT3G42628 | 1.54  | 1.79E-02 | 9.9.9xVQpSIDAQLR |                | PEP carboxylase 2/3 (PPC2/3)/PEP carboxylase-related | Chloroplast, cytoplasm, cytosol, plasma membrane |
| AT3G53420, AT2G37170, AT2G37180 | 1.82  | 2.64E-03 | 277277277SLGpSFpSAANV |                | Plasma membrane intrinsic protein 2A/B/C (PIP2A/B/C) | Chloroplast, membrane, plasma membrane, vacuole |
| AT3G22850        | 1.42        | 8.53E-03 | 232pVQpSVQWNSK | Rxx[pS] | Aluminum induced protein with YGL and LRDR motifs | Cytoplasm, cytosol, nucleus, plasma membrane |

Note: Both wild-type and ctr1-1 mutant Arabidopsis were grown in the presence of ACC. 
*Given by two-tailed Student’s t test. All phosphopeptides listed were selected using two-tailed Student’s t test ([pS]/H11021 p < 0.05). The phosphorylated amino acids (Ser, Thr, or Tyr) are denoted by p, and oxidized Met is denoted by oX.
family, or subfamily would recognize similar patterns/motifs in substrates for modification, the corresponding predictor in GPS 2.1 was assigned for each Arabidopsis kinase if available (see “Experimental Procedures”) (60). In the end, 932 kinases were selected with GPS predictors (supplemental Table S6).

Each ethylene-regulated phosphosite was analyzed with GPS 2.1, and the default threshold (medium) (60) was chosen with false positive rates of 6% and 9% for serine/threonine kinases and tyrosine kinases, respectively. The site-specific kinase–substrate relations were then determined based on the kinase-predictor mapping list (supplemental Table S6) and further filtered using the existing PPI databases to reduce false positive predictions (see “Experimental Procedures”). As a result, a total of 23,674 experimentally identified PPIs and 717,810 predicted PPIs in Arabidopsis were taken from online databases (supplemental Table S6; see “Experimental Procedures”). A predicted site-specific kinase–substrate relation was accepted only if the relationship between kinase and substrate was supported by either experimental results or predicted PPIs.

A total of 14 site-specific kinase–substrate relations covering 11 kinases and 11 phosphosites on 10 substrate proteins were eventually predicted (supplemental Table S6, Fig. 6). Firstly, in the case of the ethylene-regulated phosphosites detected from rcn1-1 mutant, calmodulin-binding receptor-like cytoplasmic kinase 2 (AT4G00330) was predicted as a protein kinase family. The other 14 site-specific kinase–substrate relations were determined based on the kinase-predictor mapping list and further filtered using the existing PPI databases to reduce false positive predictions (see “Experimental Procedures”). As a result, a total of 23,674 experimentally identified PPIs and 717,810 predicted PPIs in Arabidopsis were taken from online databases (supplemental Table S6; see “Experimental Procedures”). A predicted site-specific kinase–substrate relation was accepted only if the relationship between kinase and substrate was supported by either experimental results or predicted PPIs.
protein kinase AT1G07650 (see Fig. 6A). Secondly, in the ctr1-1 experiment, the CTR1 up-regulated phosphorylated α/β-hydrolase superfamily protein AT5G38520 was predicted as a substrate of a protein kinase superfamily protein AT1G54820, LHCB1.1 and LHCB 1.5 as substrates of protein kinase 2B (APK2B, AT2G02800), glutaredoxin family protein AT1G64500 as a substrate of both SNF1-related protein kinase 2C (AT1G78290) and CBL-interacting protein kinase 1 (CIPK1, AT3G17510), PIP2A as a substrate of altered seed germination 5 protein kinase (ASG5, AT1G20650), and finally PIP3 (AT4G35100) as a substrate of D6 protein kinase (AT5G55910) (see Fig. 6B). Lastly, in the case of the putative substrates of PP2A phosphatase obtained from homologous alignment, both octicosapeptide/Phox/Bem1p domain superfamily protein kinase AT5G57610 and calmodulin-domain protein kinase 9 (AT3G20410) were predicted as protein kinases that modify the flowering bHLH 3 protein (FBH3, AT1G51140). In addition, both cyclin-dependent kinase B1 (AT3G54180) and inflorescence meristem receptor-like kinase 2 (AT3G51740) were predicted as protein kinases modifying ribosomal protein S13 (AT3G60770), and the inflorescence meristem receptor-like kinase 2 was also predicted as a protein kinase modifying tetratricopeptide repeat-like superfamily protein AT4G37210 (see Fig. 6C).

