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# Quantitative and Functional Phosphoproteomic Analysis Reveals that Ethylene Regulates Water Transport via the C-Terminal Phosphorylation of Aquaporin PIP2;1 in *Arabidopsis*

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## ABSTRACT

**Ethylene participates in the regulation of numerous cellular events and biological processes, including water loss, during leaf and flower petal wilting. The diverse ethylene responses may be regulated via dynamic interplays between protein phosphorylation/dephosphorylation and ubiquitin/26S proteasome-mediated protein degradation and protease cleavage. To address how ethylene alters protein phosphorylation through multi-furcated signaling pathways, we performed a $^{15}$N stable isotope labelling-based, differential, and quantitative phosphoproteomics study on air- and ethylene-treated ethylene-insensitive *Arabidopsis* double loss-of-function mutant *ein3-1/eil1-1*. Among 535 non-redundant phosphopeptides identified, two and four phosphopeptides were up- and downregulated by ethylene, respectively. Ethylene-regulated phosphorylation of aquaporin PIP2;1 is positively correlated with the water flux rate and water loss in leaf. Genetic studies in combination with quantitative proteomics, immunoblot analysis, protoplast swelling/shrinking experiments, and leaf water loss assays on the transgenic plants expressing both the wild-type and S280A/S283A-mutated PIP2;1 in the both *Col-0* and *ein3eil1* genetic backgrounds suggest that ethylene increases water transport rate in *Arabidopsis* cells by enhancing S280/S283 phosphorylation at the C terminus of PIP2;1. Unknown kinase and/or phosphatase activities may participate in the initial up-regulation independent of the cellular functions of *EIN3*/*EIL1*. This finding contributes to our understanding of ethylene-regulated leaf wilting that is commonly observed during post-harvest storage of plant organs.**

**Key words:** ethylene signaling, quantitative PTM proteomics, aquaporin, $^{15}$N stable isotope labeling in *Arabidopsis (SILIA)*, mass spectrometry, water transport

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## INTRODUCTION

The volatile plant hormone ethylene regulates a broad spectrum of molecular and cellular processes, and its biological impact in plants range from seed germination to senescence and wilting of leaf and flower petal (Abeles et al., 1992; Ma et al., 2008; van Doorn and Woltering, 2008). Based on many years of molecular genetics studies, a mechanistic paradigm of ethylene signaling has been established in higher plants, which has been widely used to address most of plant ethylene responses (Lin et al., 2009; Kim et al., 2012; Merchante et al., 2013; Binder et al., 2014). The ethylene signaling cascades start from the membrane-associated ethylene receptors, Ethylene Response 1 (ETR1), Ethylene Response 2 (ETR2), Ethylene Insensitive 4 (EIN4), Ethylene Response Sensor 1 (ERS1), and Ethylene Response Sensor 2

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(ERS2) (Hua and Meyerowitz, 1998). The physical interaction of ethylene molecule with each receptor complex triggers a suppression of the kinase activity of a downstream signaling component, Constitutive Triple Response 1 (CTR1) (Kieber et al., 1993), which is a putative Raf-like mitogen-activated protein kinase kinase kinase (MAPKKK). It physically interacts with ethylene receptors (Huang et al., 2003). In the absence of ethylene, CTR1 kinase directly inhibits the molecular function of endoplasmic reticulum (ER) membrane-localized Ethylene Insensitive 2 (EIN2) protein by phosphorylating this positive regulator of ethylene response (Ju et al., 2012; Qiao et al., 2012). Upon ethylene binding to various ethylene receptors, the CTR1 kinase activity is modified by ethylene and EIN2 is dephosphorylated thereafter. Consequently, the C terminus of EIN2 is cleaved off from its N-terminal domain that has a putative metal ion channel function, and migrates into the nucleus to stabilize ethylene response transcription factors, Ethylene Insensitive 3 (EIN3) and Ethylene Insensitive 3-Like 1 (EIL1) (Ju et al., 2012; Qiao et al., 2012). Finally, the EIN2 C terminus-activated EIN3 and EIL1 transcription factors (Guo and Ecker, 2003; Potuschak et al., 2003) upregulate downstream transcriptional activities of a large number of Ethylene Response Factors (ERFs; Ohme-Takagi and Shinshi, 1995), leading to various plant ethylene responses. However, recent research findings have begun to reveal multiple signaling pathways (Lu et al., 2001; Binder et al., 2004; Yoo et al., 2008; Li et al., 2009; Zhu et al., 2013) and multiple levels of molecular mechanisms regulating the complex ethylene responses, which include both post-transcriptional regulation (Zhang et al., 2015) and post-translational modification (PTM) (Zhao and Guo, 2011). The ethylene-dependent and ubiquitin/26S proteasome-mediated protein degradation or stabilization of both ethylene receptors and signaling components, EIN2 and EIN3, have also been shown to participate in regulation of the sophisticated ethylene responses (Kendrick and Chang, 2008; Qiao et al., 2009). Thus, both multiple ethylene signaling pathways and multiple levels of molecular mechanisms were proposed to regulate the stem and etiolated seedling gravicurvature, both of which are dose- and time-dependent (Lu et al., 2002; Binder et al., 2004; Li, 2008). The dual-and-opposing mode of action has also been applied to address the delay of *Arabidopsis* bolting in both ethylene-insensitive mutant *etr1-1* and constitutive ethylene response mutant *ctr1-1* (Ogawara et al., 2003; Achard et al., 2007; Zhu et al., 2013).