The cascades of enzyme–substrate relationships defined by the GPS predictor were further substantiated by data presented in the literature. The previous work has already revealed that more than half of the predicted kinases are regulated by ethylene (35). Calmodulin-binding receptor-like cytoplasmic kinase 2 (kinase of leucine-rich repeat transmembrane protein kinase AT1G07650), SNF1-related protein kinase 2C (kinase of glutaredoxin family protein AT1G64500),

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**Fig. 6.** Bioinformatic analysis of ethylene signaling-related kinase-substrates networks. A, phosphoproteins up- or down-regulated by ethylene and the putative kinases involved in the early stage (1 min) of ethylene-regulated protein phosphorylation. B, phosphoproteins up- or down-regulated by CTR1 MAPKKK in the presence of ethylene and the putative kinases involved in ethylene- and CTR1 kinase-regulated protein phosphorylation. C, prediction of putative substrates of PP2A. Green box: PP2A; green circle: CTR1 kinase; red octagons: authentic positively regulated substrates; pink octagons: predicted positively regulated substrates; gray ellipses: authentic negatively regulated substrates; purple ellipses: predicted negatively regulated substrates; peach circles: kinases found from the literature; lime circles: kinases predicted by GPS; dashed lines with arrows: putative positive regulation; dashed lines with stop ends: putative negative regulation; solid lines with arrows: kinase phosphorylation reported in the literature; double lines with arrows: direct kinase phosphorylation predicted by GPS; separated arrows: authentic positive regulation; separated stops ends: authentic negative regulation.
and calmodulin-domain protein kinase 9 (kinase of FBH3) are enhanced by ethylene, and protein kinase superfamily protein AT1G54820 (kinase of α/β-hydrolase superfamily protein AT5G38520), CIPK1 (kinase of glutaredoxin family protein AT1G64500), ASG5 (kinase of PIP2A), and inflorescence meristem receptor-like kinase 2 (kinase of RPS13 and tetratricopeptide repeat-like superfamily protein AT4G37210) are suppressed by ethylene (35). Interestingly, FBH3 is also a putative PP2A phosphatase substrate as determined by this work (supplemental Table S3). Thus, calmodulin-domain protein kinase 9 and PP2A may constitute a modifying/demodifying enzyme pair that regulates the phosphorylation of FBH3 in an antagonistic fashion. Furthermore, the glutaredoxin family protein AT1G64500 may be directly phosphorylated by CIPK1 according to the GPS predictor. Interestingly, the expression of CIPK1 happens to be regulated by CTR1 (91). In our quantitative phosphoproteomics experiment on ethylene-treated wild-type/crrl-1 pairs, CTR1 was found to enhance the phosphorylation of glutaredoxin family protein AT1G64500 (Fig. 6B, Table III). It is therefore likely that CTR1 may regulate the phosphorylation of glutaredoxin family protein AT1G64500 by regulating CIPK1.

In short, via the application of both GPS predictor analysis and bioinformatics analysis of rapidly ethylene-regulated and CTR1-regulated phosphoproteins, we have established several ethylene-signaling networks that have not been reported in the literature previously (Fig. 6). The integration of quantitative phosphoproteomics and PPI GPS prediction is able to provide useful information for directing the future functional validation of these phosphoproteins and kinases in the context of cell signaling.

**DISCUSSION**

*In vivo* stable isotope metabolic labeling, especially heavy nitrogen (15N) labeling, has been successfully applied to characterize PTMs in various organisms (66, 92, 93). Given the availability of a huge collection of Arabidopsis mutant lines, we chose to apply 15N stable isotope metabolic labeling-based quantitative PTM proteomics to analyze two highly selected and well-characterized ethylene response mutants, crrl-1 and rcm1-1 (or eer1-1), to identify phosphorylation events induced by both short-term and long-term ethylene treatment. Ethylene was found to quickly trigger phosphorylation alteration of proteins located in various cellular compartments ranging from the cytosol to the nucleus (Table II). The 1-min ethylene-induced protein phosphorylation profile is dramatically different from that resulting from constant ethylene treatment of the wild type and crrl-1 (Table III). The successful identification of novel rapidly ethylene-induced phosphosites may be attributed to the SILIA-based quantitative phosphoproteomic approach (48), in which all protein molecules including proteases and phosphatases are instantly denatured by a high concentration of urea (8 M) once the frozen cells are dissolved into the protein isolation solution. A second contributor to this success was the use of a mutant defective in phosphatase (PP2A) activity. The application of quantitative phosphoproteomics on various phosphatase-deficient Arabidopsis mutants may become a powerful way to profile more novel phosphosites and contribute to the establishment of phosphor-relay-mediated signaling networks in Arabidopsis, especially given that Arabidopsis contains more than 150 phosphatases (94). The usefulness of this approach is further confirmed by the identification of several putative substrates for PP2A from the integration of quantitative phosphoproteomic and bioinformatic analysis (supplemental Table S3).

Lastly, the success may be attributed to the employment of the stable isotope metabolic labeling strategy. Given that the total amount of cellular proteins is similar in each pair of plant tissues (air versus ethylene-treated rcm1-1 and ethylene-treated wild type versus crrl-1), this 15N metabolic labeling technique emphasizes the mixing of two differentially labeled total cellular proteins at the initial step, which allows targeted proteins 14N/15N differentially coded under two separate physiological conditions to go through identical protein separation, peptide generation and isolation, peptide ionization, and ion intensity quantification processes so that variations occurring from each step mentioned above are eliminated, unlike with the iTRAQ technique (36, 58, 59).

Molecular genetics and biochemical data have demonstrated that both ethylene response 1 and ethylene response sensor 1 physically interact with CTR1 kinase, and these interactions are required in ethylene signaling (9, 10). Once an ethylene receptor–CTR1 kinase complex interacts with ethylene, its signal is transduced downstream through altered kinase activities and phosphor-relay to other signaling components. However, the finding that CTR1 kinase interacts separately with both ethylene receptor and EIN2 and consequently phosphorylates EIN2 (11, 12) to inactivate the central positive regulator of ethylene responses still does not explain how the ethylene receptor–CTR1 kinase complex physically transduces the ethylene binding of receptor complexes to both histidine (or aspartic acid) kinase activity of the receptor and serine/threonine kinase activity of CTR1, because it is still uncertain how many ethylene receptor–CTR1 kinase complex-dependent substrates are present in plant cells. The physical association of Arabidopsis PP2A with the kinase domain of CTR1 in *vitro* has further complicated the mode of action of ethylene receptor–CTR1 kinase complexes. It has been hypothesized that the increased sensitivity and amplitude of plant responses to ethylene observed in the rcm1 (or eer1-1) loss-of-function mutant are achieved through the enhanced effectiveness of CTR1 kinase inactivation resulting from the binding of ethylene, which in turn lowers the threshold of induction required for the development of the ethylene response phenotype (45). Moreover, in the regulation of CTR1 MAPKKK on the stability of EIN3, the role of the mitogen-activated protein kinase kinase 9–mitogen-activated protein kinase 3/6 cascade is still controversial (37, 95); protein phosphorylation-mediated ethylene signaling may include all these...
possible kinase activity outputs and multiple signaling pathways. Our quantitative phosphoproteomics performed on wild-type Arabidopsis and ctr1-1 plants, which were of a similar morphological phenotype (Fig. 1) and exposed to identical ethylene treatments, revealed distinct and differential phosphopeptide profiles between them (Table III). Given the existing model by which ethylene inactivates CTR1 kinase activities, leading to the dephosphorylation of EIN2 S645 and S924 (11, 12), our differential phosphoproteomics results strongly suggested that kinase activities of the ethylene receptor–CTR1 complex may still exist after ethylene treatment (Table III). Alternatively, it is likely that the physical presence of CTR1 protein enhances the phosphorylation of some phosphosites indirectly. It is also possible that there are ethylene-induced kinase activities other than that of CTR1 kinase, which transduces the ethylene binding of the receptor to downstream protein phosphorylation events in parallel with CTR1 kinase. Furthermore, it is also possible that the ethylene signaling from the binding of ethylene to protein kinase activity output may occur through alteration of the protein substrate specificity of CTR1 kinase by the ethylene-modified receptor complex. Nevertheless, these multiple protein phosphorylation-mediated signal transduction pathways may integrate with EIN2-mediated transcriptional activities to constitute an intricate dual-and-opposing effect of ethylene on the production of phosphorylated isoforms that have been shown to be required for downstream protein phosphorylation events in parallel with CTR1 kinase. This finding suggests possible linkages between ETH11 (64, 65) and CTR1 kinase-up-regulated and chloroplast-located phosphoproteins, it has been reported that phosphorylation of the T451 amino acid of PTAC16 and the S48 amino acid of LHCb1.1–1.3 (S47 of LHCb1.4 or S46 of LHCb1.5) are STN7-dependent and -independent, respectively (85). Thus, it is likely that there is more than one pathway from CTR1 kinase to chloroplast. Even though the changes are relatively small, it was revealed via Student’s t test from the rcnl-1 experiment that the phosphorylation levels of both T451 of PTAC16 and S48 of LHCb1.1–1.3 (S47 of LHCb1.4 or S46 of LHCb1.5) in 1-min ethylene-treated plants are lower than those in air-treated plants (see supplemental Tables S4 and S5). This may be caused by reduced kinase activity of CTR1 under ethylene treatment. This observation clearly indicates that the kinase activity of CTR1 is reduced but not completely inhibited. The light-harvesting chlorophyll a/b-binding protein family is required for stomatal movement (102). Because the expression of LHCb1.1 is repressed by both glucose and fructose in wild-type Arabidopsis but not in ctr1-1 mutant, it is concluded that the sugar-mediated repression of LHCb1.1 is CTR1-dependent (103, 104). Because CTR1 kinase also enhances the phosphorylation of LHCb1.1, it is likely that the production of the phosphorylated isoform of LHCb1.1 can be dually and oppositely affected by CTR1 kinase or CTR1-regulated kinase/phosphatase in the cell signaling systems (Fig. 7D).