Transcriptomics studies have confirmed both the early and the late differential gene expression under ethylene treatment (Yang and Poovaiah, 2000; Zhong and Burns, 2003; De Paepe et al., 2004; Qiao et al., 2012). Similarly, quantitative phosphoproteomics studies have also demonstrated a time-dependent and differential phosphorylation in both etiolated and light-grown plants (Chen et al., 2011; Yang et al., 2013). The kinase activities of both ethylene receptors and CTR1 MAPKKK (Gao et al., 2003; Wang et al., 2003; Kendrick and Chang, 2008) are presumably transmitted through multi-furcated MAPK cascades to regulate the stability of EIN3 (Ouaked et al., 2003; Yoo et al., 2008). Recently, dephosphorylation of serine 62 in an Ethylene Response Factor 110 (ERF110) has been found to be independent of the function of EIN2 but dependent on that of CTR1 (Li et al., 2009). Its phosphorylated isoform is necessary for the initiation of bolting (Zhu et al., 2013).

To address the interesting phenomena of ethylene-stimulated multiple-pathway phosphor relay, a $^{15}$N stable isotope metabolic labeling (*SIML*)-based quantitative and differential phosphoproteomics (Huttlin et al., 2007) has been adopted in recent years to investigate both the wild-type and two ethylene response mutants, *eer1-1 (rcn1-1)* and *ctr1-1*, to identify ethylene-altered phosphoproteins (Yang et al., 2013). The $^{15}$N stable isotope labelling in *Arabidopsis* (*SILIA*)-based phosphoproteomics approach (Guo and Li, 2011) is one type of *SIML* similar in principle to the *SILAC* approach (stable isotope labeling by amino acids in cell culture) (Yao et al., 2001; Ong et al., 2002). An important advantage of *SIML*-based quantitative PTM proteomics over the label-free proteomics approach is that the *in vitro*-derived variability resulting from peptide preparation, chromatography separation, and mass spectrometry (MS) analysis is eliminated when the control and treated protein samples, labelled with light and heavy nitrogen isotope, respectively, are mixed at the beginning, and processed and measured together (Kline and Sussman, 2010; Schulze and Usadel, 2010). In the present study, we applied the *SILIA*-based quantitative phosphoproteomics to the ethylene-insensitive double mutant *ein3eil1* to investigate the *EIN3/EIL1*-independent alternative phosphor-relay pathway. Interestingly, this quantitative and functional phosphoproteomics study has revealed that the C-terminal phosphorylation of a water channel subunit, PIP2;1, is upregulated by an *EIN3EIL1*-independent ethylene signaling pathway at the initial stage of ethylene treatment, and its water transport activity is enhanced by phosphorylation at the C-terminal S280 and S283 sites.

## RESULTS

### Phosphoproteomic Analysis of Ethylene-Treated *ein3eil1*

To discover the proteins whose phosphorylation is regulated by ethylene through signaling pathway(s) independent of the transcription factors EIN3 and EIL1, we performed *SILIA*-based quantitative and differential phosphoproteomics on ethylene-insensitive *Arabidopsis* mutant *ein3eil1* plants that were treated by air and ethylene, respectively (Supplemental Figure 1). A dataset containing a total of 8979 peptides was obtained from the ethylene-treated and untreated plants according to the cutoff given by a false discovery rate (FDR) of 1% (see Methods), in which 5180 (57.7%) were phosphopeptides. Eventually, 742 non-redundant phosphopeptides were yielded, from which 535 repetitively (i.e., at least twice) detected phosphopeptides (either $^{14}$N-coded or $^{15}$N-coded), representing 387 different phosphoproteins of unique GenBank accession number. These phosphopeptides were selected as reliable identifiers and used in the subsequent analyses (Supplemental Table 1).

Of the 535 repetitively identified non-redundant phosphopeptides, singly and doubly phosphorylated peptides accounts for 515 (96.3%) and 20 (3.7%), respectively (Supplemental Figure 2). Only phosphorylation events that occurred on