Finally, FBH3 (AT1G51140) is one of four flowering bHLH proteins. The overexpression of FBH3 causes early flowering
Fig. 7. Proposed models for ethylene signaling networks. A, a model of the ethylene signaling network is reconstructed by integrating the phosphoproteins regulated by either ethylene or CTR1 protein with the well-known linear ethylene signaling pathway. B, dual-and-opposing effect of ethylene on arginine/serine-rich splicing factor (ATRSP41, AT5G52040). C, dual-and-opposing effect of ethylene on plasma membrane intrinsic protein 2A (PIP2A, AT3G53420). D, dual-and-opposing effect of CTR1 on light harvesting chlorophyll A/B binding protein 1.1 (LHCBI.1, AT1G29920). E, dual-and-opposing effect of ethylene on flowering gene bHLH 3 (FBH3, AT1G51140). Octagons, circles, boxes, and hexagons represent receptors, kinases, phosphatases, and other components of the ethylene signaling network, respectively. Arrows represent positive effects, whereas stop ends represent negative effects. Red and gray lines represent protein phosphorylation and protein dephosphorylation, respectively, and cyan lines represent the regulation of gene expression. Black line represents the effect on the synthesis of hormones. Solid lines represent direct regulation, and double solid lines represent direct or indirect regulation. Dashed lines represent putative interactions. A dashed line with a question mark means the regulation is assumed.
in Arabidopsis (105). FBH3 was suggested as one of the putative substrates for PP2A in our differential phosphoproteomic analysis, and it was also predicted as a substrate of calmodulin-domain protein kinase 9 (AT3G20410), the expression of which happened to be enhanced by ethylene (35). Thus, again ethylene is proposed to have a dual-and-opposing effect on the phosphorylation state of FBH3 through the opposing enzyme activities of kinase against phosphatase on the phosphosite (Fig. 7E).

Although significant changes in phosphopeptide profiles have been uncovered by our PTM proteomic analysis, no single case has been observed in our experiments in which a complete phosphorylation/dephosphorylation event (e.g. only the 1\textsuperscript{15}N-coded peak in forward experiments or the 1\textsuperscript{15}N-coded one in reciprocal experiments) occurred in either differential hormone treatments or different genotypes.

GPS, as a predictor for site-specific kinase–substrate relations, has been used in many investigations on protein phosphorylation in animals (60). In the present study, we analyzed the results of quantitative phosphoproteomics with GPS to predict site-specific protein kinases involved in ethylene signaling (35, 91). Some of these putative kinases (supplemental Table S6) have been successfully constructed in models of ethylene signaling networks (Fig. 7). These findings indicate that GPS not only can be used in the prediction of plant kinase–substrate relationships and establish the PTM networks during cell signaling and development (Figs. 6 and 7). The resulting quantitative proteomics and molecular systems-biology-based cell signaling networks may serve as guidelines for further experimental validation and functional studies.

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